A Mutagenic Primer Assay for Genotyping of the *CRHR1* Gene Rare Variant rs1876828 (A/G) in Asians: A Cost-Effective SNP Typing

Neeraj Sharma,¹ Shally Awasthi,^{1*} and Shubha R Phadke²

¹Department of Paediatrics & Translational Medicine Unit, King George's Medical University, Lucknow, Uttar Pradesh, India

²Department of Medical Genetics, SGP GIMS, Lucknow, Uttar Pradesh, India

Background: Today, the genetic and genomic research entered in a new era of high-throughput genotyping technology. However, mutagenic polymerase chain reaction–restriction fragment length polymorphism (PCR–RFLP) is still a choice of genotyping method in molecular epidemiological research. It has been extensively used for the detection of risk alleles, if the target SNP has no natural discriminating restriction site. We undertook this study to develop a mutagenic primer assay for a *CRHR1* rare gene variant: rs1876828 (A/G) and to determine their allele frequency in north Indian children. **Methods:** The mu-

tagenic primers were designed and assay conditions were optimized to perform mutagenic PCR–RFLP in 550 subjects. The efficiency of assay and results were validated by sequencing. **Results:** This study demonstrated that the mutagenic primer assay is feasible and applicable to discriminate *CRHR1* gene rare variant rs1876828 (A/G) and the “frequency of allele ‘G’” was 100% in north Indian asthmatics as well as normal subjects. **Conclusion:** This method can be used for both large- and small-scale study of complex genetic, where *CRHR1* gene plays the pivotal roles. *J. Clin. Lab. Anal.* 30:169–174, 2016. © 2014 Wiley Periodicals, Inc.

Key words: corticotropin-releasing factor; genetic variant; mismatching; PCR–RFLP; single nucleotide polymorphism

INTRODUCTION

The *CRHR1* gene (NM_004382: 17q12–q22) is a member of the corticotropin-releasing factor family (1). It is a G protein coupled receptor, essential for the activation of signal transduction pathways and regulation of diverse physiological processes, including stress, reproduction, immune response, and obesity (2). This receptor is highly expressed in the cortex, cerebellum, hippocampus, olfactory bulb, and the anterior pituitary (3). However, low level of expression was also found in the skin, ovary, testis, and adrenal gland. This receptor plays a pivotal role in the regulation of the hypothalamic–pituitary–adrenal axis (HPA) in response to stress. It act as primary receptor and mediates the action of corticotropin-releasing hormone (*CRH*) on the pituitary to release adrenocorticotrophic hormone. Adrenocorticotrophic hormone stimulates the production of an important immune-regulatory molecule cortisol in the adrenal cortex (4, 5).

It is hypothesized that the single nucleotide polymorphisms (SNPs) in *CRHR1* receptor gene may alter the CRH pathway and endogenous steroid levels. This can influence the disease pathogenesis and responses during corticosteroid administration in patients. Recent studies have identified the genetic variant: rs1876828, having statistically significant effect during inhaled corticosteroid (ICS) treatment in asthmatic patients. The SNP rs1876828 minor allele (A, 22% frequency in Caucasians) has been associated with increased FEV1, following corticosteroid therapy in Caucasian asthmatics (homozygous minor had FEV1 change $23.7 \pm 9.75\%$ vs. FEV1 change $5.14 \pm 1.31\%$

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*Correspondence to: Dr. Shally Awasthi, Department of Paediatrics, King George's Medical University, Lucknow, Uttar Pradesh 226003, India. E-mail: shally07@gmail.com

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for homozygous major individuals; (6, 7)). The role of this gene has been also implicated in corticosteroid response in the treatment of depression, anxiety, leukemia, and chronic obstructive pulmonary disorder (6–10). These findings support to provide personalized treatment plans by determining the most efficacious treatments through genetic test.

The completion of human genome project increased the search of genetic variations, having impact on the pathophysiology and treatment of disease. This also increased the demand of a reliable, time saving, cost effective, and easy to use genotyping methodology. Today, many different methods have been developed for SNP genotyping by PCR, likewise allele-specific PCR, primer extension oligonucleotide ligation, and direct DNA sequencing (11–13). Recent advancement in molecular technology for SNP typing (Invader method, Taqman method, Gene Chips, MALDI-TOF method) improved the output, as well as reliability of data (14–20). To date, the genotyping of rs1876828 polymorphism has been performed via a SEQUENOM MassARRAY MALDI-TOF mass spectrometer and direct sequencing (6–8). However, instrumentation and reagents are expensive for most of the laboratory with medium facility. It generally also requires well-trained hands.

