Journal of Sciences, Islamic Republic of Iran 15(2): 103-108 (2004)
University of Tehran, ISSN 1016-1104

Application of Molecular DNA Markers (STRs) in Molecular Diagnosis of Down Syndrome in Iran

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Abstract

Down syndrome is one of the most common causes of mental retardation observed in approximately 1/700 live birth. The use of two or more STR markers related to chromosome 21 facilitates the diagnosis of Down syndrome within about six hours from the collection of the samples. This is the first study has been performed in Iranian population to assess the diagnostic value of using small tandem repeat (STR) markers assays for the rapid detection of chromosome 21 trisomy and compare its application to the conventional cytogenetic analysis. Eighty seven cases with Down syndrome and 120 normal controls have been tested by conventional karyotyping and molecular technique using five STR markers (D21S11, D21S1414, D21S1440, D21S1411, D21S1412) located on the long arm of chromosome 21. The heterozygosis of these tested markers has been calculated in tested Iranian population. All tested cases had free chromosome 21 trisomy in cytogenetic analysis from them 3 cases were mosaic for chromosome 21 trisomy. In molecular analysis 85% generated three allelic pattern, 14% dialleilc and 1% monoallelic pattern by using five molecular markers. Diagnosis of chromosome 21 trisomy was achieved for 99% of cases by molecular method, from them 3.5% of cases were mosaic and provided diallelic pattern. We have found a specificity of 100% and sensitivity of 99% for molecular diagnosis of Down syndrome using highly polymorphic STR markers. This technique is cheaper, quicker, no need of alive or sterile samples and useful for wide screening of newborns in nurseries. Molecular method accurately determines trisomy 21 especially when cytogenetic approach is not suitable in the case of fixed or dead tissues, small number of cells such as those taken from early amniocentesis or cells separated from maternal blood for noninvasive prenatal diagnosis. Although molecular diagnosis of Down syndrome targeted specifically to chromosome 21 trisomy but it can exclude the presence of the most frequent chromosomal disorder. The result of this investigation has documented the diagnostic advantages of this approach to perform even for prenatal test or in parallel with cytogenetic analysis.

Keywords: Down syndrome; STR marker; Chromosome 21 trisomy; Polymorphism

Introduction

Down syndrome is one of the most common chromosome aneuploidies which causes mental retardation, severe learning difficulty, increased incidence of heart abnormalities, tendency to leukemia

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and premature Alzheimer-like dementia. Down syndrome is mainly caused by chromosome 21 trisomy happened due to non-disjunction event at meiotic cell division. This extra chromosome 21 is usually maternal in origin and occurs more frequently with advanced maternal age. A few cases of Down syndrome (approximately 5%) are due to chromosome rearrangements [1-6].

The birth rate of Down syndrome still remains high, 1/230 pregnancies and 1/700 live birth [7-11]. The high prevalence is mainly due to lower level of care considered for 2/3 of Down syndrome pregnancies that are under 35 years of mother age and low level of information behind etiology of Down syndrome for instance about advanced maternal age. To reduce significantly the birth prevalence of Down syndrome, a wide-ranging screening of pregnant women has been suggested [11-12]. However, conventional methods such as cytogenetic analysis for diagnosis of chromosomal abnormalities often need lengthy laboratory procedures, and expertise, are expensive as well as significant delay in obtaining a diagnosis. Applying of fluorescence *in situ* hybridization technique from late 1980s using fluorescently labeled DNA probe has facilitated analysis of chromosome abnormalities. It has been used in diagnosis of chromosome 21 trisomy for prenatal diagnosis in interphase nuclei of uncultured amniocytes [13]. However, this technique needs costly probes, florescence microscope facilities and trained expertise and has not routinely been used for postnatal and prenatal diagnosis of Down syndrome throughout laboratory services in Iran. An alternative method, which is rapid, inexpensive and reliable, is using short tandem repeats (STRs) markers related to chromosome 21. This test is even suitable for large scale screening of all pregnant women [11-16].

STRs are hypervariable regions of the genome with repeat unites of 2 to 7 bp in length. The high length polymorphism of STRs is from variations in the number of tandem repeated units results in producing allele's length variation in most samples. Through the process of polymerase chain reaction (PCR), polymorphic STR markers can be amplified and examined in a family to find out the progeny of chromosome 21 alleles. Informative loci are representative of existing chromosome 21 in most cases would show either three or two alleles with 1:1:1 or 2:1 ratio in dosage respectively. The quantitative nature of this technique allows the amount of PCR product to be determined in the case when parents share same alleles [11-15]. Analysis of STRs by fluorescently labeled primers has preformed in some recent reports [11-15]. Although this technique would be preferential for the large scale analysis for instance in the case of large screening of many samples in a short time period [11-15], for analysis of a few samples in long term would be inconvenient due to need for running ABI sequencer machine in each experiment.

