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A Novel Multiplex Real-Time PCR Assay Using TaqMan MGB Probes for Rapid Detection of Trisomy 21

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***Abstract*—**Cytogenetic analysis still remains the gold standard method for prenatal diagnosis of trisomy 21 (Down syndrome, DS). Nevertheless, the conventional cytogenetic analysis needs live cultured cells and is too time-consuming for clinical application. In contrast, molecular methods such as FISH, QF-PCR, MLPA and quantitative Real-time PCR are rapid assays with results available in 24h. In the present study, we have successfully used a novel MGB TaqMan probe-based real time PCR assay for rapid diagnosis of trisomy 21 status in Down syndrome samples. We have also compared the results of this molecular method with corresponding results obtained by the cytogenetic analysis. Blood samples obtained from DS patients (n=25) and normal controls (n=20) were tested by quantitative Real-time PCR in parallel to standard G-banding analysis. Genomic DNA was extracted from peripheral blood lymphocytes. A high precision TaqMan probe quantitative Real-time PCR assay was developed to determine the gene dosage of DSCAM (target gene on 21q22.2) relative to PMP22 (reference gene on 17p11.2). The DSCAM/PMP22 ratio was calculated according to the formula; ratio=$2^{-\Delta\Delta C_T}$. The quantitative Real-time PCR was able to distinguish between trisomy 21 samples and normal controls with the gene ratios of 1.49±0.13 and 1.03±0.04 respectively (p value <0.001). These results represent the presence of 3 copies of target gene in DS samples Vs 2 copies in normal controls. The results of quantitative Real-time PCR were in complete agreement with results of cytogenetic analysis. This study confirms previous reports regarding successful implementation of quantitative Real-time PCR for detection of trisomy 21. However, the assay has been improved by using MGB probes and more accurate data analysis. This assay, in particular, when performed in combination with another molecular assay such as QF-PCR or MLPA, can be used as a reliable technique for rapid prenatal diagnosis of trisomy 21.

***Keywords*—**Trisomy 21, Real-time PCR, MGB-TaqMan Probes, Gene Dosage.

I. Introduction

DOWN syndrome (DS), the most common fetal aneuploidy, is caused by an extra copy of chromosome 21 (trisomy 21) affecting 1 in 700-1000 live births [1]. Detection of this chromosomal aberration is one of the major focuses of prenatal diagnostics. Since the 1970's, chromosomal cytogenetic analysis using Gimsa banding of metaphase preparation and Fluorescent In Situ Hybridization (FISH) (1980's) are used for direct prenatal diagnosis of DS [2]. Currently, these methods are still considered as gold standard techniques for prenatal diagnosis of DS. The cytogenetic analysis requires intact, living cells that may only be obtained by an invasive procedure such as chorionic villous sampling (CVS) and amniocentesis. The cytogenetic assay takes 2 weeks to be completed that might be too long for high risk pregnant women with high gestational age.

By the development of polymerase chain reaction and human genome sequencing, molecular genetics methods have been implemented for the detection of DS. Quantitative fluorescent polymerase chain reaction (QF-PCR) has been introduced as a reliable molecular method for widespread clinical application. This assay, which is based on analysis of short tandem repeats (STRs) polymorphic loci on chromosome 21, should be definitely designed for a target population. Commercial QF-PCR kits e.g., Aneufast™ are available for simultaneous assessment of fetal samples for most common chromosomal abnormalities including chromosome 13, 18, 21, X, and Y [3-6]. However, the need of capillary electrophoresis equipment on a DNA sequencing system for STR fragments analysis has limited its use. A more recent technique, Multiplex Ligation-dependent Probe Amplification (MLPA) has been also suggested as an alternative rapid assay for detection of major chromosomal abnormalities [7]. The advent of real-time PCR has revolutionized the measurement of nucleic acid copy numbers in recent years. Quantitative Real-time PCR has been successfully used to determine chromosome 21 specific gene dosage in Down syndrome [8]. Here, we describe a novel multiplex TaqMan probe-based real time PCR assay for rapid diagnosis of trisomy 21.

II. Material and Methods

A. Subjects

Genomic DNA were extracted from peripheral blood lymphocytes of Down syndrome cases (n=25) referred to Pasteur institute of Iran. A written consent was obtained from their parents according to the Pasteur Institute ethical committee guidelines. Twenty normal control samples were also included in the study. 2ml of peripheral blood was used for DNA extraction according to standard salting out protocol.

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The concentration and quality of the DNA was measured using NanoDrop® ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) at 260 and 280 nm. DNA samples with the A260 / A280 ratios of more than 1.7 were selected for quantitative analysis. Sample aliquots were stored at −20 °C and fresh working solutions (10 ng/μL) were prepared immediately before each experiment.

