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Detection of common deafness mutation by maternal plasma cell-free DNA

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Abstract. – OBJECTIVES: The aim is to investigate the use of the ligase detection reaction (LDR) microarray to examine the difference of the single nucleotide between the pregnant woman and the fetus by cell-free DNA in the maternal plasma in congenital deafness.

MATERIALS AND METHODS: The proband and the couples' venous blood samples and the amniotic fluid/ chorionic villi collected from seven deafness families for prenatal diagnosis were analyzed. The cell-free DNA from maternal plasma was examined to determine if they carried the mutations of GJB2 235delC.

RESULTS: Three samples were found to carry the mutation of GJB2 235delC. It is in agreement with the sequencing results. The affected fetuses were suggested to take invasive procedure for confirmation.

CONCLUSIONS: The chip may be a potential method to screen for congenital deafness based on maternal plasma DNA.

Key Words:
Prenatal diagnosis, LDR microarray, Congenital deafness, Maternal plasma DNA.

Introduction

Congenital deafness, mainly autosomal recessive hereditary, is a common clinical genetic disorder with the prevalence of 1-3‰ in newborn[1]. Seventy percent of them are nonsyndromal[2].

Mutation in the GJB2 (gap junction beta-2 protein) gene is the most common cause and contributes to half of the genetic deafness[3]. The GJB2 gene encodes a transmembrane protein called connexin 26 (Cx26), functioning in potassium recycling in the inner ear through the formation of gap junctions[4]. The 235delC mutation is common in Asian population, while the 35delG and 167delT mutation are common in the Caucasian, Jewish populations respectively. Besides, mutation in the other genes encoding connexins, such as GJB6 for Cx30, GJB3 for Cx31, GJA1 for Cx43 and GJB1 for Cx32 are also identified as the molecular etiology for deafness.

A mutation in the SLC26A4 gene is the second common cause and contributes to Pendred Syndrome (PS) and DFNB4 deafness presenting with enlarged vestibular aqueduct syndrome (EVAS)[5]. The SLC26A4 gene codes an anion (chloride/iodide/bicarbonate) transporter called pendrin, expressing in the kidneys, inner ear, and thyroid. SLC26A4 mutations are responsible for approximately 5-7% of the genetic deafness cases. The IVS7-2A > G and 2168A > G are the most common mutations in the East Asian population, including those in China, Japan and Korea[6].

The mutations of MT-RNR1, encoding mitochondrial 12S ribosomal RNA (12s rRNA), may increase the susceptibility to aminoglycoside ototoxicity and is transmitted by maternal inheritance. The prevalence of the 1555A > G mutation is 3.2%-7.5% in Chinese. The mtDNA1494C > G mutation is prevalent in 0.45% of hearing-impaired children[7].

Genetic test is effective for the prevention of congenital deafness. Detection of nine hotspots in four common hearing loss related genes, including GJB2 (35delG, 176_191del16, 235delC,

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299_300delAT), SLC26A4 (IVS7-2A > G, 2168A > G), GJB3 (538C > T), and 12s rRNA (1555A > G, 1494C > G), by a hereditary hearing loss microarray screening method is carried out in Chinese neonates (http://zfxxgk.beijing.gov.cn/columns/81/5/297059.html).

The traditional invasive prenatal testing includes conventional amniocentesis offered at 15-20 weeks, chorionic villus sampling (CVS) at 11-13+6 weeks, and cordocentesis after 20 weeks, respectively.

The procedure-related fetal loss rate is about 1 in 200, or lower depending on the center. Non-invasive prenatal screening through fetal cell-free DNA is a safe option. Recently, massively parallel sequencing (MPS) based on maternal plasma DNA has been proved to be accurate for the screening for fetal trisomies with the low false positive rate[8]. Single molecule counting technologies, such as digital Polymerase Chain Reaction (PCR) and MPS, are potential screening methods for the single gene diseases. But they are expensive, while the data analysis is complicated[9].

DNA microarray technology has been advocated as one of the most powerful approaches for highly parallel, large-scale single-nucleotide polymorphism (SNP) analysis. In recent years, several approaches for SNPs genotyping with DNA microarrays have been designed. These methods are based on nucleic acid hybridization or hybridization coupled with an enzyme-mediated reaction, either by primer extension or ligation[10]. Ligase detection reaction (LDR) microarray is of high sensitivity to detect the low abundance of cell-free fetal DNA in maternal circulation and has the potential to become an efficient method[11].

In this study, a LDR microarray platform was developed to test the commonest deafness mutation GJB2 235delC in maternal plasma of high-risk pregnancies. The difference of the concentration of single nucleotide in cell-free DNA between the pregnant women and the fetuses was compared.

Materials and Methods

Collection of Samples

The amniotic fluid or chorionic villi was collected from 7 singleton pregnancies for prenatal diagnosis. The median gestation was 17+3 (13-24) weeks. Five mL of venous blood from the proband and the couples were obtained respectively. Institutional Review Board approval was obtained at all participating centers and informed consent was obtained from all subjects.

The blood samples were anti-coagulated with EDTA. After a first centrifugation at 1,600 g for 10 minutes and a second centrifugation at 16,000 g within 4 hours after sampling, the supernatants were collected and stored at -80 refrigerator. Cell-free DNA was extracted from the maternal plasma by QIAamp Circulating Nucleic Acid Kit (Qiagen, Valencia, CA, USA) and the DNA fragment size was analyzed by Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA).

