Advanced Studies in Biology, Vol. 4, 2012, no. 1, 11 - 17

Application of Multiplex Real-Time PCR Assay Using TaqMan MGB Probes on Amniocyte Samples for Prenatal Diagnosis of Trisomy 21

Mehrdad Hashemi[1], Reza Mahdian[2], Maliheh Entezari[3] and Ahmadreza Kamyab[1]

1. Scientific Society of Cellular and Molecular
Centeral Commission of Scientific, Literary and Artistic Science
and Research branch, Islamic Azad University, Tehran, Iran
([*]corresponding author, e-mail: mhashemi@iautmu.ac.ir)

2. Biotechnology Research Center, Molecular Medicine Department
Pasteur Institute of Iran, Tehran, Iran

3. Department of Genetics, Islamic Azad University
Tehran medical Branch, Tehran, Iran

Abstract: 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. Therefore diagnosis and prevention of live-born affected fetus is a health care priority.We have successfully used a novel MGB TaqMan probe-based real time PCR assay for rapid diagnosis of trisomy 21. Amniocyte samples was obtained from 59 pregnant women who had the high risk criteria of having fetus with trisomy 21. Quality and quantity of DNA was measured by the optical absorbance DSCAM gene (target genes) and PMP22 (reference gene) were selected and specific primers and probes for these genes and were designed. The target/reference genes ratio was calculated using comparative cycle threshold method .The results of gene dosage analysis showed the target/reference genes ratio of 1.61 ± 0.09 and 1.03 ± 0.05 in trisomy 21 and normal sample for amniocyte samples respectively that showed statistically significant difference between two groups. Therefore, Real-Time PCR technique can be used as an accurate, rapid and sensitive method for prenatal diagnosis of trisomy 21.

Keywords: DOWN syndrome, Real time PCR, PMP22 gene

Introduction

Aneuploidy is the most common chromosomal aberration with clinical importance in the human being which exists in 3 to 4% of recognized pregnancies. Probability of occurrence these abnormalities increases with mother's age (1,2). 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 (3).

Cytogenetic analysis requires 15 to 25 days that might be too long for high risk pregnant women with high gestational age (4). 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 (5-7). QF-PCR for example, is based on STR polymorphism analysis and therefore may not be appropriate for every population (8).The advent of real-time PCR has revolutionized the measurement of nucleic acid copy numbers in recent years. (9).

In the present study, we have successfully used a novel MGB TaqMan probe-based real time PCR assay for rapid diagnosis of trisomy 21.

MATERIAL AND METHODS

Amniocyte samples were obtained from 59 pregnant women who had the high risk criteria of having fetus with trisomy 21. Informed written consent was obtained from Down's patients parents according to the guidelines of scientific research ethical committee of Pasteur Institute of Iran. Twenty normal control samples were also included in the study. Genomic DNA was extracted using QIAamp® DNA Micro Kit (QIAGEN, Germany).

DSCAM gene located on Down syndrome critical region (DSCR;21q21.3-21q22.12) of chromosome 21 were selected as target genes for the assays. Peripheral Myelin Protein (*PMP22*) gene located on chromosome 17 (17p11.2-12) was selected as reference gene. Specific primer and probe sets were designed using Primer Express® software v.3.0 (Applied Biosystems, Foster City, CA, USA).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.

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	

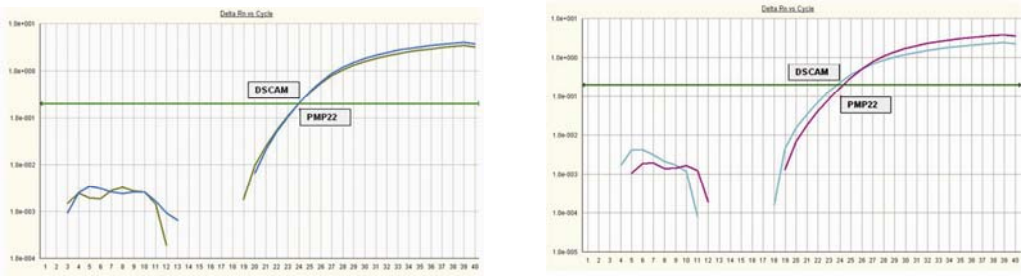
TABLE 1.Characteristics of the primers and MGB-TaqMan probes used in the Real-time PCR assays

The probe-based counterpart of the assay was set up using dual-labeled MGB probe. The multiplex reactions were performed in triplicate containing; 12.5µl of TaqMan® gene expression Master Mix (Applied Biosystems, Foster city, CA, USA), 0.5µl of *DSCAM* and *PMP22* TaqMan probes (200nM), 1 µl of forward and reverse primers (400nM) for target and reference genes, 5 µl of genomic DNA (20 ng) and 5 µl H_2O. Each experiment included unknown samples, trisomy 21 and normal controls.

Results

Quantitative analysis was performed by the measurement of C_T values during the exponential phase of amplification as described previously (10). Using the formula; *Gene dosage ratio* = $2^{-\Delta\Delta CT}$, the DSCAM/PMP22 ratio was calculated for each sample , *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)]*. Statistical analysis and graph preparation were performed using SPSS software. 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 (Fig 1). 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. The results of gene dosage analysis showed the target/reference genes ratio of 1.61 ± 0.09 and 1.03 ± 0.05 in trisomy 21 and normal sample for amniocyte samples respectively that showed statistically significant difference between two

groups.(p<0.001).These results were in agreement to the results of cytogenetic analysis.



a **b**

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).

