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New Assay of Detecting Common Mutation Causing Sanjad-Sakati Syndrome Using Real-Time Fluorescence PCR and Melting Curve Analysis

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Authors' contributions

This work was carried out in collaboration between all authors. Author MHA conceived of the study, and participated in its design and coordination and drafted the manuscript. Author HA carried out all technical aspects. Author FI helped conceive the study and drafted the manuscript. All authors read and approved the final manuscript.

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Short Communication

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ABSTRACT

Sanjad-Sakati Syndrome (SSS) is an autosomal recessive disorder reported mainly in Middle Eastern populations. The mutation c.155_166del in exon 3 of the TBCE gene is the most common cause of SSS in the population. Each double stranded DNA product has a specific melting temperature (Tm) at which50% of the DNA is single stranded. By using melting curve analysis we present a new assay for rapid genotyping of SSS (less than one hour) to be used in prenatal and preimplantation genetic diagnosis (PGD) settings.

Keywords: Melting curve analysis; real time PCR; sanjad sakati syndrome; TBCE mutation.

1. INTRODUCTION

Sanjad-Sakati Syndrome (SSS)(OMIM 241410) is an autosomal recessive disorder reported almost exclusively in Middle Eastern populations [1]. Symptoms of SSS are congenital hypoparathyroidism, mental retardation, facial dysmorphism and extreme growth failure. The

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frequency of SSS in Arab population estimated to be 1 in 40,000-100,000 live births [2]. This entity was originally described by Sanjad [3] et al. in 1988 and 1991 as a new syndrome. In 1999 the disorder was mapped to chromosome 1 as similar to autosomal recessive Kenny-Caffey syndrome [4]. In 2002 the causative gene of SSS was identified [1]. The syndrome is caused by alterations in the tubulin-specific chaperone E (TBCE) gene in chromosome 1q43-44.The gene TBCE encodes one of several chaperon proteins required for the proper folding of ά-tubulin subunits and the formation of ά-β-tubulin heterodimers.

According to the Human Gene Mutation Database (HGMD®) [http://www.hgmd.org/], there are three reported mutations in the TBCE gene (NM_001287801). These are c.66delAG (HGMD;CD022747)[1], c.155_166delgccacgaaggga (HGMD; CD022748)[1], and c.1113T>A; p.C371X (HGMD; CM022675)[1]. Through years of work experience at the Saudi Diagnostics Laboratory in addition to other reports [1,2,5] we found that the mutation c.155_166del in the second coding exon (exon 3) of the TBCE geneis the most common cause of SSS in Arabian Peninsula. The mutation resulted from a 12bp deletion of nucleotides 155_166 in the TBCE gene.

High resolution Melting Curve Analysis (hrMCA) in conjunction with real-time PCR was introduced in 1997 [6]. Using the LC green dye, melting curve analysis offers the opportunity to identify and characterize PCR products with respect to their melting behavior [7]. Each double stranded DNA product has a specific melting temperature (Tm) at which 50% of the DNA is single stranded. Continuous monitoring of the denaturation process detects rapid loss of fluorescence near the melting point Tm and results in single, sharp melting peaks when plotted as the first negative derivative of fluorescence versus temperature (-dF/dT).

As the Tm of a fragment is a function of the fragment length and the G+C content it can be used to distinguish mutant products from wild type ones.

Techniques such as PCR sequencing and Fluorescent PCR were used to diagnose for SSS in prenatal and preimplantation genetic diagnosis (PGD) laboratories [2]. To reduce turnaround time from days to hours in prenatal and PGD settings, we introduce here anew hrMCA technique that would detect the common mutation c.155_166del that causes SSS.

2. MATERIALS AND METHODS

Genomic DNA from fifty two samples representing 26 families (26 whole bloods, 3 amniotic fluid and 23 chorionic villi) was extracted using Gentra DNA isolation Kit (QIAGEN, USA). These samples were diagnosed previously in the period of 2011-2013 in the prenatal section of the Saudi Diagnostics Laboratory at King Faisal Specialist Hospital and Research Centre, Riyadh, Saudi Arabia. These samples were used to compare and validate a new real-time PCR associated with high resolution melting curve analysis (hr-MCA) assay versus gold standard sequencing based PCR. Sample collection and enrollment in this study was approved by our institutional review board (IRB) according to guidelines of the declaration of Helsinki.

Oligonucleotide primers for PCR amplification of genomic DNA were designed using Primer 3 software (http://frodo.wi.mit.edu/) and synthesized by Metabion International AG (Munich, Germany).

For real-time PCR, following primers Fw-gagagaggaaagcatgatgg and Rv-tcataatgaaaagttacctgcattt were used to amplify 70bp amplicon for normal allele and 58bp for affected allele. A total of 10µl reaction mix contain: 10ng genomic DNA, 1X PCR Buffer containing 3mM $MgCl_2$, 200 µM each dNTP, 500 ng/µl BSA, 5µM LC green dye,0.5 units Ampli Taq Gold DNA poly merase and 0.5µM of each primer. The amplification was carried out on the LightCycler® system (Roche Diagnostics GmbH, Germany) with programmed transitions of 20ºC/s using the following conditions: one cycle of 95ºC for 10 minutes; 40 cycles of 95ºC for 10 seconds, 58ºC for 10 seconds, and 72ºC for 15 seconds.

Melting curve analysis was performed on the LightCycler®. After amplification, the samples were heated momentarily to 95ºC and cooled to 40ºC then melted from 65ºC to 85ºC raising at 0.05ºC per second. After DNA extraction; results were available in less than one hour.

