

Rapid Prenatal Diagnosis of Trisomy 21 by Real-time Quantitative Polymerase Chain Reaction with Amplification of Small Tandem Repeats and S100B in Chromosome 21

Young Ho Yang,^{1,2,3} Mi Suk Nam¹, and Eun Suk Yang¹

¹Department of Obstetrics and Gynecology, Yonsei University College of Medicine, Seoul, Korea;

²Division of Prenatal Genetics Clinic, Yonsei University College of Medicine, Seoul, Korea;

³The Genetic Laboratory of the Medical Research Center, Yonsei University College of Medicine, Seoul, Korea.

Trisomy 21 (Down syndrome) is the most common congenital anomaly, and it occurs in one out of 700-1000 births. Current techniques such as amniocentesis and chorionic villi sampling (CVS) require lengthy laboratory culture procedures and high costs. This study was undertaken to establish a rapid prenatal diagnosis of trisomy 21 using real-time quantitative polymerase chain reaction (PCR) of fetal DNA from amniotic fluid. Real-time quantitative PCR was performed with DNA templates obtained from 14 normal blood samples, 10 normal amniotic fluid samples, 14 Down syndrome blood samples, and 7 Down syndrome amniotic fluid samples. Primers for D21S167 and S100B of chromosome 21 were used. Primers that direct the amplification of the 165-bp fragment of the insulin-like growth factor (IGF)-1 gene on chromosome 12 using a PCR primer were included to generate an internal standard for quantitation. The relative levels of D21S167 and S100B were 2.6 and 2.4 times higher in the blood of Down syndrome patients than those in the control group. The differences between these two groups were statistically significant (p -values were 0.0012 and 0.0016, respectively). The relative levels of D21S167 and S100B were 2.1 and 2.7 times higher in the amniotic fluid of Down syndrome fetuses than those in the control group. The difference between these two groups was statistically significant (p -values were 0.0379 and 0.0379, respectively). Prenatal diagnosis of trisomy 21 by real-time quantitative PCR using STR (small tandem repeats) amplification of D21S167 and S100B is a useful, accurate and rapid diagnostic method. Furthermore, it may also be useful for prenatal diagnosis with fetal DNA from maternal blood, and for preimplantation genetic diagnosis and prenatal counseling.

Key Words: Real-time quantitative PCR, trisomy 21, prenatal diagnosis

INTRODUCTION

Down syndrome is the most common chromosomal abnormality. Trisomy 21 is found in 95% of Down syndrome patients, whose clinical characteristics are mental retardation, an abnormal heart, characteristic facial features, the simian crease, and a high risk of leukemia.¹ Prenatal diagnosis of Down syndrome has been performed by chorionic villi sampling, amniocentesis and cordocentesis, but disadvantages of these methods include high cost, difficulties in cell culture, and significant time requirements. These limitations have prompted us to investigate alternative methods for the diagnosis of trisomy 21.^{2,4}

Recently, diverse methods have been developed to detect additional copies of chromosome 21, such as fluorescence in situ hybridization (FISH) and polymerase chain reaction (PCR).^{5,6}

Primers for the analysis of chromosome 21 have been developed. Mansfield⁷ and Yang et al.⁸ have reported that the D21S11 gene on chromosome 21 could be used for the target of PCR, and von Eggeling⁹ and Yang et al.⁸ have reported that the S100B gene on chromosome 21 could be used for the PCR primer. Findlay et al.^{10,11} have presented data demonstrating that fluorescent PCR amplification of D21S167, located on 21q22.2 could be applied to the prenatal diagnosis of

Received June 23, 2004

Accepted December 30, 2004

Reprint address: requests to Dr. Young Ho Yang, Department of Obstetrics and Gynecology, Yonsei University College of Medicine, 134 Shinchon-dong, Seodaemun-gu, Seoul 120-752, Korea. Tel: 82-2-2228-2233, Fax: 82-2-313-8357, E-mail: ob@yumc.yonsei.ac.kr

trisomy 21.

Lee et al.¹² have devised a method, termed the homologous gene quantitative polymerase chain reaction (HGQ-PCR), which uses one pair of primers. This method can directly identify the additional copy of chromosome 21 by simultaneously amplifying two highly homologous genes of the human liver-type phosphofructokinase located on chromosome 21 (PFKL-CH21) and the human muscle-type phosphofructokinase located on chromosome 1 (PFKM-CH1). This quantitative method may be used for the prenatal diagnosis of Down syndrome caused by trisomy 21.

