

Development of a High-resolution Melting Analysis Method Based on SYBR Green-I for rs7216389 Locus Genotyping in Asthmatic Child Patients

Zahra Vali ^{1,2}, Abbasali Raz ^{1*}, Hanieh Bokharaei ¹, Mohammad Nabavi ³,
 Mohammad Hassan Bermanian ³, Mina Sharifi Yazdi ⁴, and Navid Dinparast Djadid ^{1*}

1. *Malaria and Vector Research Group (MVRG), Biotechnology Research Center, Pasteur Institute of Iran, Tehran, Iran*

2. *Department of Pediatrics, Shahid Sadoughi Hospital, School of Medicine, Shahid Sadoughi University of Medical Sciences, Yazd, Iran*

3. *Department Allergy and Immunology, Hazrate Rasoul Akram Hospital, Tehran University of Medical Sciences, Tehran, Iran*

4. *Boo-Ali Polyclinic of Social Security Organization, Kashan, Iran*

Abstract

Background: Asthma is caused by the combination of different factors. Current concepts of asthma pathogenesis emphasize on gene-environment interactions. Mega-genome scanning projects revealed that different Single Nucleotide Polymorphisms (SNPs) are related to asthma susceptibility. rs7216389-T is one of them that is related to childhood asthma and its effect on childhood asthma severity has been proved in different nations, however no study has been performed in Eastern Mediterranean and Middle East countries yet.

Methods: To perform population genetic studies, a rapid and high-throughput screening method is necessary. High-resolution melting analysis is a rapid, powerful and accurate method, which is suitable for this type of studies. Therefore, it has been decided to develop a high-resolution melting method for rs7216389 locus genotyping in Iranian asthmatic children. In the current study, a high-resolution melting analysis method based on SYBR Green-I was developed to check the frequency of rs7216389-T mutation in Iranian asthmatic children for the first time.

Results: Second and third classes of intercalating dyes are commonly used for high-resolution melting method. However, in this study, SYBR Green-I was used for rs7216389 locus genotyping for the first time. Our results for 60 samples showed that SYBR Green-I has good efficacy for rs7216389 locus genotyping through high-resolution melting method in comparison with PCR-RFLP and sequencing.

Conclusion: Comparison of our results based on HRM analysis with PCR-RFLP showed that our developed method is rapid, accurate, high-throughput and economic to study the rs7216389 locus in asthmatic children and it is applicable for other similar population genetic studies.

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* **Corresponding authors:**
 Abbasali Raz, Ph.D., and
 Navid Dinparast Djadid, Ph.D.,
 Malaria & Vector Research
 Group (MVRG), Biotechnology
 Research Center (BRC),
 Pasteur Institute of Iran,
 Tehran, Iran
Tel: +98 21 66480780
Fax: +98 21 66465132
E-mail:
 raz.biotech@gmail.com;
 navidmvr@gmail.com
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Introduction

Asthma is a complex and multi-factorial inflammatory disease of lung airways, which results in airflow obstruction. Different genet-

ic and environmental factors are involved in asthma susceptibility and manifestations. Numerous studies have been performed to de-

termine the relationship between asthma and genetic factors. It has been revealed that more than 200 genes and loci are linked to asthma and its related phenotypes¹. A clear pattern of inheritance has not been seen in asthma and it seems that asthma heritability is between 36-79%²⁻⁴. Due to this heterogeneity, the study of asthma genetics is very problematic. Therefore, several genome-wide linkage studies have been performed on asthmatic patients thus far. In 2007, a genome-wide association revealed that rs7216389-T (a sequence variant on chromosome 17q21) is associated with the risk of childhood asthma⁵.