The aim of this study was to evaluate the feasibility, sensitivity and specificity of five highly polymorphic molecular STR markers on the long arm of chromosome 21 and even in the Down syndrome critical region in the diagnosis of Down syndrome in Iranian patients.

Material and Method

This study was carried out on approximately 86 families having a Down child taken from care centers or schools for mentally retarded children invited to participate in this project. A medical doctor and a genetic specialist consulted all chosen families. Informed consent was obtained from all participants and the study was approved by the ethics committee of the center. A control group consists of 100 normal controls were considered for comparison of Mendelian inheritance and density of molecular marker alleles. Five milliliters of venous blood was obtained from cases in 15 ml falcon tubes. Samples were subsequently divided into two tubes, one containing Na-Heparin anticoagulant to use for cytogenetic analysis and another containing EDTA anticoagulant to use for PCR analysis. The karyotype of all Down cases was assessed by conventional cytogenetic analysis according to Gosden *et al.* 1992 [16]. For DNA analysis standard phenol-chloroform DNA extraction procedure [17] was used for extraction of DNA from collected blood samples. PCR amplification was performed using primer sequences for five STR markers (D21S11, D21S1414, D21S1440, D21S1411, D21S1412) specific for chromosome 21. Two marker sequences were obtained from Pertl *et al.*, 1996 [11] and other primer sequences were obtained from the Genome Database (http://www.gdb.org/) (Table 1). PCR amplification was carried out in separate assays in a total volume of 25 µl contained 50 ng genomic DNA, 200 µM dNTPs, 20 pmol of each primer, 2.5 µl of 10XTaq DNA polymerase buffer, 1.5 mM $MgCl_2$, and 0.5 U Taq DNA polymerase (Roche, Germany). PCR thermal cycle was performed in 32 cycles. Each cycle consists of 95°C denaturizing for 30 s, 60°C annealing for 1 min and 72°C extension for 1 min. The thermal cycles were started with an initial denaturizing of 95°C for 5 min and a final 72°C extension for 10 min.

Amplified PCR products were separated by polyacrylamide gel electrophoresis to classify different alleles according to the size of the DNA sequence. The

Table 1. Name, primer sequence and heterozygosity percentage obtained in this study for five STR markers used for detection of chromosome 21 trisomy

Marker	Primer sequences	Heterozygosity	Reference
D21S11(F)	5′tat tgt agt caa ttc ccc aag tga3′	0.96	Pertl *et al.*, 1996 [11]
D21S11(R)	5′gtt gta tta gtc aat gtt ctc cag3′		
D21S1414(F)	5′aaa tta gtg tct ggc acc cag ta3′	0.88	Pertl *et al.*, 1996 [11]
D21S1414(R)	5′caa tcc ccc aag tga att gcc ttc3′		
D21S1440(F)	5′gag ttt gaa aat aaa gtg ttc agc 3′	0.93	Genome Database
D21S1440(R)	5′ccc cac ccc ttt tag ttt ta 3′		
D21S1412 (F)	5′cgg agg ttg cag tga gat g3′	0.8	Genome Database
D21S1412(R)	5′ggg aag gct atg gag gag a3′		
D21S1411(F)	5′atg atg aat gca tag atg gaa g 3′	0.93	Genome Database
D21S1411(R)	5′aat gtg gtg cct tcc acg c3′		

PCR products (5µl) were mixed with 1µl of loading dye (0.25% bromophenol blue, 0.25% xylene cyanol, 30% glycerol); size marker Ladders (Boehringer Mannheim DNA molecular weight marker X) and processed at 100 Volts for 2 h on 2% agarose gel, 0.5 XTBE buffer. After running gels, they were stained with 0.5 µg/mL ethidium bromide to visualize PCR bands on UV light transilluminator to determine the quality of PCR products.

Evaluation of polymorphism was carried out using denaturing polyacrylamide gel electrophoresis to separate different alleles more carefully. The denaturing PAGE gel consisted of 8% polyacrylamide (29:1 acrylamide/bisacrylamide), 7 M urea, 1 XTBE, 500 µl 10% ammonium persulfate and 50 µl TEMED. The gel was allowed to polymerize at room temperature for 1 h and then was run at 100 Watts. Twenty-five microliter of the PCR products was mixed with 45 µl of loading buffer and denatured at 95°C for 8 min. Denatured samples were cooled on ice and 8 µl of each sample was loaded. The gel apparatus was DNA sequencing unit (30×45 cm) connected to 2000 V power supply clamps. The gel was run at 50°C constant temperature and 100 watts limiting power for about 3 h (BioRad Power/Pak 3000 power supply). Gels were stained and alleles were visualized using standard silver staining procedure.