Recently, Grove¹³ has developed a real-time quantitative PCR, enabling the rapid and reliable detection of specific DNA or RNA. However, there are few reports about prenatal diagnoses using this method.

This study was undertaken to establish a prenatal diagnosis of trisomy 21 by real-time quantitative PCR using blood samples of Down syndrome patients and amniotic fluids from mothers bearing a Down syndrome fetus.

MATERIALS AND METHODS

Samples

Peripheral venous blood samples were obtained from Down syndrome patients, and amniotic fluids were obtained from pregnant women between the gestational ages of 8 and 41 weeks, for whom prenatal and genetic counseling were indicated.

Processing of blood and amniotic fluid samples

DNA templates were obtained from 14 samples of normal whole blood, 10 normal amniotic fluid samples, 14 Down syndrome whole blood samples, and 7 Down syndrome amniotic fluid samples.

DNA purification

DNA was extracted from 300µl whole blood samples and 10 ml amniotic fluid samples using the Dizard genomic DNA purification kit (Promega).

Primer design

Primers were designed using Primer Express V. 1.5.¹⁴

Sequences were obtained from the NCBI database (www.ncbi.nlm.nih.gov, accession number NT-011515[S100B] and X00173[IGF-1]).

TaqMan probes were custom-synthesized by PE Applied Biosystems.

IGFIF, 5'-CCTGCCCTCCATAGGTTCT-3';

IGFIR, 5'-GGTGACCCCTGTCCCAGTT-3';

IGFIP, 5'-(VIC)AAATGAGATCACCCCCTCACT TGG(TAMRA)-3',

D21S167F, 5'-TTCCATGTACTCCTGCATTCACA AA-3';

D21S167R, 5'-GCTGGTAAATGGGCTGTGTGTA G-3';

D21S167P, 5'-(FAM)CGTGTATGTGTGAACCTAG TCTACTGAGG(TAMRA)-3';

S100BF, 5'-TGGGCCCTCCTGCTGAA-3';

S100BR, 5'-GTTTGAAATCCACTCATGCAATG-3';

S100BP, 5'-(FAM)AGTGCCCTAAGCACAGGTGT ACGG(TAMRA)-3'

Real-time quantitative PCR

Real-time quantitative PCR analysis was performed by the use of a PE Applied Biosystems 7700 Sequence Detector (SDS ver. 1.9.1). The TaqMan Universal PCR Master Mix and TaqMan DNA Template Reagent were supplied by PE Applied Biosystems.

Each reaction contained 200 nM of each amplification primer and 100 nM of the corresponding TaqMan probe. Thermal cycling was initiated with a two-minute incubation at 50°C, to allow the uracil N-glycosylase to act, and this was followed by a first denaturation step of 15-seconds at 95°C, an annealing/extension of one minute at 60°C, and amplification detection.

Amplification data was collected by the 7700 Sequence Detector and stored in a Macintosh computer, and then analyzed using the Sequence Detection System software (SDS ver. 1.7) developed by PE Applied Biosystems. IGF-1 was used as an internal control to evaluate the quantitation of the template DNA. On the other hand, the concentration of each target DNA was calculated using a standard curve.

Statistical significance was determined according to the Wilcoxon rank sum test, and differences between two groups were considered significant when $p < 0.05$.

RESULTS

The relative DNA level of D21S167 in the blood was higher in Down syndrome subjects compared with the control group (6.16 versus 2.32). The relative quantity of S100B in the blood of Down syndrome subjects was also higher when compared with the control group (15.62 versus 6.47). The relative DNA levels of D21S167 and S100B were 2.6 and 2.4 times higher in the blood of Down syndrome patients than in the control group. The difference of DNA levels between these two groups was statistically significant (p -values: 0.0012 and 0.0016, Fig. 1).