The correlation between 17q21 locus, childhood and persistent asthma has been supported by different studies; however, its mechanism is unclear⁶⁻¹². The association of this SNP has been studied in Europe, America, Africa and Asia and it was shown that the relationship between this locus and childhood asthma is ethnic dependent. According to these studies, in Europe and East Asian countries (such as China, Japan and Korea), there is an apparent correlation between rs7216389-T mutation, onset age and severity of childhood asthma^{5,7,13-15}. However, this correlation has not been observed in African and Mexican patients^{13,16}. As no study had been performed in West Asian countries, it has been decided to check this correlation in Iranian asthmatic children.

Different methods have been developed to evaluate the association of SNPs and specific phenotypes. PCR Restriction Fragment Length Polymorphism (PCR-RFLP), real-time PCR (with specific fluorescent probes) and Denaturing Gradient Gel Electrophoresis (DGGE) are the most commonly used techniques which are suitable for these types of studies. PCR-RFLP is time consuming and needs a specific restriction enzyme for each SNP. DGGE requires an expert and specific equipments as well. Furthermore, real-time PCR with specific fluorescent probes is expensive and needs an expert for probe designing and data analysis.

Recently, High-Resolution Melting Analy-

sis (HRM or HRMA) has been introduced as a rapid, efficient and high-throughput post-PCR analysis technique to investigate the variance in nucleic acid sequences and scanning the genetic mutations in population without requirement for specific fluorescent probes. This technique enables researchers to discover new genetic variants (without sequencing), rapid detection and categorization of genetic mutations or genetic variation in population. Furthermore, in comparison with other real-time PCR methods, low-cost fluorescent dyes are used in HRM technique, which requires less optimization rather than other methods such as TaqMan, Fluorescence Resonance Energy Transfer and molecular beacon.

Different types of double stranded DNA (dsDNA) intercalating dyes with particular specifications are used for real-time PCR. HRM dyes have distinctly different specifications from other dyes, which are commonly used for standard quantitative PCR. Some requirements such as amplification efficiency and signal/noise ratio, which are important in quantitative PCR, are not necessary for HRM. However, for HRM analysis, dyes should provide the detailed information of melting behavior of an amplicon and the T_m of amplified target should not be changed. In addition, dyes should not be bound preferentially to purines or pyrimidines bases and inhibit DNA amplification ideally. According to their specifications, dsDNA intercalating dyes are divided to three main classes which are non-saturating, saturating and release-on-demand dyes. In fact, second (SYTO9[®] and LCGreen[®]) and third (EvaGreen[®]) classes of dsDNA intercalating dyes are suitable for HRM analysis^{14,15}.

The cost of second and third classes of intercalating dyes is more than the first class (SYBR[®] Green I). Some reports indicated that SYBR[®] Green I has good efficacy for HRM analysis and it can be used for allelic discrimination^{17,18}. Therefore, adopting first class dyes for allelic discrimination by HRM method is rational and economic. In fact, in this study it was intended to develop and evaluate

SYBR[®] Green I efficiency for genotyping rs7216389 locus variants in Iranian asthmatic children and compare its efficiency with PCR-RFLP technique and Solis Biodyne qPCR mix (its intercalating dye is EvaGreen[®]) in parallel.

Materials and Methods

Blood sample collection and DNA extraction

This study was designed for evaluating the correlation between rs7216389-T mutation and the onset age and severity of childhood asthma⁵. Therefore, asthmatic children under 18 were selected for sampling after examination by asthma and allergy specialist and their grading was performed according to the Global Initiative for Asthma (GINA) in Hazrate Rasoul Akram hospital, Immunology and Allergy Department¹⁹. Two ml of whole blood was taken from 60 patients after the informed consent was obtained from their parents. Samples were kept at -20°C until DNA extraction and DNAs were extracted by DNAeasy kit (QIAGEN) according to the manufacturer's instruction.

