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Comparison of TaqMan Real-Time and Tetra-Primer ARMS PCR Techniques for Genotyping of Rs 8066560 Variant in Children and Adolescents with Metabolic Syndrome*

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article

Abstract

Background. Single nucleotide polymorphisms (SNPs) are major contributors to susceptibility or resistance to various human diseases. Metabolic syndrome (MetS) is a collection of risk factors, including abdominal obesity, dyslipidemia and increased blood pressure. MetS is more common among Iranian children and adolescents in comparison to other ethnicities. Sterol regulatory element binding factors (SREBFs) involve in the regulation of carbohydrate and lipid metabolism. The tetra-primer amplification refractory mutation system PCR (tetra-primer ARMS-PCR/T-ARMS-PCR) is a fast and economical means of assaying SNPs, requiring only PCR amplification and subsequent electrophoresis for the determination of genotypes.
Objectives. This study aims to optimize and compare the results of tetra-primer ARMS-PCR for genotyping of rs8066560 in Iranian children and adolescents being afflicted with metabolic syndrome with the TaqMan assay.
Material and Methods. In this study, a total of 50 individuals with 9–19 years of age, including 25 healthy subjects and 25 MetS cases were studied. The tetra-primer ARMS-PCR was used to genotype the rs8066560.
Results. After PCR optimization, we could successfully detect the rs8066560 polymorphism in all the studied subjects. Furthermore, we observed complete concordance between tetra-primer ARMS-PCR assay and TaqMan method`s results.
Conclusions. Tetra-primer ARMS-PCR can be utilized as a cost-effective, rapid and reproducible method for SNP genotyping especially while performing large-scale epidemiological/association at studies (**Adv Clin Exp Med 2015, 24, 6, 951–955**).

Key words: metabolic syndrome, polymorphism, genotype.

A single nucleotide polymorphism (SNP) is a DNA sequence variation at a single position happening around once per 250–1000 bp [1, 2]. SNPs include 90% of sequence variants in human genome [1]. Patterns of SNPs that each individual harbors will influence the risk for major common diseases like cancer, autoimmune disorders, cardiovascular diseases and diabetes [3].

Metabolic syndrome (MetS) is a collection of risk factors, including abdominal obesity, dyslipidemia and increased blood pressure [4]. MetS is more common among Iranian children and adolescents

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in comparison to other ethnicities [5, 6]. This metabolic disorder increases the incidence of heart attack and type 2 diabetes mellitus (T2DM) resulting in increased death rate worldwide [7].

Multiple genetic factors are involved in the incidence and development of MetS [8]. Sterol regulatory element binding factors (SREBFs) are a group of transcription factors involving in the regulation of carbohydrate and lipid metabolism. In mammals, there are three members of SREBF family, SREBF-1a, -1c and -2 [9]. The association between various SNPs of SREBF-1 and the components of MetS have been studied [10–21]. Recently, we genotyped a SNP, rs8066560, in *SREBF-1* gene in order to investigate its association with MetS, hyperglycemia and insulin resistance in Iranian children and adolescents [22, 23].

In our previous studies, the TaqMan genotyping method was successfully used to determine the two alleles of rs8066560 [22, 23]. Here, in order to rapidly screen for this SNP in MetS individuals, we developed a tetra-primer amplification refractory mutation system PCR (tetra-primer ARMS--PCR/T-ARMS-PCR) and compared the results of this genotyping technique with that of the TaqMan method.

Material and Methods

Samples

A total of 50 individuals, including 25 healthy subjects and 25 MetS cases were recruited from two referral centers in Isfahan province, Iran. The diagnosis of cases was made on the basis of the Adult Treatment Panel (ATP) III criteria modified for the pediatric age group [5]. Genomic DNA from 100 μL fresh peripheral blood samples containing EDTA was extracted by Diatome kit (Isogen Laboratory, Russia) and stored at –20˚C for genotype analysis.