The relative DNA level of D21S167 in the amniotic fluid of Down syndrome fetuses was higher than that of the control group (6.37 versus 3.00). The relative DNA level of S100B in the amniotic fluid of Down syndrome fetuses was also higher when compared with the control group (19.14 versus 7.04). The relative DNA levels of D21S167 and S100B were 2.1 and 2.7 times higher in the amniotic fluid of Down syndrome

fetuses than those in the control group. The difference of DNA level between the Down syndrome group and the normal control group was statistically significant (p -value 0.0379 and 0.0379, Fig. 2).

DISCUSSION

Currently, trisomy 21 can be detected using molecular genetic techniques such as polymerase chain reaction (PCR) or fluorescent in situ hybridization (FISH). One such method for detecting trisomy 21 is PCR using the specific primers for chromosome 21. Keuren et al.¹⁵ have reported that the genetic marker D21S167 was located on 21q11.2-q22.2, and that the genetic marker D21S11 was located on 21q21 of chromosome 21. Trisomy 21 was defined as either a triallelic signal or a diallelic pattern, whereas disomy was indicated by the heterozygote pattern observed by fluorescent PCR using the D21S167 and D21S11 markers.^{10,11}

This method was applied to other trisomies such as trisomy 18 and trisomy 13, using the D18S51 genetic marker for trisomy 18, and the D13S631 and D13S258 genetic markers for trisomy 13. Other trisomies have also been successfully detected.¹⁰

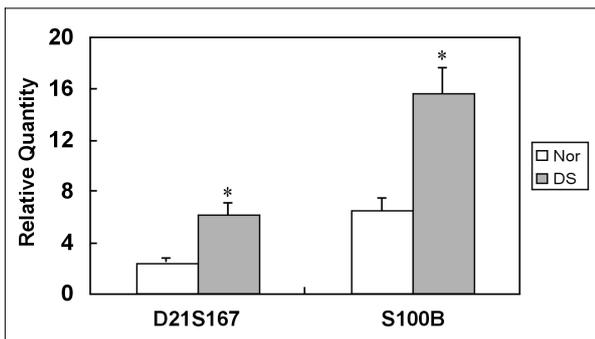


Fig. 1. Relative DNA levels of D21S167 and S100B determined by real-time quantitative PCR in the blood of Down syndrome and normal control groups. The relative DNA levels of D21S167/IGF-1 in blood were 2.3 ± 0.4 in the normal control group ($n=14$) and 6.1 ± 0.8 in the Down syndrome patients group ($n=14$), and the relative DNA levels of S100B/IGF-1 were 6.4 ± 1.0 in the normal control group ($n=14$) and 15.6 ± 2.1 in the Down syndrome group ($n=14$, mean \pm SEM). Brackets indicate SEM. $*p < 0.01$. (Nor, Normal; DS, Down syndrome).

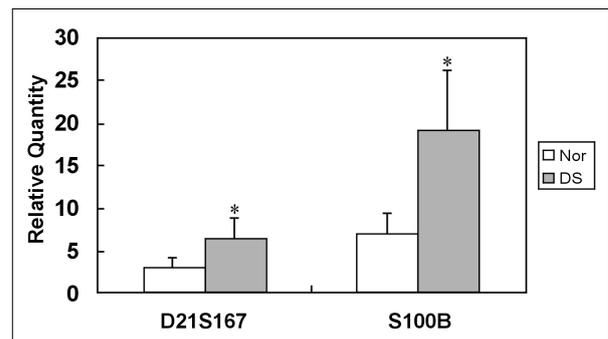


Fig. 2. Relative DNA levels of D21S167 and S100B determined by real-time quantitative PCR in the amniotic fluid of the Down syndrome and normal control groups. The relative DNA levels of D21S167/IGF-1 in amniotic fluid were 3.0 ± 1.1 in the normal control group ($n=10$) and 7.0 ± 2.5 in the Down syndrome patients group ($n=7$), and the relative DNA levels of S100B/IGF-1 were 6.3 ± 2.5 in the normal control group ($n=10$) and 19.1 ± 7.0 in the Down syndrome group ($n=7$, mean \pm SEM). Brackets indicate SEM. $*p < 0.05$. (Nor, Normal; DS, Down syndrome).

