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Brief communication (Original)

Rapid diagnosis of trisomy 21 by relative gene copy using real-time quantitative polymerase chain reaction

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Background: Trisomy 21 or Down syndrome (DS) is the most common aneuploidy disorder. Fetal karyotypic analysis remains the criterion standard for prenatal diagnosis of DS, although the method is time consuming and requires skilled personnel. Real-time quantitative polymerase chain reaction (qPCR) can be used to determine a difference in the amount of gene copy by calculation of the difference between the cycle threshold (ΔC_T) of a tested gene and a reference gene.
Objectives: To develop a rapid qPCR diagnostic method for trisomy 21.
Methods: Ten DS patients with the known karyotype of trisomy 21 were enrolled. Their parents were included as controls. *D21S11* locus on chromosome 21 and SM locus on chromosome 16 from each subject were amplified by qPCR. The *D21S11*/SM ΔCT and $2^{-\Delta\Delta CT}$ values were compared between DS patients and their parents.
Results: The *D21S11*/SM ΔC_T values of the DS patients were higher than their respective controls except for one family. The mean $2^{-\Delta\Delta CT}$ value between patients and mothers was 1.88 ± 0.95 (95% CI 1.20–2.56), and between fathers and mothers as controls was 1.06 ± 0.68 (95% CI 0.58–1.54).
Conclusion: The diagnostic method of trisomy 21 by using qPCR is feasible, although false negative results may occur. Using more index genes is recommended to increase the sensitivity and specificity.

Keywords: Down syndrome, gene copy difference, prenatal diagnosis, real-time quantitative polymerase chain reaction, trisomy 21

Down syndrome (DS) is the most common genetic condition occurring approximately in one of 800 live births [1]. Patients with DS have cognitive impairment and complications from anomalies of several organ systems [2, 3]. Trisomy 21 is the most common abnormal karyotype found in 95% of patients [4].

Prenatal diagnosis is generally offered for pregnant women who are at high risk of having an offspring with DS [5]. Until recently, a definite prenatal diagnosis of DS is usually made by a conventional cytogenetic analysis of fetal-derived cells from chorionic villi or amniotic fluid. Although the method yields highly accurate results and also can detect other numerical chromosomal aberrations, it requires a cell culture process and therefore is laborious and time-consuming.

Several methods in molecular genetics, such as interphase fluorescent in situ hybridization (FISH) and quantitative fluorescence polymerase chain reaction (QF-PCR) have been developed to overcome the disadvantages of the cytogenetic analysis [6-10]. Because these methods do not require a cell culture, they provide a more rapid diagnosis. The methods have been applied for prenatal diagnosis of common chromosomal disorders including trisomy 13, 18, and 21. Recently, chromosomal microarray analysis has also been reported [11]. Although interphase FISH, QF-PCR, and chromosomal microarray analysis provide shorter turnaround laboratory time than conventional cytogenetic analysis, the methods are not commonly available and their costs are high.

Real-time quantitative PCR (qPCR) has been used to detect an alteration in the gene copy number, by using the difference in cycle threshold (ΔC_T). C_T is a cycle number of PCR required before the amplified product is detected. A lower C_T number implies that there are more DNA templates at the beginning of the reaction. ΔCT between an amplification of two genes from the same DNA templates in the same reaction indicates that the copy numbers of the genes are different.

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Zimmermann et al. developed a qPCR method to diagnose trisomy 21 by coamplification of an amyloid gene on chromosome 21 and a glyceraldehydes 3-phosphate dehydrogenase (GADPH) gene on chromosome 12 as a control. The method could correctly diagnose nine out of ten cases of trisomy 21 and nine out of eleven cases with a normal karyotype [12]. There are later successful reports of using qPCR for diagnosis of trisomy 21 [13, 14]. However, one study shows conflicting results [15].

In this study, we aimed to develop a qPCR method for a diagnosis of trisomy 21. C_T was used to estimate the relative amount of the short tandem repeat (STR) locus *D21S11* on chromosome 21 and the DNA fragment within an α-globin gene cluster (designated SM). The SM fragment is located within an α-globin gene cluster, approximately 10 kilobases 5′ to α-globin 2 gene (*HBA2*). The DNA fragment is used as a reference marker because of its good consistency for amplification by PCR in our laboratory and that it is not in the region that is deleted in common α-thalassemia. We used ΔC_T to indicate a difference between the cycle threshold (C_T) of the STR marker *D21S11* and the SM segment. The relative amount of *D21S11*/SM is calculated by using the $2^{-\Delta\Delta CT}$ method ($\Delta\Delta C_T$ = ΔC_T sample–ΔC_T control) as described by Livak and Schmittgen [16]. Because changes of the C_T are associated with exponential change of the amplified PCR product, it is suggested that the $2^{-\square\square CT}$ value may be used to determine the relative amount of a gene compared to a reference gene. The value in trisomy 21 is postulated to be 1.5 while in a normal sample is 1.0.