The utility of a conventional polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) genotyping method has been proved by several authors (21). It is based on activity of specific restriction enzyme to cleave the PCR product in the region having point mutation. Since the flanking sequence around the rs1876828 cannot be recognized by any restriction enzyme, we performed mismatch PCR-RFLP genotyping using mutagenic primer. The basic principle of this technique is similar as PCR-RFLP and most suitable in context with simplicity, sensitivity, reliability, and required investment in instrumentation (22). In brief, this method involves artificial changes of nucleotides in primer near the SNP, either in forward primer or reverse primer. It creates endonuclease recognition site along with variant in PCR products. Further, the presence of specific allele can be determined by the similar principle of classical PCR-RFLP method (21). The aim of this study was to develop a mutagenic primer-based highly sensitive PCR-RFLP genotyping assay for the *CRHR1* (rs1876828: A > G), a rare gene variant, and to determine its frequency in north Indian asthmatics and normal children.

MATERIALS AND METHODS

Study Subjects

Asthmatics and healthy controls were identified from the outdoor and indoor patients at King George's Med-

ical University, Lucknow, Uttar-Pradesh, India, during September 2010 to May 2013. All subjects were Indian residing in north India (based on self-reported race/ethnicity). Ethical approvals were obtained from the institutional ethics committee (no.2824/R-cell-11). Asthma was clinically diagnosed by physicians according to the NIH (National Institute of Health—National Heart, Lung and Blood Institute) Guidelines 2007 (23). Before enrolment in the study, the purpose of the research and the experimental protocols were explained to the parent/guardian of each participant, and their prior written informed consent was obtained.

Sample and DNA Extraction

Blood samples (3.0 ml) were collected in ethylenediamine tetraacetic acid (EDTA) containing vial from all the participants. Genomic DNA was extracted from 1 ml blood using salting out method (24), and rest of the amount was stored at -70°C for the future applications. The quality of DNA was conformed through 1.0% agarose (Sigma-aldrich, St Louis, MO, USA) gel electrophoresis, and quantification was done using NanoDrop (Thermo Scientific, Wilmington, DE19810, USA).

Selection of Gene and SNP for the Study in Asthmatics

The gene *CRHR1* and SNP (rs1876828) were selected for the study on the basis of their role in steroid biology and pharmacogenetic influences on corticosteroid therapy in asthmatic (6, 7). The flanking nucleotide sequences of rs1876828 were retrieved from NCBI's (National Centre for Biotechnology Information) sequence database (www.ncbi.nlm.nih.gov).

Mutagenic Primer and Choice of Restriction Endonuclease

A web-based freely available tool NEBcutterV2.0 (<http://tools.neb.com/NEBcutter2/>) conformed the lack of natural restriction site to determine rs1876828 (A > G) variations. As described by Peyret et al. (1999) and Bui and Liu (2009), the purine–pyrimidine mismatch in the primer sequences are more stable and have weaker destabilization effect. In contrast the purine–purine or the pyrimidine–pyrimidine mismatch can alter geometry in double helix and reduced hydrogen bonding (25,26). Therefore to illustrate mutagenic RFLP, the nucleotide sequences of forward primer were replaced (AG to TC) at third and fourth base near the SNP site (Fig. 1). This forward primer was designed in such a way that it can anneal adjacent to site of variation on plus strand and generate a “*BstBI*” restriction site for one of the reported allele “A.”

Fig. 1. The principle of mutagenic restriction fragment length polymorphism (RFLP) for the SNP rs1876828.

The mutagenic forward and natural reverse primers were 5'-AGC AGC ATA CCC CTA GGG ACC TTC GA-3' and 5'-CTG CAG CCG ACC TTT GAC GCC TC-3', respectively. Here, the length difference between the cut and uncut band is only limited by the length of the forward primer minus the cut part at its 5' end. The expected fragment sizes of digested PCR products were determined by NEBcutterV2.0 and they were 23 bp + 194 bp + 217 bp for "AG" genotype, 23 bp + 194 bp for "AA" genotype, and 217 bp for "GG" genotype (Fig. 1).

PCR and Mutagenic Restriction Fragment Length Polymorphism Assay

Gradient PCR assay was performed with designed primer set to optimize the annealing temperature. In brief, this temperature was considered when highest amount and specific size of PCR product were seen on agarose gel. Each PCR was carried out in a total volume of 10 μ l reaction mixture, with 30 ng of genomic DNA, 3 pmol of each primer, 1 μ l 10 $\times$ buffer, 1.5 mM MgCl₂, 200 μ M dNTPs, and 0.5 U Taq polymerase (New England Biolabs, Ipswich, MA), using Veriti thermal cycler (Applied Biosystems, Foster City, CA). PCR conditions were as follows: initial denaturation of 95°C for 3 min, followed by 35 cycles of 94°C for 1 min, 61–65°C for

45 sec (gradient step), and 72°C for 45 sec, followed by a final extension of 72°C for 2 min. Amplified PCR products were separated on ethidium bromide (EtBr) stained 2% (wt/vol) agarose gels and 217 base pair products were visualized under UV light. Five microliters of PCR product were digested overnight (16 h) at 65°C with 5 U of allele-specific restriction enzyme *BstBI* (New England Biolabs). Overnight incubated PCR-amplified DNA was visualized in 2% (wt/vol) agarose gels after EtBr staining.

