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Methods paper

Rapid detection of Down's syndrome using quantitative real-time PCR (qPCR) targeting segmental duplications on chromosomes 21 and 11☆

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ABSTRACT

Objective: Development of a qPCR test for the detection of trisomy 21 using segmental duplications.
Methods: Segmental duplications in the *TTC3* gene on chromosome 21 and the *KDM2A* gene on chromosome 11 were selected as molecular markers for the diagnostic qPCR assay. A set of consensus primers selected from the conserved regions of these segmental duplications were used to amplify internal diverse sequences that were detected and quantified with different probes labeled with distinct fluorescence. The copy numbers of these two fragments were determined based on the ΔCq values of qPCR. The results of qPCR for prenatal and neonatal screening of Down's syndrome were compared with the conventional karyotype analysis by testing 82 normal individuals and 50 subjects with Down's syndrome.
Results: The ΔCq values of segmental duplications on chr21 and 11 ranged between 0.33 and 0.75 in normal individuals, and between 0.91 and 1.18 in subjects with Down's syndrome. The ΔCq values of these two segmental duplications clearly discriminated Down's syndrome from normal individuals ($P < 0.001$). Furthermore, the qPCR results were consistent with karyotype analysis.
Conclusion: Our qPCR can be used for rapid prenatal and neonatal screening of Down's syndrome.

1. Introduction

Down's syndrome, also known as trisomy 21, is the most common human genetic disease caused by an abnormal number of chromosomes. The neonatal incidence rate of Down's syndrome is 1/600–1/800. The main clinical manifestations of Down's syndrome are serious congenital mental retardation and congenital malformations of various organs. Most Down's syndrome patients have difficulty taking care of themselves in daily life. Currently, no effective treatment is available for this disease. Therefore, prenatal diagnosis is important for the detection of Down's syndrome.

Conventionally, amniotic fluid cell culture and karyotype analysis have been widely used for prenatal diagnosis of Down's syndrome (Caspersson et al., 1970). However, this method is time-consuming, usually taking over two weeks or longer to get results. In addition, amniotic fluid cell culture has a high rate of failure that limits its application in clinical diagnosis of Down's syndrome. Fluorescence in situ hybridization (FISH) is also used for prenatal diagnosis of Down's syndrome without the need of a cell culture. The FISH diagnosis results can be obtained within 24 h (Caine et al., 2005; Ho et al., 2012). However, FISH is cumbersome and labor-intensive, and requires extensive technical expertise. These drawbacks have limited its application for a large amount of clinical samples. Therefore, novel methods for rapid prenatal diagnosis of Down's syndrome with convenience, rapid and low labor-intensity are needed.

With the rapid development of molecular biology and genome biology, a number of molecular approaches have been developed for the rapid identification of aneuploidy. Among these novel molecular approaches, quantitative fluorescent polymerase chain reaction (QF-PCR) and multiplex ligation-dependent probe amplification (MLPA) were widely used in clinic diagnosis of genetic diseases caused by aneuploidy. These two approaches are highly accurate and robust, and allow processing of 96 samples at once in an automated system, with results being available in less than 48 h. However, both approaches are required to determine the fragment length of PCR products. The high

Abbreviations: FISH, fluorescence in situ hybridization; qPCR, quantitative real-time PCR; SNP, single-nucleotide polymorphism; MLPA, multiplex ligation-dependent probe amplification; CNV, copy number variation; QF-PCR, quantitative fluorescent polymerase chain reaction; Chr, chromosome; KDM2A, human lysine (K)-specific demethylase 2A; TTC3, human tetratricopeptide repeat domain 3; FAM, 6-carboxyfluorescein; TET, 5-tetrachloro-fluorescein; Cq, quantification cycle; CV, coefficient of variation; SD, standard deviation.

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cost of instruments and reagents of capillary electrophoresis limits the wide application of QF-PCR and MLPA in prenatal diagnosis of Down's syndrome (Slater et al., 2003; Atef et al., 2011; Willis et al., 2012).

Quantitative real-time PCR (qPCR) does not require post-PCR steps or electrophoresis in the diagnosis of aneuploidy. The experiment is rapid and easy to conduct. It can be done automatically and the data are directly extracted from the real-time PCR machine, reducing the impact of subjective factors. The diagnosis results can be available within 3 h. In addition, dozens of specimens can be analyzed at the same time, which allows the test to be used in large-scale prenatal and neonatal screenings. The *DSCR3* gene on chromosome 21 and the *GAPDH* gene on chromosome 12 are used in qPCR assay for relative quantification of gene copy number for the detection of Down's syndrome (Zimmermann et al., 2002). In this qPCR assay, two sets of primers were used to amplify two different fragments on the *DSCR3* and *GAPDH* genes. Slight changes of annealing temperature or quality of template DNA can cause false negative or false positive results (Helmy et al., 2009). Therefore, selection of appropriate primers for consistent amplification efficiency is critically important for the sensitivity and specificity of aneuploidy identification.