Primer designing

Two different techniques (PCR-RFLP and HRM) were used in this study for rs7216389 locus genotyping. HRM-R530: 5'-GCCCCACACATCCTCCACGAAC-3', RFLP-F: 5'-CA AAGACTGCAGTGAGCCAAGATC-3' and HRM-F371: 5'-CAGTTCTGTCGCTGTTGT TTGTATG-3' primers were designed according to NG_015804.1 GenBank accession number. All primers were designed by Gene Runner software (version 3.05, 1994, Hastings Software Inc.) and their specificity for PCR was checked by nucleotide BLAST (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>).

PCR-RFLP

For PCR-RFLP analysis, PCR reactions were performed in 25 µl total volume by RFLP-F and HRM-R530 primers for 60 samples. The reaction mixture contained 400 nM of each primer, 1 unit of Taq DNA polymerase, 0.2 mM of each of dNTPs, 0.001% gelatin, 2.5 µl of 10×PCR buffer, 1.5 mM of MgCl₂

and 100 ng of DNA as template. All PCR reaction components were obtained from Cinnagen Company. The amplification program was as follows: initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 50s and extension at 72°C for 1 min; and an additional final extension at 72°C for 10 min. Once amplification was completed, PCR products were analyzed by agarose gel electrophoresis.

Next, 5 µl of each PCR product was added to 5 µl of the restriction master mix which was composed of 1 µl of 10×buffer, 0.1 µl *NSi* I restriction enzyme that cuts the T allele of rs7216389 locus (New England Biolab, UK) and 3.9 µl of H₂O. Digestion mixture was incubated at 37°C overnight and the digested products were analyzed by agarose gel electrophoresis. By amplification, a 230 bp fragment was amplified and after digestion, two fragments with 90 bp and 140 bp lengths were produced. Finally, different detected genotypes (normal, mutant and heterozygote genotypes) were sequenced for confirmation and those were used as reference genotypes for HRM analysis in the next steps.

High-resolution melting curve PCR analysis

HRM experiments were performed by specific amplification of a 160 bp fragment with HRM-F371 and HRM-R530 primers. HRM curve acquisition and analysis were performed on Rotor-Gene Q instrument (QIAGEN). SYBR Green I homemade Real-time PCR master mix (which was prepared according to Karsai *et al* and Monika Jung *et al*'s descriptions) was used in this study to evaluate the effect of different factors on PCR efficiency, melting behavior of amplicons and efficacy of SYBR Green I for HRM analysis^{20,21}. The molar concentration of SYBR Green I was calculated by Zipper *et al*'s values²². Master mix was prepared as 2× and its aliquots were kept at -20°C and kept at 4°C after thawing. The stability of our homemade real-time PCR master mix in the mentioned storage condition was checked on one sample, triplicate formats and different time frames.

To find the best concentrations of SYBR Green I and $MgCl_2$ for HRM analysis and allelic discrimination, serial dilutions of these ingredients (0.1-1 μM of SYBR Green I and 0.5-3 mM of $MgCl_2$) were checked. Because SYBR Green I has inhibitory effect on Taq DNA polymerase activity, the maximum concentration of this ingredient for HRM analysis was determined first. Then, the effect of different concentrations of $MgCl_2$ on melting behavior of three pre-defined genotype samples by PCR-RFLP and sequencing was evaluated. PCR cycling parameters for real-time PCR and HRM curve acquisition were followed by: one cycle of initial denaturation at $94^\circ C$ for 5 min, 40 cycles of $94^\circ C$ for 10 s, $60^\circ C$ for 15 s and $72^\circ C$ for 20 s. For HRM preparation step, PCR products were heated at $72^\circ C$ for 2 min and then cooled at $50^\circ C$ for 1 min. Afterward, the melt step of HRM analysis was performed with different programs with increments of 0.07, 0.08, 0.09, 0.1, 0.15 and $0.2^\circ C/s$ from $77^\circ C$ to $95^\circ C$ in different experiments. The fluorescence data were acquired at the end of each extension step during PCR cycles and each steps of HRM with auto-gain optimization menu. To control the data quality, PCR amplification plots were analyzed by evaluation of the C_t value and end point fluorescence level. Samples with C_t value over 30 and end point fluorescence level less than 50% of average fluorescence of other samples were omitted from HRM analysis²³. When the results were compared, HRM was performed by raising the temperature from $78^\circ C$ to $93^\circ C$, with an increment of $0.1^\circ C/s$, in order to obtain the melting profile data in the next experiments. Initially, for HRM analysis, melting plots were normalized by adapting the start and end fluorescence signals of all samples to the same level²³. Then, normalized HRM data were subjected to gene scanning analysis by Rotor-Gene Q- Pure Detection software, version 2.0.2 (Build 4) (QIAGEN) to identify the temperature shift changes in melting curves, which indicate the presence of variation in target sequence.

