ORIGINAL ARTICLE

The application of sequence specific primer and RT-PCR to LRRK2 gene polymorphism typing

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ABSTRACT

Objective: To establish a new detecting method for disease susceptibility loci R1628P and G2385R of Parkinson's disease (PD) related gene LRRK2.
Methods: Sequence specific primers were designed to make a genotyping of DNA markers with known genotypes by use of quantitative fluorescence real-time PCR (RT-PCR). 100 cases of PD samples with unknown genotypes were tested, and verified by use of polymerase chain reaction linked restriction fragment length polymorphism (PCR-RLFP).
Results: The genotyping results of DNA markers proved to be correct, and 100 cases of samples to be tested had a completely consistent genotyping result with PCR-RLFP genotyping result.
Conclusions: Sequence specific primer and quantitative fluorescence RT-PCR can successfully make a genotyping for disease susceptibility loci R1628P and G2385R of LRRK2.

Key Words: Parkinson's disease, LRRK2 gene, Sequence specific primer, Quantitative fluorescence RT-PCR, Genotype

1. INTRODUCTION

Parkinson's disease (PD) is a common degenerative disease of the nervous system, and its incidence increases with age. LRRK2 gene consists of 51 exons, and its encoding product is a protein made up of 2,527 amino acids. This type of protein is with GTPase activity and protein kinase activity, and is found to be closely related with the formation of Lewy bodies that are associated with the occurrence of PD. LRRK2 has a certain effect on the occurrence of familial and sporadic PD. Currently, it has been identified that this gene has more than 20 disease-related loci, in which, R1628P and G2385R locus mutations are highly prevalent in sporadic PD patients in Eastern Asian countries and regions including China, Singapore, Japan, Taiwan of China and so on.[1,2] Meanwhile, the research on LRRK2 gene function, mutation and its correlation to PD has gradually become a hot spot in academic circles. This research will apply RT-PCR technology to the genotyping of R1628P and G2385R loci of LRRK2 gene to establish a convenient, rapid and high-throughput detecting platform and provide a new method for the basic research and clinical diagnosis of PD.

2. MATERIALS AND METHODS

2.1 Main reagents

SYBR® Prime EX TaqTM Real time PCR kits were purchased from TaKaRa; proteinase K was bought from Sigma; other analytic reagents were made by Amresco; Sangon Biotech (Shanghai) Co., Ltd. was commissioned to synthesize PCR primers.

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2.2 The source of samples and DNA extraction

100 cases of patients with sporadic PD in Chinese Han population in Northern China were selected as research objects and diagnosed by experts in Neurological Medicine according to UK Parkinson's Brain Bank criteria. The research was approved by Ethics Committee and all patients signed informed consent forms. 1 ml of venous blood was taken from each subject, with the addition of trisodium citrate for the use of anticoagulation. The blood sample was centrifuged at 4°C for 15 min, with leukocytes collected. The conventional SDS-proteinase K/phenol-chloroform method was used to extract genomic DNA.

2.3 DNA markers with known genotypes

In the preliminary study, leukocyte genomic DNA samples of R1628P and G2385R loci of LRRK2 gene were verified in the laboratory. According to the results, there were 5 cases of GG genotypes and 5 cases of GC genotypes in R1628P locus; and there were 5 cases of GG genotypes, 5 cases of GA genotypes and AA genotypes in G2385R locus.

2.4 Genotype detection of R1628P and G2385R loci of LRRK2 gene

Relative quantification method was used to make a genotyping of the gene polymorphism of R1628P and G2385R loci of LRRK2 gene with the help of PCR reaction induced by sequence specific primers (PCR-SSP), taking SYBR Green Real time PCR as the technology platform, human β2-microglobulin (B2M) as the reference gene, the wild base corresponding to the heterozygote in DNA markers as the control group. See Table 1 for primer sequences. The genotyping results were listed as follows: R1628P locus, primer U1 corresponded to wild base G; primer U2 corresponded to mutation base C; G2385R locus, primer L1 corresponded to wide base G; primer L2 corresponded to mutation base A. The total volume of PCR reaction system was 20 μl, including 6.0 μl of dd H_2O, 10 μl of SYBR® Prime Ex TaqTM (2×), 0.8 μl of upstream primers and 0.8 μl of downstream primers (10 μmol/L), 0.4 μl of ROX fluorescent dye II (50×) and 2.0 μl of genomic DNA (10 ng/μl). PCR reaction occurred in real-time quantitative PCR amplifier (ABI 7500). The standard procedures of two-step PCR amplification were: 95°C, 30 s; 95°C for 5 s, 60°C for 34 s, 40 cycles. Each sample to be tested was made in triplicate, and positive control and negative control were made for each PCR reaction.