TABLE I
CHARACTERISTICS OF THE PRIMERS AND MGB-TAQMAN PROBES USED IN THE REAL-TIME PCR ASSAYS

Name	Sequence	Reporter dye	Amp. size
DSCAMF	CCGGGCAGTCTAATTCCAGAAC	None	100
DSCAMR	AGTATGTGCACTCAGAAACCAGCTG	None	
DSCAMP	TGCACTTGACTTCCAGG	FAM-MGB	
PMP22F	GGAGGAGAGAAGGCTTGAATGC	None	103
PMP22R	GTTCCACATGCACACAGAAACG	None	
PMP22P	TGTCTAAGGTTGAGTTCAT	VIC-MGB	

A. Quantitative Real-time PCR

DSCAM (Down syndrome cell adhesion molecule) gene located on Down syndrome critical region (DSCR) of chromosome 21 (21q22.2) and PMP22 (Peripheral myelin protein) gene located on 17p11.2-12 were selected as target and reference genes, respectively. Primers and probes were designed using Primer Express software Ver.3.0 (Applied Biosystem, Foster City, CA). The selected primers and probes underwent an extensive search in the NCBI/BLAST databases to avoid any significant homology with other known nucleotide sequences. DSCAM probe was synthesized with the reporter dye 6-carboxyfluorescein (FAM) covalently linked to the 5' end (Applied Biosystems, UK). PMP22 specific probe was conjugated at the 5' end with the reporter dye VIC. The probes were minor groove binder (MGB)-DNA probes with enhanced hybridization to the target DNA at higher Tm temperature compared to the conventional TaqMan probes .The characteristics of the primers and the probes used in this study are summarized in table 1. Real time PCR reaction was performed as described previously [9], with minor modifications to optimize multiplex reactions. The PCR master mix (Applied Biosystems, UK) was optimized for TaqMan probe based reactions and contained AmpliTaq Gold DNA Polymerase, AmpErase UNG, dNTPs with dUTP, Passive Reference, and optimized buffer components. PCR was carried out in optical grade 96-well plates (MicroAmp, Applied Biosystems, Singapore) at reaction volume of 25 μl including 12.5 μl TaqMan universal master mix (Applied Biosystems, UK), 400 nM of each primer, 200 nm of the specific probes and 10 ng of genomic DNA. Reaction mixture was kept at 50˚C for 2 min (for optimal AmpErase UNG activity), at 95˚C for 10 min (for deactivation of AmpErase UNG and activation of AmpliTaq Gold), followed by 40 cycles including 95˚C for 15 seconds and 60˚C for 1 min (for probe/primer hybridization and extension). Each real-time PCR run included three normal control samples in triplicate. To calculate the PCR efficiency of each gene, serially diluted concentrations of template DNA were prepared. Simultaneous real-time PCR was run for DSCAM (target) and PMP22 (reference) genes using concentrations of 100, 50, 25, 12.5, and 6.25 ng DNA per reaction. All the reactions were run in triplicates for drawing standard curves and repeated, at least twice, for gene dosage analysis experiments.

B. Data analysis

Quantitative analysis was performed by the measurement of C_T values during the exponential phase of amplification as described previously [9]. The C_T parameter was defined as the cycle number at which the amplification curve crossed a fixed threshold line. In each assay, mC_T was the mean C_T value of triplicate amplifications. Relative quantity of DSCAM gene normalized to PMP22 gene was determined using comparative ΔC_T method. ΔC_T was calculated as the difference between the C_T values of DSCAM gene and the C_T value of PMP22 gene of the same sample. The data were analyzed using the formula: *Gene dosage ratio* $= 2^{-\Delta\Delta C_T}$, *where* $-\Delta\Delta C_T = [mC_{T\ DSCAM}$ *(normal sample)* $- mC_{T\ PMP22}$ *(normal sample)]* $- [mC_{T\ DSCAM}$ *(test sample)* $- mC_{T\ PMP22}$ *(test sample)]* [10]. Three normal samples were used as calibrator in each experiment and the gene dosage ratios were determined relative to the mean ΔC_T value of these samples. Data processing was performed using the ABI Prism 7300 Sequence Detection System and the SDS software Ver. 1.2.3 (Applied Biosystems, UK). Statistical analysis and graph preparation were performed using Microsoft® Excel 2007 software. The p value of < 0.05 was considered statistically significant for t-Test analysis.