To compare the efficiency of SYBR Green I for rs7216389 locus genotyping by HRM method, our homemade real-time master mix and Solis Biodyne qPCR mix were used in parallel and in the same condition in which their intercalating dye was EvaGreen[®]. To perform this comparison, HRM was done by raising the temperature from $78^\circ C$ to $93^\circ C$, with an increment of $0.1^\circ C/s$ and final analysis was performed separately for EvaGreen and SYBR Green I by Rotor-Gene Q- Pure Detection software, version 2.0.2 (Build 4) (QIAGEN) due to the difference in specifications between SYBR Green I and Eva Green.

Results

PCR-RFLP

For genotyping of the samples by PCR-RFLP, PCR was performed on extracted patients' DNAs by RFLP-F and HRM-R530 primers. Then, the quality of amplified products was analyzed by agarose gel electrophoresis and their genotyping was performed by *NSI* I enzyme digestion reaction. Finally, the genotype of samples was determined by agarose gel electrophoresis and their digestion profile analysis (Figure 1). Sixty samples were analyzed in this study among which 17 samples were heterozygote and 29 samples and 14 samples had T and C alleles, respec-

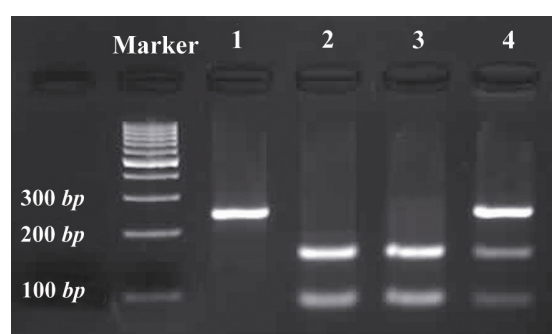


Figure 1. PCR-RFLP analysis by *NSI* I restriction enzyme digestion: To determine the samples' genotype by PCR-RFLP, PCR was performed by RFLP-F and HRM-R530 primers, digestion reactions were performed by *NSI* I enzyme and samples' genotype was determined by AGE analysis. *NSI* I enzyme cuts the mutant allele. The genotype of number 1 is normal, numbers 2 and 3 are mutant and number 4 is heterozygote

tively. These samples were used for HRM optimization and evaluation of SYBR Green-I efficacy and accuracy for rs7216389 locus genotyping by HRM method in the next steps.

High-resolution melting curve PCR analysis

SYBR Green I is a non-saturating intercalating dye and it has an inhibitory effect on Taq DNA polymerase activity in high concentration. Therefore, the effect of serial dilutions (0.1-1 μ M) of SYBR Green I on amplification of 10 ng DNA of one sample was evaluated to select the maximum concentration, which can be used for HRM analysis. This evaluation was performed by studying the effects of SYBR Green I on threshold cycle (C_t) value,

amplification plot specifications and agarose gel electrophoresis. Our results revealed that 0.66 μ M of SYBR Green I is the best concentration in 20 μ l final volume.