Materials and methods

Our institutional ethics committee approved the study protocol. Ten children who attended the Genetic clinic at Chiang Mai University Hospital, Chiang Mai, Thailand, who had trisomy 21 as previously confirmed by conventional cytogenetic analysis, were included. Their parents were included as controls. Informed consent was obtained from the parents.

Detailed history taking and physical examination of all parents was performed to exclude chromosome disorders. Four milliliters of blood sample in EDTA was collected from each subject. Genomic DNA was extracted from leukocytes by using a QIAamp DNA Blood Mini kit (Qiagen, Hilden, Germany) as per the manufacturer's protocols.

For a marker on chromosome 21, *D21S11* locus (GenBank: M84567.1) was amplified by : forward primer (5′>3′) CCCCAAGTGAATTGCCTTCT and reverse primer (5′>3′) AGTCAATGTTCTCCAGAG ACAGAC. The primers for a marker on chromosome 16, SM fragment (GenBank: NG_000006.1, positions 23261–23355) were: forward primer (5′>3′) CAGG CTGCGATGAGAACATA and reverse primer (5′>3′) CTAGGCAGGAAAGCGTCTTG. The PCR mixture (25 μL) contained 5 μL DNA, 0.3 μmol/L of each primer, 0.2 mmol/L DNTPs, 1.25 Units of Platinum Taq DNA polymerase (Invitrogen, Brazil), 1.5 mmol/L of $MgCl_2$, and 1.5 μmol/L of SYTO9 dye, in 1× PCR buffer. The PCR cycles consisted of a 2 minutes initial denaturation followed by 44 cycles of 95°C for 15 seconds, 60°C for 15 seconds, and 72°C for 15 seconds on a Bio-Rad Real time thermal cycler CFX96 (Bio-Rad Laboratories, Hercules, CA, USA). The PCR protocol was modified from a method previously described by Pornprasert et al. [17]. The reactions were performed in duplicate and the C_T value of each PCR was measured automatically. The average C_T value was used for calculation. Standard curves for D21S11 and SM were established by using serial dilutions of genomic DNA.

For statistical analysis, we discriminated the specimens into two groups, which were patients and controls (fathers and mothers of patients). The ΔC_T was calculated from the difference between the C_T values of *D21S11* and SM (*D21S11* C_T–SM C_T) in each individual. Then $2^{-\Delta\Delta CT}$ values were calculated between patients and mothers, and between fathers and mothers as controls. A paired *t* test was used for comparison of ΔC_T and $2^{-\Delta\Delta CT}$ values between patients and their parents. $P < 0.05$ was considered statistically significant.

Results

The standard curves for *D21S11* and SM were shown in **Figure 1**. The PCR amplification efficiencies were 92.1% for *D21S11* and 93.7% for SM. There was a high linear correlation between the C_T and log value of the DNA concentration.

The calculated *D21S11*/SM ΔC_T values of each individual and $2^{-\Delta\Delta CT}$ values between the patients and their parents were analyzed with SPSS Version 12. The result showed ΔC_T from the DS group were higher than the control group except for one family. The mean ΔC_T from DS group, father and mother were 4.21 ± 0.61, 3.37 ± 0.49, and 3.46 ± 0.77 respectively.

The differences were significant both between the DS and father group (95% CI 0.29–1.38, $P = 0.007$) and between the DS and mother group (95% CI 0.24-1.27, $P = 0.009$).

The mean $2^{-\Delta\Delta CT}$ values between patients and mothers was 1.88 ± 0.95 (95% CI 1.20-2.56), and between fathers and mothers as controls was 1.06 ± 0.68 (95% CI 0.58-1.54).

Discussion

Rapid diagnosis of DS is needed in the setting of prenatal diagnosis, especially when couples present later in the second trimester. In this study, a qPCR to detect relative gene copy ratio of *D21S11* STR locus on chromosome 21 and SM fragment on chromosome 16 as a control was established. Theoretically, by using the $2^{-\Delta\Delta CT}$ values of *D21S11*/SM gene, which reflects the relative gene copy of *D21S11*, the relative copy number of D21S11 in trisomy 21 should be 1.5. From this study, the $2^{-\Delta\Delta CT}$ mean value of *D21S11*/SM gene in the DS group was 1.88 ± 0.95, while in controls the value was 1.06 ± 0.68. The values were higher than 1.5 and 1.0 which may be explained by different amplification efficiencies of *D21S11* and SM. The qPCR test can correctly diagnose nine of ten trisomy 21 patients.