III. RESULTS

A. Validation of real-time PCR assays

Validation experiments were performed to determine the amplification efficiencies of DSCAM and PMP22 genes. Briefly, the log concentration of serially diluted template DNA was plotted against the corresponding C_T values acquired for each gene. The slope of the trend lines were -3.23 and -3.30 for DSCAM and PMP22 genes, respectively (Figure 1). Acceptable range for this parameter is $-3.6 < \text{slope} < -3.1$, where slope of -3.3 corresponds to PCR efficiency of 100%. The reliability of the PCR reaction efficiencies was also assessed by plotting ΔC_T values ($C_{T\ DSCAM} - C_{T\ PMP22}$) against the log amount of input DNA. Through a wide range of template DNA input (6-100 ng), the absolute value of the trend line slopes for both genes were ≤ 0.1, which indicated the validity of the relative quantitative assay by $\Delta\Delta C_T$ method. Furthermore, gel electrophoresis analysis of the PCR products revealed a single band with expected size (100 bp for DSCAM, and 103 bp for PMP22).

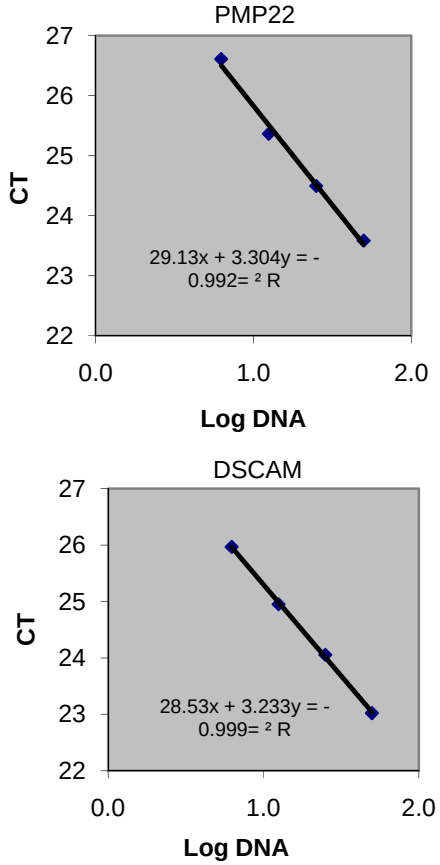

Fig. 1 Validation of real-time PCR assays for analysis of DSCAM and PMP22 gene dosage. Simultaneous quantitative real-time PCR was run for DSCAM (target) and PMP22 (reference) genes using various amounts (from 6 to 50 ng) of template DNA per reaction. C_T values were determined and plotted against log input DNA for both genes. The resulting best fit line equations were: *y=-3.30x+28.909*, and *y=-3.23x+29.135* for PMP22 and DSCAM genes, respectively. Best fit line slopes were approximately equal with high linear correlation (R^2 = *0.992 and 0.999*).

B. Discrimination between normal and trisomy 21 samples

G-banding analysis of the subject revealed the trisomy 21 status (47, xy +21) in all Down syndrome cases and normal karyotype in normal controls. As expected from validation experiments, the amplification curves of DSCAM and PMP22 genes crossed the threshold line at approximately the same point in normal samples (Figure 2). This was translated into ΔC_T values of 0-0.5 cycle with the mean value of 0.3±0.1 in data analysis. Whereas, in trisomy 21 samples, the difference between C_T value of DSCAM and PMP22 genes ranged from 0.7-1.1 cycle resulting in mean ΔC_T values of 0.8±0.2. Using the formula; *Gene dosage ratio* = $2^{-\Delta\Delta C_T}$, the DSCAM/PMP22 ratio was calculated for each sample. The mean ratio was determined in both groups as follows: 1.03±0.04 for normal samples, and 1.49±0.13 for trisomy 21 cases. After setting up the assay for known trisomy 21 and normal samples, a blind analysis were performed to further confirm the assay consistency. Every sample were labeled with an identification code and tested by the real time PCR assay in blind. All 25 trisomy samples were assigned as having high DSCAM/PMP22 ratio ranging from 1.32 to 1.79. Normal samples showed significantly different ratios (0.94-1.07, $p<0.001$). These results were in agreement to the results of cytogenetic analysis

IV. Discussion

A reliable and fast assay is needed for prenatal diagnosis of Down syndrome, in particular, in high risk pregnant women with high gestational age. Currently, prenatal diagnosis of this genetic anomaly mainly relies on the karyotyping of cultured fetal cells. The 2-week period needed for cell culture and subsequent analysis has proven to be associated with considerable parental anxiety and medical problems in those situations which require therapeutic intervention [2]. In the last decade, there have been major advances made in the screening and performing of prenatal diagnosis for Down syndrome. Although researchers have developed innovative molecular methods such as FISH, QF-PCR and MLPA for this purpose, some limitations still remain problematic [3]-[7]. QF-PCR for example, is based on STR polymorphism analysis and therefore may not be appropriate for every population.