The S100B gene has been used for quantitative PCR to detect the DNA levels of chromosome 21.8 D21S11 and S100B were useful markers for the detection of trisomy 21 by quantitative PCR, and the prenatal diagnosis of trisomy 21 by PCR-associated STR analysis of S100B proved a useful, innovative, accurate and rapid method for diagnosis.⁹ In the analysis of S100B, the ratio of S100B to IGF-1 was 1.4:1.6 in Down syndrome samples, while the ratio of S100B in all the normal samples was close to 1:1 (Data not shown).

Quantitative polymerase chain reaction was introduced to measure the concentration of fetal DNA in maternal plasma and serum.¹⁶⁻²¹ Lee et al.¹² have reported that the human liver-type phosphofructokinase gene located on chromosome 21 (PFKL-CH21) at position q22.3 in trisomy 21 is 1.5-fold higher by homologous gene quantitative PCR (HGQ-PCR) than that of PFKM-CH1 located on chromosome 1, and PFKM-CH10 located on chromosome 10. These results gave the first indication that this quantitative method could be used for the prenatal diagnosis of Down syndrome caused by trisomy 21.

Honda²⁰ found that fetal gender was determined by conventional PCR by detecting a Y-chromosomal sequence (DYS14) in maternal blood with 95% sensitivity, whereas in real-time quantitative PCR, the total sensitivity after the fifth week was 100%. Bianchi²² reported that large amounts of cell-free fetal DNA were present in amniotic fluid when using real-time quantitative PCR of fetal DNA in the amniotic fluid. Bianchi found 0 GE/ml of the Y-DNA in the amniotic fluid for the female fetus, whereas the mean amount of male DNA in the male fetus was 3427 GE/ml. Lo et al.²³ demonstrated that fetal DNA was present in maternal serum and plasma, and that the detection of fetal DNA sequences was made possible by recent developments in molecular biology. This would provide physicians and researchers a new screening test for fetal chromosomal disorders.

The recent introduction of real-time quantitative PCR, which is the most sensitive method for DNA and RNA detection, makes possible the rapid and accurate quantitation of specific DNA and mRNA. This system has a number of advantages: a high throughput and fast turnaround time, an accurate target quantitation over a wide concentration

range within three to four hours, low cost, and it is possible to optically monitor the process continuously in the laboratory.

This study was designed to use D21S167 on 21q22.2 and S100B on 21q22.3 of chromosome 21 as targets of PCR. The blood of Down syndrome patients and the amniotic fluids of mothers conceiving Down syndrome fetuses were analyzed and compared with the normal control groups by real-time quantitative PCR. Based on the observation that D21S167 and S100B levels are high in the blood of Down syndrome patients, this study was designed to show that the levels of D21S167 and S100B in the amniotic fluid of Down syndrome patients are higher than in normal control groups. For our first attempts, 1 ng of DNA from blood and amniotic fluid was used as a template for PCR.

However, in the amniotic fluid, target genes were not amplified because the amount of DNA was too small. Therefore, we next used 100 ng of DNA from the amniotic fluid, which produced a good result.

The prenatal diagnosis of trisomy 21 by real-time quantitative PCR using STR (small tandem repeats) amplification of D21S167 and S100B is a useful, accurate and rapid diagnostic method. This method can also be employed in the diagnosis of trisomy 13 and 18. Furthermore, it may be useful for prenatal diagnosis using fetal DNA from maternal blood, and for preimplantation genetic diagnosis and prenatal counseling.

REFERENCES

1. Hook EB. Rates of chromosome abnormalities at different maternal ages. *Obstet Gynecol* 1981;58:282-5.
2. Globus MS, Loughman WD, Epstein CJ, Halbasch G, Stephen JD, Hall BD. Prenatal genetic diagnosis in 3000 amniocentesis. *N Engl J Med* 1979;300:157-63.
3. Yang YH, Kim MS, Park YW, Kim SK, Cho JS, Jeong HJ. Chorionic villus sampling: Experience of first 510 cases in Korean. *Korean J Obstet Gynecol* 1993;36:906-15.
4. Yang YH, Park YW, Kim SK, Cho JS, Jeong MJ, Kim HS, et al. Chorionic villus sampling: clinical experience of the initial 750 cases. *J Obstet Gynecol Res* 1996;22: 143-9.
5. Yang YH, Kang JY, Yang ES, Jang SY, Cho JS, Park YW, et al. Clinical usefulness of fluorescence in situ hybridization.