Next, the effect of serial dilutions of MgCl₂ (0.5-3 mM) on melting behavior of three pre-defined genotype samples (with different genotypes) in 0.66 μ M of SYBR Green I was checked. Our findings revealed that 2 mM MgCl₂ is the best concentration for rs7216389 locus genotyping with our homemade master mix.

Furthermore, the stability and reproducibility of results with our homemade real-time master mix was checked by the study of T_m , melting profile and C_t value of the same sam-

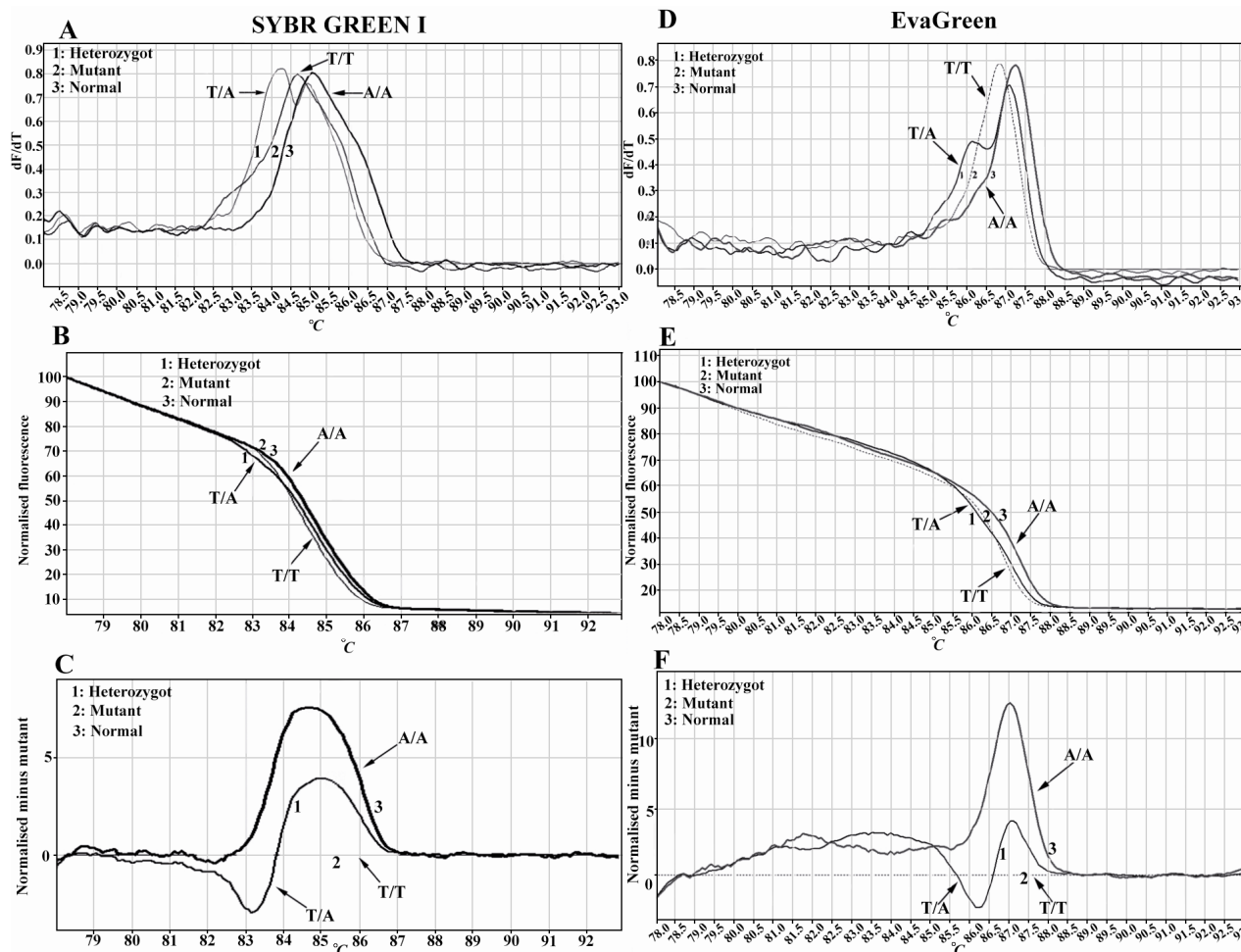


Figure 2. HRM analysis of rs7216389 locus with SYBR Green I and EvaGreen: After PCR amplification, HRM analysis was performed and it was followed by normalization of the data. Results were analyzed in the normalized fluorescence versus temperature plot. Figure 2 (A-C) is related to SYBR Green I and Figure 2 (D-F) is related to EvaGreen plots. Numbers 1, 2 and 3 in the plots are related to heterozygote, mutant and normal samples respectively. Figure 2 (A-C) are related to melting, normalized and difference plot of SYBR Green I. Figure 2 (D-F) are related to melting, normalized and difference plot of EvaGreen. Our results showed that the developed method with SYBR Green I and EvaGreen is applicable for rs7216389 locus genotyping. Furthermore, the comparison of EvaGreen and SYBR Green I results showed that SYBR Green I can be used for rs7216389 locus genotyping

Table 1. Evaluation of stability and reproducibility of the results in our homemade SYBR Green-I real-time PCR master mix in different time frames after thawing

Gene	DNA concentration	Week 1		Week 2		Week 3		Week 4		Week 5		Week 6	
		C _t	T _m	C _t	T _m	C _t	T _m	C _t	T _m	C _t	T _m	C _t	T _m
ORMDL3	10 ng	25.19	83.240	24.96	83.353	25.33	83.172	25.46	83.195	25.31	83.121	25.96	83.087
		25.22	83.243	25.11	83.362	25.29	83.181	25.55	83.204	25.42	83.117	25.87	83.078
		25.16	83.239	25.12	83.358	25.31	83.169	25.58	83.201	25.38	83.120	25.91	83.082