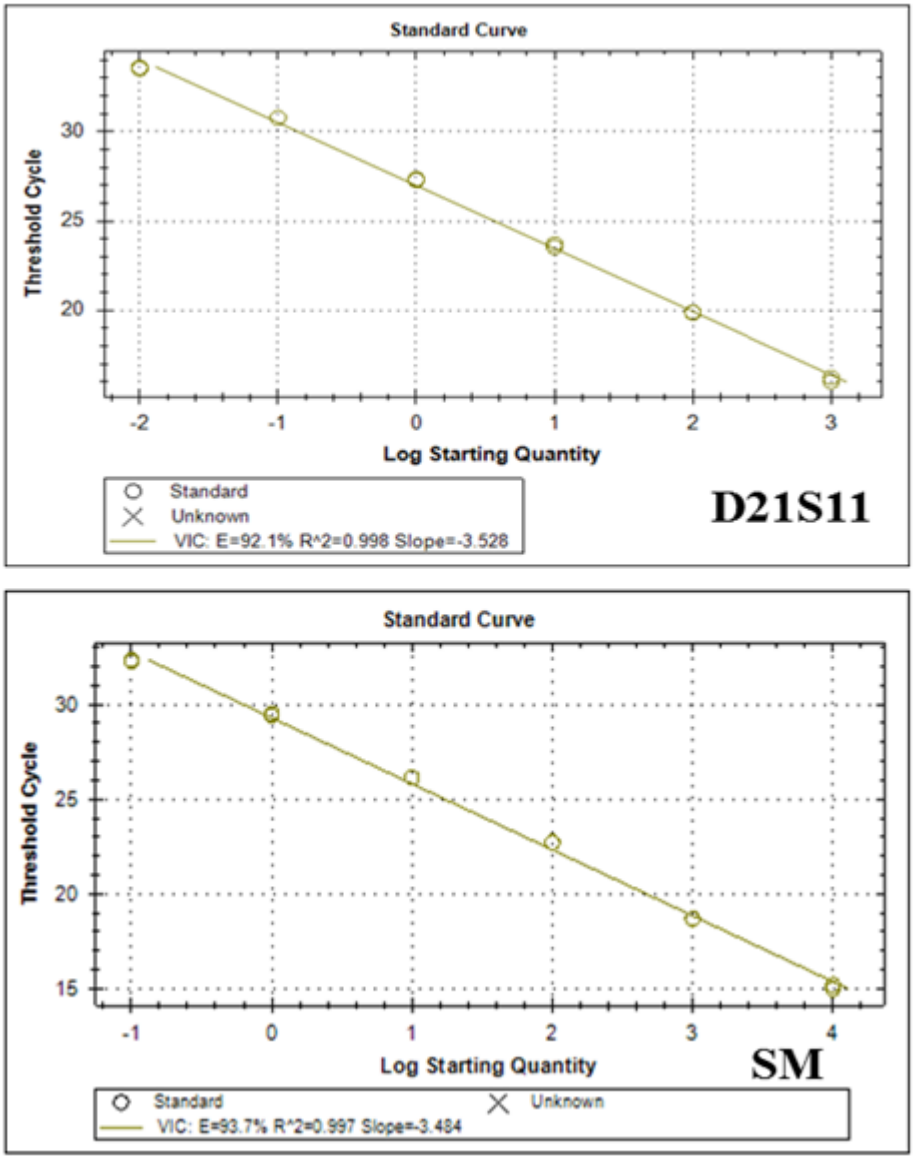

Figure 1. Standard curves for *D21S11* and SM real-time polymerase chain reaction

Hu et al. reported using qPCR to determine DSCR3/GAPDH ratio to diagnose trisomy 21. The DSCR3 is a gene within the Down syndrome critical region. The DSCR3/GAPDH ratio could correctly determine trisomy 21 and normal controls in all five trisomy 21 fetuses and 34 normal fetuses, and seven children with trisomy 21 and 74 healthy controls [13]. Zhu et al. reported using $\Delta\Delta C_T$ of DSCR4 on chromosome 21 and RABIF on chromosome one as a reference. The $\Delta\Delta C_T$ values in 12 trisomy 21 fetuses were higher than those of 551 normal fetuses and a clear cut-point could be established between the two groups [14].

Helmy et al. also reported the use of DSCR3/GAPDH ratio to diagnose trisomy 21 [15]. However, the ratio between the trisomy 21 and control groups (7 DS patients and five controls, and three DS fetuses and 18 normal controls) were not found to be different, although the amplification efficiencies of both genes were similar. The authors recommended further studies before the tests may be routinely used.