3. RESULTS

3.1 Genotype detection of DNA markers

The relative expression of homozygotes in DNA markers was twice that of heterozygotes. According to the detection, it was found that homozygote G/G and heterozygote G/C genotypes were visible at LRRK2-R1628P locus, and homozygotes G/G, A/A and heterozygote G/A were visible at LRRK2-G2385R locus. The genotyping results obtained in this experiment proved to be consistent with known genotypes.

3.2 Genotype detection of samples to be tested

After the successful genotyping of DNA markers, the same method as above was used to make a genotyping of samples to be tested, and the classic polymerase chain reaction linked restriction fragment length polymorphism (PCR-RFLP) was utilized to verify the accuracy of the genotyping results. It was found that the genotyping results obtained by these two genotyping methods coincided. Thus it can be seen that the genotyping method of LRRK2 gene polymorphism established by this research proves to make an accurate genotyping. There was no heterozygote C/C formed after the variation of R1628P locus in 100 cases of PD samples in this research.

Table 1. The information of primer sequences used for amplification

Primer Name	Primer Sequence 5′ - 3′
LRRK2 R1628P U1	CAAAACACCCTAAGGGCATTATTTCGCG
LRRK2 R1628P U2	CAAAACACCCTAAGGGCATTATTTCGCC
LRRK2 R1628P L	CTAGGAGCTTAAAATACTGTGACATGTAGTTCT
LRRK2 G2385R U	GAACTAATATAAGGTTGTATTACACGTAGAAATT
LRRK2 G2385R L1	CCTTAAAAAGTGCACGCAGTCTATTAGTCC
LRRK2 G2385R L2	CCTTAAAAAGTGCACGCAGTCTATTAGTCT
B2M U	TCACGTCATCCAGCAGAGAATG
B2M L	CAGTGGGGGTGAATTCAGTGTAG

4. DISCUSSION

RT-PCR technology is characterized by outstanding advantages of real-time monitoring, extensive detection range, high sensitivity and high specificity through the accumulation of fluorescence signals to monitor the whole reaction process and make PCR amplification and end-product detection performed in a sealed environment. Due to no follow-up detection steps, this convenient and rapid method can effectively avoid cross contamination, make multiple detections, reduce a large number of testing time and costs and greatly overcome the deficiency of the traditional PCR technology. This research is intended to design sequence specific primers aimed at the specific locus of target gene, optimize the reaction system and cycle parameters (including the amount of primers and the amount of templates) and successfully establish SYBR Green Real time PCR technology platform which can detect R1628P and G2385R loci of LRRK2 gene and be applied to the basic research and clinical diagnosis of disease susceptibility loci of PD related gene LRRK2. SYBR Green applied to this research is a type of dye which can

bind to double-stranded DNA minor groove binder (MGB). After binding, the fluorescence intensity increases a hundred-fold. Fluorescence signal is proportional to the products continuously generated with the progress of PCR, resulting in the formation of the fluorescence amplification curve. The increase in fluorescence intensity is related to the initial template. It is applicable to make a quantitative detection of target nucleic acids. Therefore, it is found in the research that the initial copy number of homozygotes is almost two times that of heterzygotes, and the accurate genotyping was realized by use of sequence specific primers. In comparison to Taqman probe with high sensitivity and specificity, in this method, utilizing SYBR Green to design fluorescent quantitative primers is easy to operation with a relatively low cost. It can also accomplish the research purpose and be appropriate for the high-throughput detection in the basic research institutes.

CONFLICTS OF INTEREST DISCLOSURE

The authors declare they have no conflicts of interest.

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