ple in different time frames in triplicate format. The C_t and T_m values of different assays have been presented in table-1. After thawing, 2× master mix was kept at 4°C and protected from light during the assays. According to the C_t and T_m values, degradation of SYBR Green I in the mentioned condition started at week 7 but previous C_t and T_m values were very close to week 0 in different time frames. Moreover, standard deviations in triplicate reactions were acceptable as well (Table 1).

In the next step, the efficacy of our homemade master mix for discrimination of different genotypes was checked. Sixty samples, in which their genotypes had been determined by PCR-RFLP in previous steps, were selected for HRM analysis experiments. After PCR amplification, HRM analysis was performed by the mentioned program, which was followed by normalization of the data. In addition, results were analyzed in the normalized fluorescence versus temperature plot. Three samples with different genotypes were assigned to three genotypes as references in each run. Genotypes of other samples were determined by the software automatically with confidence more than 98.2% (Figure 2). Next, the genotypes of samples were compared by two different methods (HRM analysis and PCR-RFLP). Our results showed that these two methods confirm each other and accuracy of the developed SYBR Green I based HRM method for rs7216389 locus genotyping is 100%.

SYBR Green I and EvaGreen® comparison for HRM analysis

Finally, our homemade SYBR Green I 2× master mix was compared with Solis Bio-dyne qPCR mix (a ready-to-use and commer-

cial master mix for HRM analysis with EvaGreen® as its intercalating dye) in parallel and same condition. In the melt curve plot (d Fluorescence/d Temperature) of EvaGreen®, for mutant and normal genotypes, two peaks at 86.800°C and 87.216°C were seen, respectively and for heterozygote samples two peaks at 86.120°C and 87.056°C were observed. In the melt curve plot of SYBR Green I, for mutant a peak at 84.615°C, for normal a peak at 84.968°C and for heterozygote genotype, two peaks at 84.231°C and 84.88°C were observed (Figure 2A).