For sequencing based PCR, following primers were used Fw-tcctccccaagtggtatctg and Rv-cagcagcaattaagccacaa. PCR was performed in a final volume of 25 µl containing approximately 20ng of genomic DNA and Qiagen (Manchester,

UK) master mix kit (including 1X PCR buffer, 100 mmol l^{-1}dNTP and 1U per reaction HotStarTaq polymerase) and 0.5 mmol l^{-1} primer. PCR products were treated with the Agencourt AMPure PCR purification system (Agencourt Bioscience Corporation, Beverly, MA, USA). PCR products were sequenced using BigDye Terminator kit version 3.1 (PE Applied Biosystems, Beverly, MA, USA) as described by the manufacturer. Sequences were analyzed using Mutation Surveyor software Version 3.24 (Soft Genetics LLC, State College, PA, USA).

3. RESULTS

Fifty two samples were genotyped in parallel for the common c.155_166del mutation in the TBCE gene using sequencing based PCR and new introduced assay melting curve analysis. Results from both techniques were identical and complete concordance was achieved by both assays (Table 1). The real-time based technique (hrMCA) was able to discriminate between wild-type DNA, Heterozygous (carrier), and homozygous (affected) samples (Fig. 1). The melting temperature for the mutant allele in hr MCA was 76ºC and 80ºC for the normal allele. To proof the validity of new assay for high throughput results; same experiment for hr MCA was used to run samples on ABI7900 (Applied Biosystems, USA) instrument (instead of LightCycler® by using LC green and Cyber green dyes. Results obtained by Cyber green were better than LC green results (Data not shown).

4. DISCUSSION

In this study two genotyping methods were compared independently using DNA samples representing all possible genotypes of the c.155_166del mutation. We have developed and validated a new real-time PCR genotyping assay, and performed a comparison against the direct sequencing reference methods.

LightCycler technology combines rapid-cycle polymerase chain reaction with real-time fluorescent monitoring and melting curve analysis. In this report we describe fast, sensitive and accurate tool to identify c.155_166del mutation.

The method is fast, cheap and accurate making it the best over other techniques. Although direct sequencing is a golden tool for mutation detection; it is still costly and time consuming. hrMCA method would be more useful for detecting thec.155_166del mutation in preimplantation genetic diagnosis (PGD) and prenatal diagnosis over other techniques described by others [2].

Table 1. Comparison of results obtained by two methods; sequencing and high-resolution melting curve analysis (hrMCA)

	Negative			Heterozygous			Homozygous		
	WB	**CVS**	**AF**	**WB**	**CVS**	**AF**	**WB**	**CVS**	**AF**
Sequencing results	**0**	**7**	**1**	**26**	**11**	**2**	**0**	**5**	**0**
hrMCA results	**0**	**7**	**1**	**26**	**11**	**2**	**0**	**5**	**0**

WB: whole blood, CVS: chorionic villus sample, AF: amniotic fluid

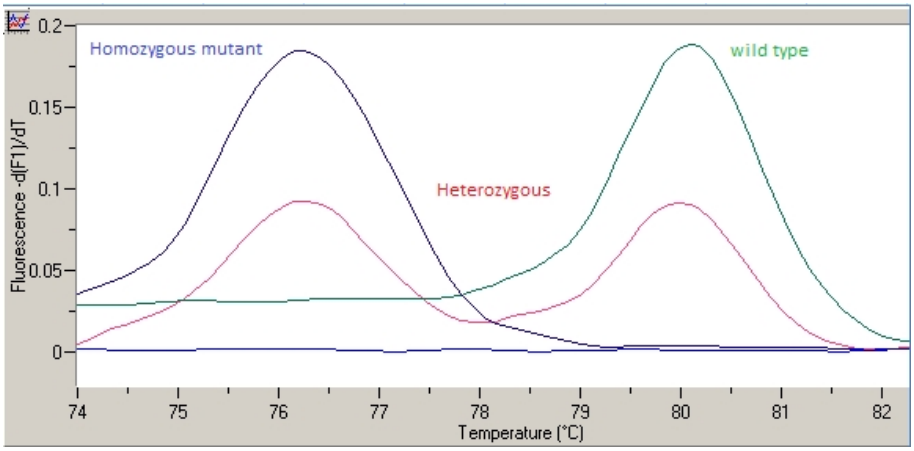

Fig. 1. Screenshot of melting curve analysis of c.155_166del mutation in the *TBCE* gene

The purple curve shows homozygous mutant and green curve shows wild type genotype, while red curve shows heterozygous. Blue line represents the buffer

In a homogeneous population due to tribal structure and high consanguinity rate such as an Arab population; carrier detection would be a useful approach to control genetic diseases. By using ABI 7900 instrument and Cyber green instead of LC green, this method can be used to rapidly detect common SSS mutation from blood samples or dried blood spots. Cyber green results were better than LC green; this is may be due to that fluoresce of Cyber green is stronger than LC green and due to the fluorescence transmission of polypropylene plates is not efficient like LightCycler capillaries [8]. The main limitation of this assay and hrMCA in general is that the technique cannot be one hundred percent accurate. Any alteration at the same region may affect detection of the c.155_166del mutation. Reverse primer resides on cosmic mutation (COSM1668579; c.182G>A) towards the 3' end. This variant c.182G>A was not observed in all 104 alleles by direct sequencing. In other hand the melting temperatures for affected and normal alleles depends also on reaction composition. Melting temperatures for normal and affected alleles may vary (+/-≤0.5ºC) in each run depending on the reaction components. Nevertheless, 4ºC differences in melting temperatures between wild type and mutant are evident to discriminate between genotypes. To overcome such limitations we recommend always running positive and negative controls in each run. Detection of mutations by real-time fluorescence PCR and melting curve analysis would be useful approach for known common mutations. In conclusion this assay may be perfectly applied as a diagnostic test or large scale method to facilitate carrier screening programs.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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