Primer Design

The tetra-primer ARMS-PCR [24] was used to detect the rs8066560. This procedure includes four primers to amplify three fragments which are easily visible using agarose gel electrophoresis. Both inner primers contain a mismatch at position -2 from the 3` end to increase the specificity of the reaction. The primers for the tetra-primer ARMS-PCR were designed on the basis of the *SREBF-1* sequence (GenBankaccession NT_010718) and primer design computer program accessible through the Internet at http://cedar.genetics.soton.ac.uk/public_html/primer1.html [24]. The blast program at http://www.ncbi.nlm.nih.gov/blast was used to check the specificity of the designed primers. The sequences of primers used in this study are represented in Table 1.

PCR Method

Polymerase chain reaction amplification was performed in a 25 μL reaction containing 0.2 mM dNTP, 2.5 μL of complete buffer (containing $MgCl_2$) and 1 Unit of DFS-Taq polymerase (BIORON, Germany), 200 ng of genomic DNA and 2.5 pmol of each outer and inner primers. Thermal conditions of the PCR were 1 cycle at 95°C for 5 min followed by 35 cycles at 95°C for 1 min, 70°C for 1 min, and 72°C for 1 min and the final extension step was performed at 72°C for 15 min. The sizes of amplified DNA were confirmed by 1.5% agarose gel electrophoresis at 90 V for 30 min and visualized by green viewer staining, and examined by ultraviolet light.

Statistical Methods

All statistical analysis was performed using the Statistical Package for Social Sciences (SPSS) v. 16.0 and reported as numbers and percentages. Comparison of allele and genotype frequencies between control and MetS groups was examined by the chi-squared test. P values of < 0.05 were considered statistically significant.

Table 1. Sequences of the designed PCR primers for genotyping rs8066560 (A/G)

	Primer sequence	**Amplicon size (bps)**
Forward inner primer (A allele)	5`GGTCCACACCCCAGTTGGCTGCAACCGGA*	203 bp
Reverse inner primer (G allele)	5`TGGGTCTGCCCAGGGTTCAGGCACCCGCC*	358 bp
Forward outer primer	5`CCCTCAAGGCCTCACAACCCAGCAGTCACC	504 bp
Reverse outer primer	5`AGCTGGGAAATAAGGGCCTGGGCTGGACG	–

* – underlined nucleotide shows the location of deliberate mismatch.

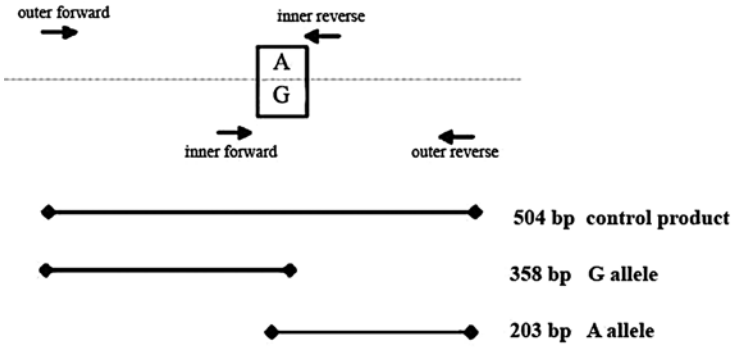

Fig. 1. Schematic diagram of the T-ARMS-PCR assay. Two outer primers generate a 504 bp amplicon being served as a (non-allele specific) control product. Outer forward and inner reverse primers generate a 358 bp amplicon specific for G allele and inner forward and outer reverse primers amplify a 203 bp specific for the A allele