Discussion

Completion of human genome project provided a unique opportunity for a better understanding of molecular mechanism of different infectious and non-infectious diseases and their characteristics in populations. Furthermore, it is very valuable for developing more advanced diagnostic technologies, new drug discoveries and modern treatment approaches. According to these molecular data, finding the correlation between different SNPs and specific diseases and phenotypes can be feasible. It has been revealed that some abnormalities are ethnic dependent. Therefore, numerous studies have been carried out to investigate the correlation of specific abnormalities with genetic factors and vice versa in different nations. To perform these types of studies, it is necessary to design a high-throughput, efficient, accurate, fast and economic method to scan the target locus in population. Nowadays different methods are in practice to study and find the correlation between the known and unknown genetic parameters with disease manifestations and pathogenesis which one of

them is HRM method. HRM method was discovered and developed by Idaho Technology and the University of Utah in 2003²⁴. This is a rapid, accurate, powerful and economic method and has numerous applications in genome-linkage and epigenetic studies^{14,15,25-27}.

Asthma is a common immunologic and allergic disorder. New findings have revealed that genetic factors are determinants of asthma susceptibility and severity in children that one of them is rs7216389 locus. It has been reported that rs7216389-T mutation is correlated with onset age and severity of childhood asthma^{5,12,27}. This correlation has been studied in some nations by different molecular techniques, but no study had been performed in West Asian countries. In this study, we developed an HRM method to investigate the correlation of rs7216389-T mutation and childhood asthma in Iranian children for the first time.

Generally, second and third generations of intercalating dyes such as SYTO9[®], LCGreen[®] and EvaGreen[®] are used in HRM studies. However, these dyes are more expensive than SYBR Green I, which is the most commonly used intercalating dye for quantitative real-time PCR experiments. SYBR Green I is a non-saturating intercalating dye; thus, it is not used for HRM analysis. But recently, it has been reported in few studies that SYBR Green I has the acceptable efficiency for HRM analysis in comparison to second and third generations of intercalating dyes. Therefore, we intended to develop a SYBR Green I based HRM analysis to investigate the correlation of rs7216389-T mutation and childhood asthma in Iranian asthmatic children.

Our study revealed that the developed method based on SYBR Green I is applicable for high-throughput scanning of rs7216389-T mutation. Our experiments on results reproducibility and master mix stability in different time frames indicated that the results are reproducible and variations of Ct values are in acceptable range and hence, the developed method is accurate. Furthermore, our results on allelic discrimination of rs7216389 locus

by SYBR Green I have shown that it is efficient and accurate for this locus genotyping.

Additionally, the efficiency of SYBR Green I and EvaGreen[®] (Solis Biodyne qPCR mix) for rs7217389 locus genotyping was compared in this study. Our data demonstrated that SYBR Green I results are acceptable and it has a good efficacy for differentiation of three different genotypes of rs7216389 locus as compared to EvaGreen[®]. T_m values of three genotypes were different with Solis Biodyne qPCR mix and our homemade real-time PCR master mix and this dissimilarity seems to be related to the difference of master mix ingredients and their concentration (such as MgCl₂, DMSO and PCR buffer components) that affects the melting behavior of amplicons.

According to our findings, it can be deduced that the developed methods based on SYBR Green I can be used for rs7216389 locus genotyping and it is a rapid, economic, accurate and high-throughput method for investigation of rs7216389 locus and childhood asthma correlation in different geographical populations.

However, it should be mentioned that HRM assay development with SYBR Green-I is more complicated than HRM development with second and third generations of intercalating dyes which is related to their specifications. Furthermore, the reproducibility of SYBR Green-I based HRM assays should be checked in different laboratories. Another limitation of SYBR Green-I based HRM analysis is that SYBR Green-I efficacy is not similar for all SNPs, especially for those which are located in GC rich regions. Furthermore, SYBR Green-I is not suitable to perform gene scanning in compare the good efficacy of second and third generation of intercalating dyes.

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