In the present study, we developed and validated a multiplex quantitative Real-time PCR assay using MGB type TaqMan probes. The assay was able to successfully discriminate trisomy 21 samples from normal controls ($p<0.001$). Although, this is not the first attempt to develop a real-time PCR assay for the detection of trisomy 21 [1], [2], [11], it has some advantages with regard to the choice of the reference gene, the type of the probes used, and data analysis method. Yali Hu *et al.* have published the first paper regarding the application of Real-time PCR for this purpose [11]. They have used GAPDH gene, which has numerous pseudogenes (~400 copies) on human genome, as reference gene. This might cause assay errors due considerable difference between target and reference gene copy numbers. We have used PMP22 gene, which has no pseudogenes, to normalize DSCAM gene dosage. Any deletional mutation or gene copy variation of this gene will result in obvious phenotypical outcome so that normal individual would certainly have two intact copy of the gene.

More importantly, instead of using standard curve method to determine target/reference gene ratio [1], [11], we have implemented the $\Delta\Delta C_T$ method. This was achieved by optimizing PCR reactions with equal amplification efficiencies for both genes. Yang Hu *et al.* reported another real time assay in which the achieved gene dosage ratios for Down syndrome to normal subjects were quite far from ideal values (2.5 Vs theoretically expected ratio of 1.5). This could happen, at least in part, because of unequal PCR efficiencies. In our hands, the mean gene dosage ratio of trisomy 21 samples relative to normal samples was 1.49±0.13.

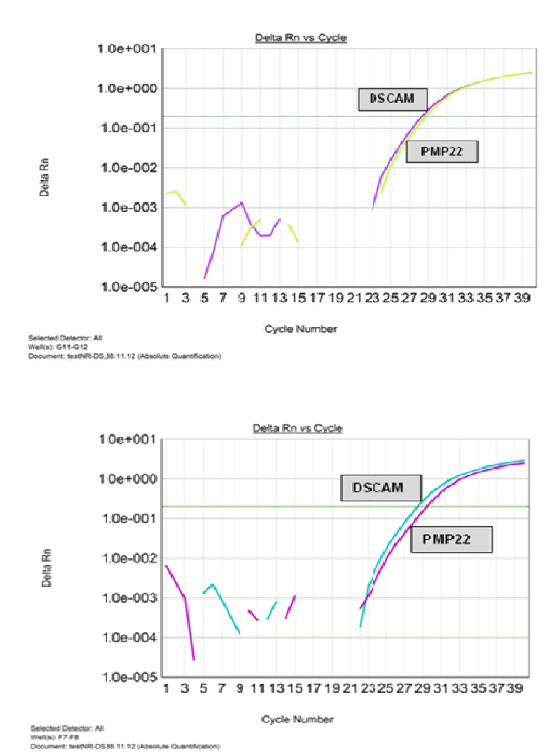

Fig. 2 Amplification curve analysis for DSCAM and PMP22 genes using Real time PCR assay. a) The amplification curves of both genes have crossed the threshold line almost at the points in normal sample (ΔC_T=0.2). **b)** As a result of the increased DSCAM gene copy number (3/2) in Down syndrome patient, the amplification curve of this gene has crossed the line about one cycle earlier than the PMP22 curve (ΔC_T=0.9).

The accuracy and reproducibility of a TaqMan probe based assay is mainly depended on the design and quality of the probes. Conventional TaqMan probes are generally dual-labeled with a fluorescent dye at 5' end and a quencher dye (mostly TAMRA) at 3' end. In order to keep the Tm of the probe high enough (8-10 C° higher than primers Tm), the probe length should be considerably longer than primers. This would result in high background fluorescence due to leakage of the fluorescent signal emitted by the reporter dye. To overcome this problem for a precise gene dosage analysis it is recommended to use MGB TaqMan probes with a special non fluorescent quencher which binds to the minor groove of the template DNA. These probes emit low levels of background fluorescent signal, and even single nucleotide mismatch would prevent their perfect hybridization to the template DNA reducing the possibility of non-specific signals. Yu-ning Zhu *et al.* have developed a real-time PCR assay with conventional TaqMan probes for diagnosis of trisomy 21 [2]. They tested the assay on a large number of samples and despite inter-assay ΔC_T variations due to small PCR efficiency differences, they were able to discriminate normal and Down syndrome group with minimal overlaps. We would assume that some of these inaccuracies are caused by slight non-specific fluorescent signals of conventional probes. These discrepancies might be avoided by using MGB probes and implementation of more restrict quality control plan. In conclusion, this study confirms previous reports regarding successful implementation of quantitative Real-time PCR for detection of trisomy 21. The assay has been improved by using a reference gene with stable genomic copy number and MGB-TaqMan probes as well. However, it is still a great challenge to use this PCR-based method to reveal difference as small as 1.5-fold quantitative differentiation. As long as validity of the assay for clinical diagnosis has yet to be evaluated, simultaneous FISH, QF-PCR or MLPA analysis could help to confirm the Real-time PCR results according to the best practice regulations in genetic laboratories [12].

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