Each sample has been determined to be homozygous or heterozygous for certain alleles as a result of the number of bands appearing on the polyacrylamide gel. The number of base pairs within a particular marker directly corresponds to the number of tandem repeats in the isolated DNA, which in turn indicates which alleles are present. Because each marker has only one copy per haploid genome, one band indicates homozygosity, while two bands indicate heterozygosity in the diploid human genome. Trisomic samples exhibit either three STR bands or two peaks with ratio of 2:1. The triallelic pattern with three peaks corresponds to three alleles, whereas the diallelic pattern with two peaks corresponds to two identical alleles and the extra product of third allele. Ultroscan XL (Pharmacia LKB biotechnology) was used to measure the density of the PCR product bands as a densitometry quantitative tool. The electrophoretograms of the PCR products were obtained for diallelic cases.

Results

All cases have been tested according to both conventional cytogenetic and molecular analyses. In cytogenetic analysis, all cases had an extra chromosome 21 while three cases showed chromosome 21 mosaics. Molecular diagnosis using five tested markers detected all of Down syndromes in triallelic or diallelic patterns except for one case in which five tested STR markers were uninformative and monomorphic. From 86 cases with Down syndrome, 85% generated three allelic pattern at least for one of five tested maker which is representative for three free chromosome 21, 14% showed diallelic pattern with 2:1 ratio and one case was monomorphic for all tested markers.

The electrophoretograms of the PCR products were obtained for diallelic cases. The frequencies of triallelic, diallelic and monoallelic for each marker have been presented in Table 2. The marker polymorphism and electrophoretogram for D21S1440 are presented in Figure 1. The ratios of the three peaks 1:1:1 and two peaks 2:1 for markers, clearly document the presence of three chromosomes 21. However, when using a normal control, a ratio of 1:1 for the tested locus was observed.