In this study, we developed a molecular method based on qPCR assay targeting segmental duplications on chromosomes 21 and 11. A highly diverse region is found in the segmental duplications, although other regions share high levels of sequence identity, which allowed us to design a set of consensus primers to amplify the internal diverse region. Since only a set of primer was used in the qPCR assay, both fragments were amplified at equal efficiency. The PCR amplicons were detected and quantified using distinct Taqman probes labeled with distinct fluorescence. In comparing our method with the conventional karyotype analysis method with 132 specimens, our qPCR assay was shown to be a rapid and sensitive molecular method for prenatal and neonatal screening of Down's syndrome.

2. Materials and methods

2.1. Statement of research ethics

This study has been approved by the Ethical Committee of the Maternal and Child Health Hospital of Qinzhou, Guangxi, China. The study subjects gave informed consent for participation in the study.

2.2. Specimens

A total of 50 specimens from Down's syndrome patients, including peripheral blood from 28 neonatal patients and uncultured amniotic fluid samples from 22 pregnant individuals were included in this study. Among the 50 Down's syndrome patients, two had unbalanced translocation 46,XX,der(14; 21)(q10; q10),+21. Peripheral blood samples from 22 normal neonatal individuals and uncultured amniotic fluid samples from 60 normal pregnant individuals were used as a control. All samples were analyzed using karyotype analysis and the results were compared with the results of qPCR.

2.3. DNA isolation

Peripheral blood and uncultured amniotic fluid were centrifuged at 3000 rpm for 10 min and cell pellets were suspended in sterilized water. DNA was isolated from uncultured amniotic fluid samples and blood samples using the QIAamp DNA Mini Kit (Qiagen) and the QIAamp DNA blood mini kit (Qiagen), respectively, according to the manufacturer's instructions. The concentration of genomic DNA was spectrophotometrically determined at an absorbance of 260 nm (Quawell). Approximately 20 ng of DNA was diluted in 25–50 μL sterilized water and stored at 4° C for qPCR assay.

2.4. Design of primers and probes

Segmental duplications are DNA fragments sharing high sequence identity and are widespread in the human genome (Bailey et al., 2001; She et al., 2004). As hotspots of copy number variance (CNV), segmental duplications play fundamental roles in both genetic diseases and genome evolution. Segmental duplications in human genomes are available from the Segmental Duplication Database (http://humanparalogy.gs.washington.edu/) at Washington University. Segmental duplications that have only two copies on the human genome, one on chromosome 21 and another on a chromosome other than a sex chromosome were considered as molecular markers in the qPCR assay. Segmental duplications meeting these criteria were then searched for in the human genome using the NCBI BLASTn program to identify DNA sequences of high identity. Only those without other homologues in the human genome were considered. Finally, segmental duplications, one located on chromosome 21 and another located on chromosome 11, were selected as molecular markers in our study for qPCR assay. These two DNA fragments have high sequence identity, but sequence diversities were observed in some regions. PCR primers were selected from regions of identical sequence and Taqman probes were designed from the flanked regions of high sequence diversity (Fig. 1). The selected primers from chromosomes 21 and 11 can maintain equal efficiency that had been verified by our previous approach (Kong et al., 2014). The ratio for a euploid sample is 2:2, and the value for a trisomic sample is 2:3, reflecting the additional target chromosome (Fig. 2).

2.5. Quantitative real-time PCR

Quantitative real-time PCR was conducted on the CFX96 real-time PCR machine (BIO-Rad Corporation). The PCR reaction was performed in a 25 μL reaction system, containing template DNA (2 μL), a set of each primer (200 nmol/L), corresponding TaqMan probes (each 100 nmol/L), and 2× TaqMan Universal PCRMaster Mix (Tiangen Biotechnology Co., Ltd.) of 12.5 μL. PCR amplification began at 95 °C for 10 min, with 10 cycles (95 °C 15 s, 65 °C 1 min). Fluorescence was not collected at this stage. This was followed by 95 °C 15 s and 65 °C 1 min. Fluorescence (FAM and TET) was collected at 65 °C, for 30 cycles. CFX Manager 2.1 (BIO-Rad Corporation) software was used to calculate the Ct values of segmental duplications. The difference of the threshold cycle value (ΔCq) of segmental duplications on chromosomes 21 and 11 was used to discriminate Down's syndrome and normal specimens.

2.6. Statistical analysis

The ratio of copy numbers of chromosomes 21 and 11 was calculated for each sample, according to the ΔCq values of the segmental duplications on chromosomes 21 and 11. The ΔCq was presented as mean value. The coefficient of variation (CV) was calculated based on the formula, $CV = SD / M \times 100\%$, where SD is the standard deviation and M is the mean value of ΔCq. The independent sample *t*-test was used to analyze the difference of ΔCq between the normal individuals and Down's syndrome subjects.

3. Results

3.1. Determination of the standard curve and the CV of qPCR assay

The genomic DNA from normal individuals were used as templates and 10-fold serial dilution was conducted to obtain five concentrations, 200 ng/μL, 20 ng/μL, 2 ng/μL, 0.2 ng/μL, and 0.02 ng/μL. Two parallel PCR reactions were prepared for each concentration and repeated 3 times. Thus, six results were generated for each concentration. The ΔCq value was calculated for each concentration to generate the CV. The CV was used to evaluate the influence of different concentrations