Results

To optimize the tetra-primer ARMS-PCR assay, we tested a known heterozygous MetS individual for whom we had previously determined the genotype for rs8066560 (A/G) by TaqMan PCR and Sanger sequencing as described [22, 23]. The genotype and allele frequencies distribution of *SREBF-1* rs8066560 are indicated in Table 2. Several factors, including primer concentrations, $MgCl_2$, PCR enhancers and PCR cycling conditions, which may influence the efficacy of PCR were optimized. We tested annealing temperatures ranging from 64 to 70°C, and observed the good quality bands at 70°C. Furthermore, we titrated the primer concentrations and obtained the best quality when equal concentrations of four primers were used. Next, we tested the effect of increasing $MgCl_2$ from 2.5 mM to 6 mM. The best amplification was obtained when the $MgCl_2$ concentration was set at 2.5 mM. Additionally, PCR amplifications were performed using various DMSO, BSA and glycerol concentrations and found that these additives will not significantly increase product yield. By changing different factors, we could detect the three expected bands, including a 203 bp band for A allele, a 358 bp for G allele and a 504 bp for two outer primers (Fig. 1) for a known heterozygous case. Additionally, randomly selected MetS and control individuals were genotyped using the optimized tetra-primer ARMS-PCR assay. We observed complete concordance between tetra-primer ARMS-PCR assay and TaqMan method. A representative tetra-primer ARMS-PCR assay analysis is shown in Fig. 2.

Table 2. Genotype and allele frequencies for rs8066560 in MetS and control groups

Group	Controls (n = 25)		Cases (n = 25)		p-value
	n	%	n	%	
Allele frequency					
A	23	46	19	38	0.418
G	27	54	31	62	
Genotype frequency					
AA	4	16	3	12	
AG	15	60	13	52	0.641
GG	6	24	9	36	

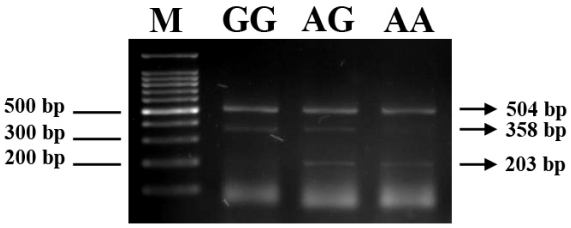

Fig. 2. Agarose gel electrophoresis of the tetra-primer PCR assay products. M represents the DNA marker. Lane 2, 3 and 4 shows a GG homozygote, an AG heterozygote and an AA homozygote individual for the rs8066560 (A/G), respectively

Discussion

In the current study, we could successfully genotype the rs8066560 (A/G) using tetra-primer ARMS–PCR. For all cases, assays of the samples gave consistent and unambiguous genotypes when compared to TaqMan assay.

In the age of personalized medicine, the demand for the genotyping methods which are simple, fast, economical and reliable is increasing [25]. SNP detection, genotyping, or allelic discrimination can be performed by methods such as PCR-restriction fragment length polymorphism (RFLP), TaqMan genotyping, amplification refractory mutation system PCR (ARMS) and direct DNA sequencing.

TaqMan Real-Time PCR needs two flurogenic probes to detect specific PCR products accumulated during PCR. Each TaqMan probe contains

a nonfluorescent quencher at the 3′ end and a reporter fluorescent dye on the 5′ end. By means of probes labeling with different, distinguishable reporter dyes, it is possible to distinct two sequences in one reaction tube. Moreover, post-PCR detection methods are eliminated. The main disadvantage of the TaqMan Real-Time PCR is that the synthesis of specific probes is necessary for different sequences which increase assay labor and material costs [26].

Although direct DNA sequencing is often considered as the gold standard for DNA sequence analysis [27], it is costly, time-consuming and cumbersome [28].

The main advantages of the PCR-RFLP technique include easy assay design using publicly available programs, inexpensiveness and no need for advanced equipment. The shortcomings include being relatively time-consuming and the need for specific restriction endonucleases [29].

Tetra-primer ARMS–PCR is a rapid, simple, robust, low-cost, and easy to use method for SNP genotyping [24] which differs from its classical version in such a way that it does not involve two separate PCR reactions for amplifying the two different alleles of an SNP. Two primers pairs are employed in tetra-primer ARMS-PCR to distinguish two alleles in one reaction tube [24, 30]. The critical part of the technique is its primer designing although even with well-designed primers, some tetra-primer ARMS-PCR assays fail to achieve allele specificity because of the non-refractory of the 3` nucleotide mismatch to PCR extension [24].

In summary, tetra-primer ARMS-PCR can be utilized as an economical, fast and reliable method for SNP genotyping even in low-tech laboratories.

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