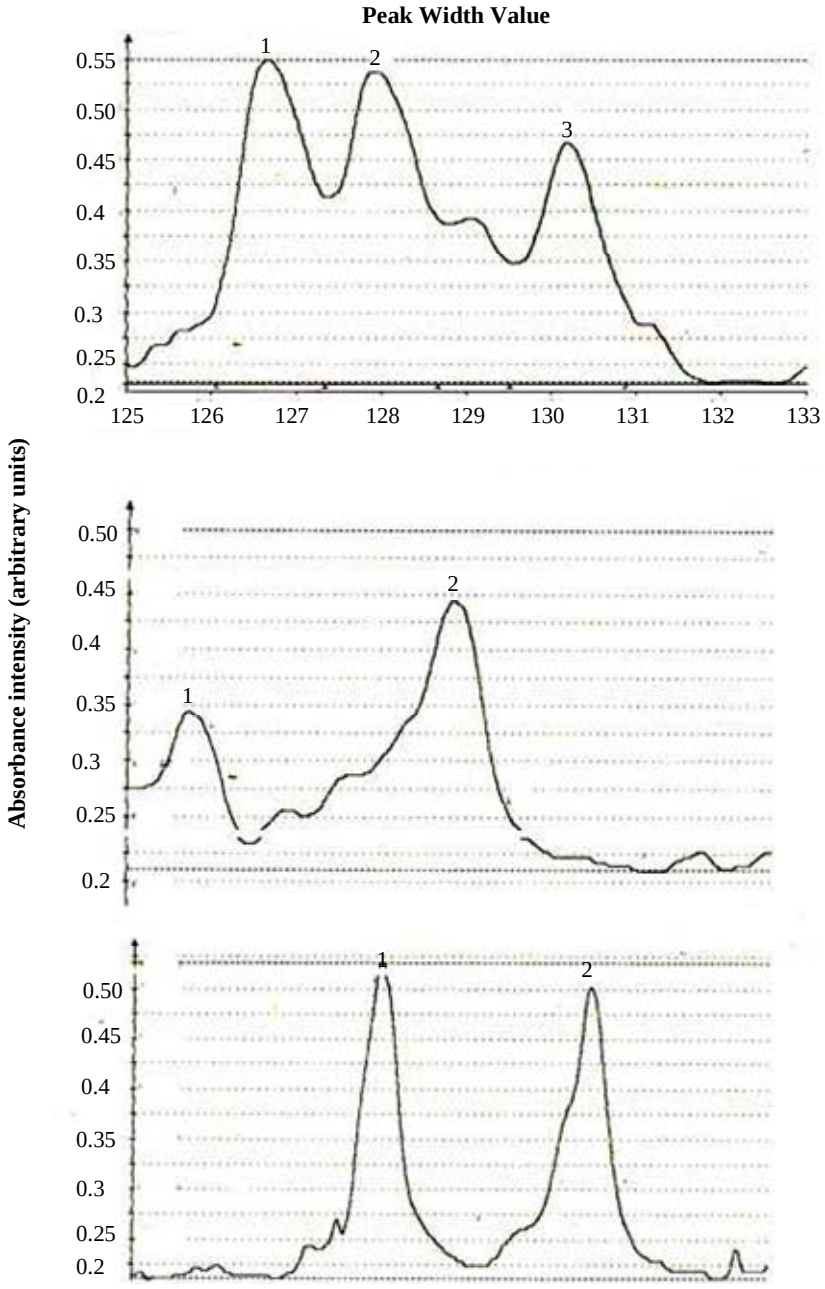

Figure 1. Electrophoretograms of D21S1440 amplified from a triallelic and diallelic patient with Down syndrome and from normal controls. Top panel shows an electrophoretogram of a case with a triallelic pattern. Middle panel shows a trisomy 21 with diallelic pattern with a dosage ratio of 2:1. Bottom panel shows a normal heterozygote disomic sample with a dosage ratio of 1:1.

Each locus pattern has been confirmed by subsequent tests for the other markers, whereas normal case always showed a disomic pattern with a ratio near 1:1. The level of heterozygosis for each tested marker is reported in Table 1. The marker D21S11, presents a high degree of polymorphism compared to other tested markers. The polymorphism of tested markers has been calculated in the tested population. This study shows accuracy and sensitivity of using STRs markers in molecular diagnosis of Down syndrome alone or in conjunction with cytogenetic analysis. It can provide more detail of parental origin and timing of chromosome error of Down syndrome.

Table 2. Tested STR markers and their frequency (percentages) in developing triallelic, diallelic and monoallelic pattern

Marker	Triallelic	Diallelic	Monoallelic
D21S11	65	31	4
D21S1414	53	33	2
D21S1440	30	63	7
D21S1412	32	36	32
D21S1411	39	44	17

Discussion

The results obtained from 86 cases and 100 normal controls present the advantages and feasibility of PCR of highly polymorphic STR markers specific for chromosome 21 as a reliable method to detect Down syndrome within a short time. This technique can successfully determine the triallelic and diallelic trisomy from normal control. In our study there was no abnormal amplification pattern.

PCR-STR assay is a useful tool for detection of Down syndrome especially when more than one marker for each chromosome is employed. The use of several highly polymorphic markers can reduce the likelihood of homozygosity and consequently the frequency of un-informative STR patterns. The selected markers used in this study are highly polymorphic in the human population and are well situated even in Down syndrome critical region (DSCR) that allows detection of translocations and partial trisomeis. In this study we only detect chromosome 21 trisomy because Down syndrome is the most common autosomal aneuploidy with higher viability compared to other autosomal aneuploidies and prevention of this syndrome has a higher priority. Molecular technique has been used to detect other chromosome aneuploidies when the specific markers related to target chromosome has been used.

Common cytogenetic studies of Down syndrome have pooled all cases regardless of parental origin or timing of the chromosome error. Important advantage of new DNA technology is strong correlation of this method to the etiology of Down syndrome in different populations and allowing a useful population-based epidemiologic categorization of Down syndrome cases by the parental origin and the timing of the chromosome error [14,15] by family study of cases. It can help to identify important environmental and maternal health-related risk factors for chromosome 21 non-disjunctions. There is no need to cell culture a common cytogenetic analysis which needs lengthy procedure. It can be performed using stored or dead tissues and samples such as fetal blood, amniotic fluid and fetal tissue in terms of prenatal diagnosis. The advantage of this method is using multiplex PCR technique to reduce the time required for performing diagnosis but it is recommended to run each marker separately especially in the case of prenatal diagnosis. It should be possible to complete the test within one day after collection of the fetal sample. It can reduce the psychological tension of parents while awaiting the cytogenetic analysis.

Acknowledgements

This study was supported by project grants from National Research Center for Genetic Engineering and Biotechnology, Ministry of Science, Research and technology, Tehran, Iran. We thank the staff of the care centers and schools for mentally retarded children, patients and their families and every body who help in taking blood samples and also Mohammad Shahroei, Nasrin Atabak for their help.

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