The current study demonstrates that the detection of gene copy difference by qPCR is a promising method for the diagnosis of trisomy 21. The method is rapid and feasible to set up in most laboratories. However, with the occurrence of false negative results, controls within each batch of a study are important. Further studies in larger population, and using of more than one index loci on chromosome 21 to decrease false negative results is suggested.

Conclusion

This study shows that using $2^{-\Delta\Delta CT}$ values of *D21S11*/SM gene to determine relative gene copy of *D21S11* is feasible for the diagnosis of trisomy 21, although false negative results may occur. Further studies with an addition of more than one index gene on chromosome 21 are suggested to consolidate the method. It can also be applied for diagnosis of other aneuploidy syndromes, such as trisomy 13 or 18.

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References

1. Hook EB. Prevalence of chromosome abnormalities during human gestation and implications for studies of environmental mutagens. Lancet. 1981; 2:169-72.
2. Gardner RJM, Sutherland GR, Shafter LG. Chromosome abnormalities and genetic counseling. 4th ed. New York: Oxford University Press; 2012.
3. Bull MJ; Committee on Genetics. Health supervision for children with Down syndrome. Pediatrics. 2011; 128:393-406.
4. Sheets KB, Crissman BG, Feist CD, Sell SL, Johnson LR, Donahue KC, et al. Practice guidelines for communicating a prenatal or postnatal diagnosis of Down syndrome: recommendations of the national society of genetic counselors. J Genet Couns. 2011; 20:432-41.
5. ACOG Practice Bulletin No. 77: screening for fetal chromosomal abnormalities. Obstet Gynecol. 2007; 109:217-27.
6. Langlois S, Duncan A. Use of a DNA method, QF-PCR, in the prenatal diagnosis of fetal aneuploidies. J Obstet Gynaecol Can. 2011; 339:955-60.
7. Sun X, Yan M, Zhang Y, Zhou X, Wang C, Zheng F, et al. Practical application of fluorescent quantitative PCR on Trisomy 21 in Chinese Han population. Mol Biol Rep. 2006; 33:167-73.
8. Pertl B, Kopp S, Kroisel PM, Hausler M, Sherlock J, Winter R, et al. Quantitative fluorescence polymerase chain reaction for the rapid prenatal detection of common aneuploidies and fetal sex. Am J Obstet Gynecol. 1997; 177:899-906.
9. Mann K, Fox SP, Abbs SJ, Yau SC, Scriven PN, Docherty Z, et al. Development and implementation of a new rapid aneuploidy diagnostic service within the UK National Health Service and implications for the future of prenatal diagnosis. Lancet. 2001; 358: 1057-61.
10. Hulten MA, Dhanjal S, Pertl B. Rapid and simple prenatal diagnosis of common chromosome disorders: advantages and disadvantages of the molecular methods FISH and QF-PCR. Reproduction. 2003; 126: 279-97.
11. Jain S, Panigrahi I, Gupta R, Phadke SR, Agarwal S. Multiplex quantitative fluorescent polymerase chain reaction for detection of aneuploidies. Genet Test Mol Biomarkers. 2012; 16:624-7.
12. Zimmermann B, Holzgreve W, Wenzel F, Hahn S. Novel real-time quantitative PCR test for trisomy 21. Clin Chem. 2002; 482:362-3.
13. Hu Y, Zheng M, Xu Z, Wang X, Cui H. Quantitative real-time PCR technique for rapid prenatal diagnosis of Down syndrome. Prenat Diagn. 2004; 24:704-7.

14. Zhu YN, Lu SM, You JF, Zhu B, Yu MY. Novel real-time PCR assay for rapid prenatal diagnosis of Down syndrome: a prospective study of 563 amniocytes. Clin Biochem. 2009; 42:672-5.
15. Helmy SM, Ismail S, Bassiouni R, Gaber KR. Sensitivity of DCSR3/GAPDH ratio using quantitative real-time PCR in the rapid prenatal diagnosis for down syndrome. Fetal Diagn Ther. 2009; 25:220-3.
16. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. Methods. 2001; 25:402-8.
17. Pornprasert S, Sukunthamala K, Sacome J, Phusua A, Saetung R, Sanguansermsri T, et al. Analysis of real-time SYBR-polymerase chain reaction cycle threshold for diagnosis of the α-thalassemia-1 Southeast Asian type deletion: application to carrier screening and prenatal diagnosis of Hb Bart's hydrops fetalis. Hemoglobin. 2008; 32:393-402.