- dization in the diagnosis of genetic disease. *Korean J Obstet Gynecol* 2002;45:1016-25.
6. Beggs AH, Kunkel LM. Improved diagnosis of Duchenne/Becker muscular dystrophy. *J Clin Invest* 1990;85:613-9.
 7. Mansfield ES. Diagnosis of down syndrome and other aneuploidies using quantitative polymerase chain reaction and small tandem repeat polymorphisms. *Hum Mol Genet* 1993;2:43-50.
 8. Yang YH, Kim IK, Oh SH, Kim CK, Kim JY. Rapid prenatal diagnosis of trisomy 21 by polymerase chain reaction associated analysis of small tandem repeats and S100B in chromosome 21. *Fetal Diagn Ther* 1998;13:361-6.
 9. von Eggeling F, Freytag M, Fahsold R, Horsthemke B, Claussen U. Rapid detection of trisomy 21 by quantitative PCR. *Hum Genet* 1993;91:567-70.
 10. Findlay I, Toth T, Matthews P, Marton T, Quirke P, Papp Z. Rapid trisomy diagnosis (21, 18 and 13) using fluorescent PCR and short tandem repeats: applications for prenatal diagnosis and preimplantation genetic diagnosis. *J Assist Reprod Genet* 1998;15:266-75.
 11. Findlay I, Matthews P, Toth T, Quirke P, Papp Z. Same day diagnosis of Down's syndrome and sex in single cells using multiplex fluorescent PCR. *J Clin Pathol* 1998;51:164-7.
 12. Lee HH, Chang JG, Lin SP, Chao HT, Yang ML, Ng HT. Rapid detection of trisomy 21 by homologous gene quantitative PCR (HGQ-PCR). *Hum Genet* 1997;99:364-7.
 13. Grove DS. Quantitative real-time polymerase chain reaction for the core facility using TaqMan and the Perkin-Elmer/Applied Biosystems Division 7700 sequence detector. *J Biomol Tech* 1999;10:11-6.
 14. Guo Z, Sharma V, Patterson D, Litt M. TG repeat polymorphism at the D21S167 locus. *Am J Hum Genet* 1990;46:776-83.
 15. Keuren M, Drabkin H, Hart I, Harker D, Patterson D, Vora S. Regional assignment of human liver-type 6-phosphofructokinase to chromosome 21q22.3 by using somatic cell hybrids and a monoclonal anti-L antibody. *Hum Genet* 1986;74:34-40.
 16. Lo YMD, Tein MSC, Lau TK, Haines CJ, Leung TN, Poon PMK, et al. Quantitative analysis of fetal DNA in maternal plasma and serum: implications for noninvasive prenatal diagnosis. *Am J Hum Genet* 1998;62:768-75.
 17. Zhong XY, Holzgreve W, Hahn S. Detection of fetal Rhesus D and sex using fetal DNA from maternal plasma by multiplex polymerase chain reaction. *Br J Obstet Gynaecol* 2000;107:766-9.
 18. Zhong XY, Hahn S, Holzgreve W. Prenatal identification of fetal genetics traits. *Lancet* 2001;357:310-1.
 19. Houfflin-Debauge V, O'Donnell H, Overton T, Benntee PR, Fisk NM. High sensitivity of fetal DNA in plasma compared to serum and nucleated cells using un-nested PCR in maternal blood. *Fetal Diagn Ther* 2000;15:102-7.
 20. Honda H, Miharu N, Ohashi Y, Ohama K. Successful diagnosis of fetal gender using conventional PCR analysis of maternal serum. *Clin Chem* 2001;47:41-6.
 21. Honda H, Miharu N, Ohashi Y, Samura O, Kinutani M, Hara T, et al. Fetal gender determination in early pregnancy through qualitative and quantitative analysis of fetal DNA in maternal serum. *Hum Genet* 2002;110:75-9.
 22. Bianchi DW, Leshane ES, Cowan JM. Large amounts of cell-free fetal DNA are present in amniotic fluid. *Clin Chem* 2001;47:1867-9.
 23. Lo YMD, Corbetta N, Chamberlain PF, Rai V, Sargent IL, Redman CW, et al. Presence of fetal DNA in maternal plasma and serum. *Lancet* 1997;350:485-7.