DISCUSSION

By the development of polymerase chain reaction and human genome sequencing, molecular genetics methods have been implemented for the detection of DS. 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 (12-14).

In the present study, the multiplex quantitative Real-time PCR assay was able to successfully discriminate trisomy 21 on amniocyte samples from normal controls (p<0.001). Yali Hu *et al.* (15) have published the first paper regarding the application of Real-time PCR for this purpose. 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 number. 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 (16) we have implemented the $\Delta\Delta C_T$ method. This was achieved by optimizing PCR reactions with equal amplification efficiencies for both genes.

The method can be used as complementary to the karyotype method, and sometimes as an alternative to it in prenatal diagnosis. . 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 (12-15).

ACKNOWLEDGMENT

This study was supported by a research grant from Scientific Society of Cellular and Molecular, Centeral Commission of Scientific, Literary and Artistic Science and Research branch, Islamic Azad University, Tehran, Iran.

REFERENCES

1- S.E. Antonarakis,R. Lyle,E.T. Dermitzakis,A. Reymond and S. Deutsch, Chromosome 21 and Down syndrome: from genomics to pathophysiology, Nat Rev Genet, 5 (2004) 725-38.

2- C.L. Anderson and C.E.L. Brown,Fetal chromosomal abnormalities: antenatal screening and diagnosis, American Family Physician, 79 (2009) 117-23.

3- Y.H. Yang, M.S. Nam and E.S. Yang, Rapid prenatal diagnosis of trisomy 21 by real-time quantitative polymerase chain reaction with amplification of small tandem repeats and S100B in chromosome 21, Yonsei Med J, 46 (2005) 193-197.

4- Y.N. Zhu, S.M. Lu, J.F. You, B. Zhu and M.Y. Yu, Novel real-time PCR assay for rapid prenatal diagnosis of Down syndrome: a prospective study of 563 amniocytes,Clin Biochem, 42 (2009) 672-675.

5- B. Pertl, U. Weitgasser, S. Kopp, P.M. Kroisel, J. Sherlock and M Adinolfi, Rapid detection of trisomies 21 and 18 and sexing by quantitative fluorescent multiplex PCR, Hum Genet , 98 (1996) 55-59.

6- V. Cirigliano, M. Ejarque, M.P. Canadas, E. Lloveras, A. Plaja, M.M. Perez, C. Fuster and J. Egozcue, Clinical application of multiplex quantitative fluorescent polymerase chain reaction (QF-PCR) for the rapid prenatal detection of common chromosome aneuploidies, Mol Hum Reprod, 7 (2001) 1001-1006..

7- U. Nicolini, F. Lalatta, F. Natacci, C. Curcio and T.H. Bui, The introduction of QF-PCR in prenatal diagnosis of fetal aneuploidies: time for reconsideration, Hum Reprod Update,10 (2004) 541-548.

8- V. Cirigliano, M. Ejarque, M.P. Canadas, E. Lloveras, A. Plaja, M.M. Perez, C. Fuster and J . Egozcue, Clinical application of multiplex quantitative fluorescent polymerase chain reaction (QF-PCR) for the rapid prenatal detection of common chromosome aneuploidies, Mol Hum Reprod, 7 (2001) 1001–1006.

9- X. Sun, M. Yan, Y. Zhang, X. Zhou, C. Wang, F. Zheng and C. Xiong, Practical application of fluorescent quantitative PCR on Trisomy 21 in Chinese Han population, Mol Biol Rep, 33 (2006) 167-173.

10- M. Hashemi., M .Behrooz Aghdam, F. Hajari Taheri, R. Mahdian, A.R. Kamyab and Sh. Shahbazi, A Novel Multiplex Real-Time PCR Assay Using TaqMan MGB Probes for Rapid Detection of Trisomy 21,Middle-East Journal of Scientific Research,;4 (2010) 398-402.

11- J. Schouten ad RJ. Galjaard, MLPA for prenatal diagnosis of commonly occurring aneuploidies, Methods Mol Biol, 444 (2008) 111-122.

12- B. Zimmermann, L. Levett, W. Holzgreve and S. Hahn, Use of real-time polymerase chain reaction for the detection of fetal aneuploidies, Methods Mol Biol, 336 (2006) 83-100.

13- M. Hayat Nosaeid, R. Mahdian, S. Jamali, F. Maryami, S. Babashah, M. Karimipoor and S. Zeinali, Validation and comparison of two quantitative real-time PCR assays for direct detection of DMD/BMD carriers, Clin Biochem, 42 (2009) 1291-1299.

14- K.J. Livak and T.D. Schmittgen, Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method, Methods, 25 (2001) 402-408.

15- Y. Hu, M. Zheng, Z. Xu, X. Wang and H. Cui, Quantitative real-time PCR technique for rapid prenatal diagnosis of Down syndrome, Prenat Diagn, 24 (2004) 704-707.

16- E. Dequeker, S. Ramsden, W.W. Grody, T.T. Stenzel and D.E. Barton, Quality control in molecular genetic testing,Nat Rev Genet, 2 (2001) 717-723.

Received: October, 2011