Validation of PCR and Mutagenic RFLP Results

Prior to begin mutagenic RFLP, PCR-amplified DNA was sequenced to determine the specificity of primers and PCR assay. About 50% of randomly selected samples were resequenced by other laboratory personal. For quality control of genotyping results, we randomly selected 20% samples for sequencing by outsource (Bangalore Genei, India Pvt., Bangalore, Karnataka, India).

Comparison With 1000 Genomes Database

Genotyping results of control subjects from north Indian subpopulation were compared with reported genotyping results using 1000 Genomes database.

Fig. 2. A sample chromatogram showing the presence of “G” allele and absence of restriction site for “BstBI” restriction enzyme, and showing “GG” genotype by both sequencing and PCR-RFLP.

RESULTS

A total of 275 unrelated asthmatics patients (92 females, mean age 71.12 ± 40.29 months and mean body mass index 15.18 ± 9.45) and 275 unrelated age and sex matched healthy controls (100 females, mean age 76.62 ± 38.17 months and mean body mass index 15.25 ± 4.96) were in-

cluded in the study. There were no statistically significant differences between the patients and control groups ($P > 0.05$) as regarding age ($P = 0.101$), sex ($P = 0.531$), and BMI ($P = 0.907$).

We described a mutagenic RFLP genotyping method based on introduction of mismatch in forward primer. NEBcutterV2.0 showed that the addition of pyrimidine

TABLE 1. Worldwide Allele Frequency of SNP CRHR1:rs1876828

Population	Frequency of rs1876828 A > G		Subpopulations	Frequency of rs1876828 A > G	
	A%	G%		A%	G%
African ^a	2	98	American of African ancestry	8	92
			Luhya in Webuya, Kenya	0	100
			Yoruba in Ibada, Nigera	0	100
Americans ^a	19	81	Colombian from Medellin, Colombia	19	81
			Mexican ancestry from Los Angeles, USA	20	80
			Puerto Rican from Puerto Rica	17	83
Europeans ^a	23	77	Utah residents with northern and western Europe ancestry	20	80
			Finish in Finland	11	89
			British in England and Scotland	25	75
			Iberian in Spain	21	79
			Toscani in Italy	36	64
East Asian ^a	0	100	Han Chinese (Beijing), China	0	100
			Southern Han Chinese	0	100
			Japanese in Tokyo, Japan	1	99
South Asian (in the present study)	0	100	North Indian, India	0	100

^a1000 Genomes database assessable at <http://www.1000genomes.org/>

(TG) at the place of purine (AG) in forward primer created an artificial restriction site (*BstBI*) for the “A” allele. However, no *BstBI* restriction enzyme recognition sequence has been generated for “G” allele at the same site. PCR primer set was designed to amplify a given DNA region along with SNP site, but not to amplify nonspecific region or paralogous region. The PCR assay conditions were optimized by performing gradient PCR. Finally, 64°C was found most suitable annealing temperature and used further in all PCR assays. This method is simple and does not require specialized equipment except a PCR machine.

Mutagenic restriction fragment length polymorphism analysis of *CRHR1* gene variant rs1876828 was performed in all recruited subjects. The obtained genotyping result showed 100% frequency of “G” allele, although no “A” allele found in studied samples. Fully concordant result was obtained, which show the nonappearance of “AA” and “AG” genotypes in studied population.

The sequencing result of a sample chromatogram is shown in figure 2. Table 1 represents the distribution of allele frequency worldwide.

DISCUSSION

The aim of this study was to setup a reliable and easy genotyping assay for the SNP rs1876828, and to find out the allele frequencies in north Indian asthmatics and healthy children. In the present study, the described simple mutagenic primer-based SNP discrimination method for *CRHR1* (rs1876828) gene variant is reliable and easy to use in laboratory with medium facility. This genotyping method made possible to genotype rs1876828 using conventional PCR-RFLP method, although it lacks any natural restriction site (22). However, this methodology was also successfully used in other molecular epidemiological studies (21).

To our knowledge, this is first study showing the absence of rs1876828 variant in north Indian asthmatics and normal children. These results are in agreement with findings from East Asian population. But, it varies in African, American, and Europeans.

Conclusively, the method described in the present study is simple, inexpensive, accurate, and applicable to identify genetic variation in small as well as large-scale epidemiological study of complex genetic disease. However, genotyping result reiterates the uniqueness of the Indian population with respect to the worldwide scenario.

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