Genotyping with Sequencing

The samples, including the maternal leukocytes, the bloods of the father and the proband, and the cells cultured from the amniotic fluid or chorionic villi have been detected using a deafness microarray-platform including nine hotspot mutations (CapitalBio, Beijing, China), following a sequencing determination, and ensure that the families need accept the detection of the mutation GJB2 235delC for the prenatal diagnosis of deafness. Gene sequences were obtained from GenBank (Accession Number NC_000013.10).

PCR Protocol

The target DNA sequences in maternal plasma samples were amplified using PCR. PCR primers for GJB2 235delC were forward primer 5'ACTTTGTCTGCAACACCCT3' (F-p1) and reverse primer 5'CTCATGTCTCCGGTAGGC3' (R-p1) and the size of the PCR products determined by agarose gel electrophoresis was 155bp. The amplification mixture in a final volume of 15μl contained 100ng cell-free DNA template, 1.5 μl 10×Buffer, 1.5 μl 10×dNTP, 0.5 mM of prier F-p1/R-p1, 1.5 μl BSA (Bovine serum albumin) and 0.3μl Tag enzymes (Biocolor, Shanghai, China). PCR was performed on the Gene Amp PCR system 9700 (Applied Biosystems, Foster City, CA, USA). The amplification procedure included denaturation for 5 minutes at 94°C, 30 cycles at 94°C for 30 seconds, annealing for 30 seconds (starting at 64°C, –0.5°C/cycle for the first 14 cycles, maintaining at 57°C for the last 16 cycles), then a final extension at 72°C for 10 minutes. The PCR products were sequenced by ABI PRISM 3100 DNA Sequencer (Applied Biosystems, Foster City, USA).

DNA Microarray Protocol

The LDR microarray was used to detect GJB2 235delC. Five probes including two discriminating probes (containing a zip-code complement sequence on the 5'-end and allele-specific to wild-type or mutant-type on the 3'-end), one common probe (containing a fluorescent label Cy3 on the 3'-end) and two corresponding zip-codes (coupled to discriminating probes at known locations on slides), which are coupled to the slides at known locations, were designed for each mutation, as described previously[12]. The discriminating probe containing a zip-code complement sequence and the common probe containing a fluorescent label can be ligated only if there is perfect complementarity at the junction. In the study, the discriminating probe for the wild is 5'-GATTGGCTCAGATTGCAGACAGGTCACATCCGGCTATGGGCCC-3', while for the mutant is 5'-ATGGGTATACAGACTCGCACGGCTCCACATCCGG -CTATGGGCC-3'. The common probe is 5'-P-TGCAGCTGATCTTCGTGTCCACG -CY3-3'. When hybridized to the universal array containing the zip-codes, the correspondent spots could be seen to give a stronger signal. The positive and negative marker probes were designed for evaluation.

The LDR microarray was performed as described previously[10]. The absolute values of the Cy3 hybridization signal from each probe were obtained using LuxScan 3.0 software (CapitalBio, Beijing, China). When the signal-to-background ratio is >10, the spot can be considered as positive. The local background was subtracted from the intensity of each spot. Each probe spots four replicates, the fluorescence intensities of four spots were averaged. The wild-to-mutant signal ratio (W/M) is the mean hybridization signal of wild-type probe (denoted as W) to mutant probe (denoted as M), and can be employed to detect the genotype of each mutation/polymorphism.

Statistical Analysis

According to our previous experience, for each mutation, the wild-type homozygote, heterozygote and mutant-type homozygote could be perfectly typed into three groups using the W/M ratio. Different probes in different amplified exons may lead to different efficiencies in hybridization showing different fluorescence intensities. The W/M ratio produced good assignment of sample genotype, with W/M > 20:1 for normal subjects, nearly 1:1 for heterozygote, and < 1:20 for homozygous mutation. If the signal ratio is between 2:1 and 8:1, the fetus is determined as high risk.

Results

The PCR product was determined by agarose gel electrophoresis. The size of the fragment is consistent with the expected. Three samples were found to carry the mutation of GJB2 235delC that matched 100% with those obtained using direct DNA sequencing. No false positive results were found. It implied that this microarray procedure can examine the differences of the single nucleotide between the pregnant women and the fetus by cell-free DNA in the maternal plasma in congenital deafness.

In the research, each test was performed at least two times, and each probe spots four replicates. Signals we obtained showed no significant differences in the same patient detecting the same mutation (data not shown). We found the results of the microarray to be reliable.

Discussion

Since Dennis Lo et al discovered the presence of fetal DNA in maternal plasma in 1997[13], great efforts have been made in the analysis of cell-free fetal DNA in maternal blood. Fetal sex determination for sex-linked disorders, fetal Rhesus D gene detection and fetal aneuploidies such as Down syndrome have been applied for clinical application and showed high diagnostic accuracy and the advantage of safer and cost-effectiveness[8,14,15]. However, the detection for single gene disease, especially for the autosomal recessive disorder, still needs a vast of work before the clinical translation because of the high cost and the complicated data analysis. To determine the risk of a fetus being affected by a single-gene disease through maternal plasma DNA analysis may need further study[16].

In this work, we developed a LDR microarray to detect two commonest deafness mutations through maternal circulation. By analyzing the M/W signal ratio of the maternal genetic DNA and the cell-free DNA in maternal plasma in one time, the risk of the fetus suffering from deafness is estimated. The microarray procedure used in this study was demonstrated to be reliable by comparing with sequencing method, and showed it can be used to detect the differences of the single nucleotide between the pregnant women and the fetus.

Compared with other gene-specific arrays, this microarray gives researchers the freedom to de-

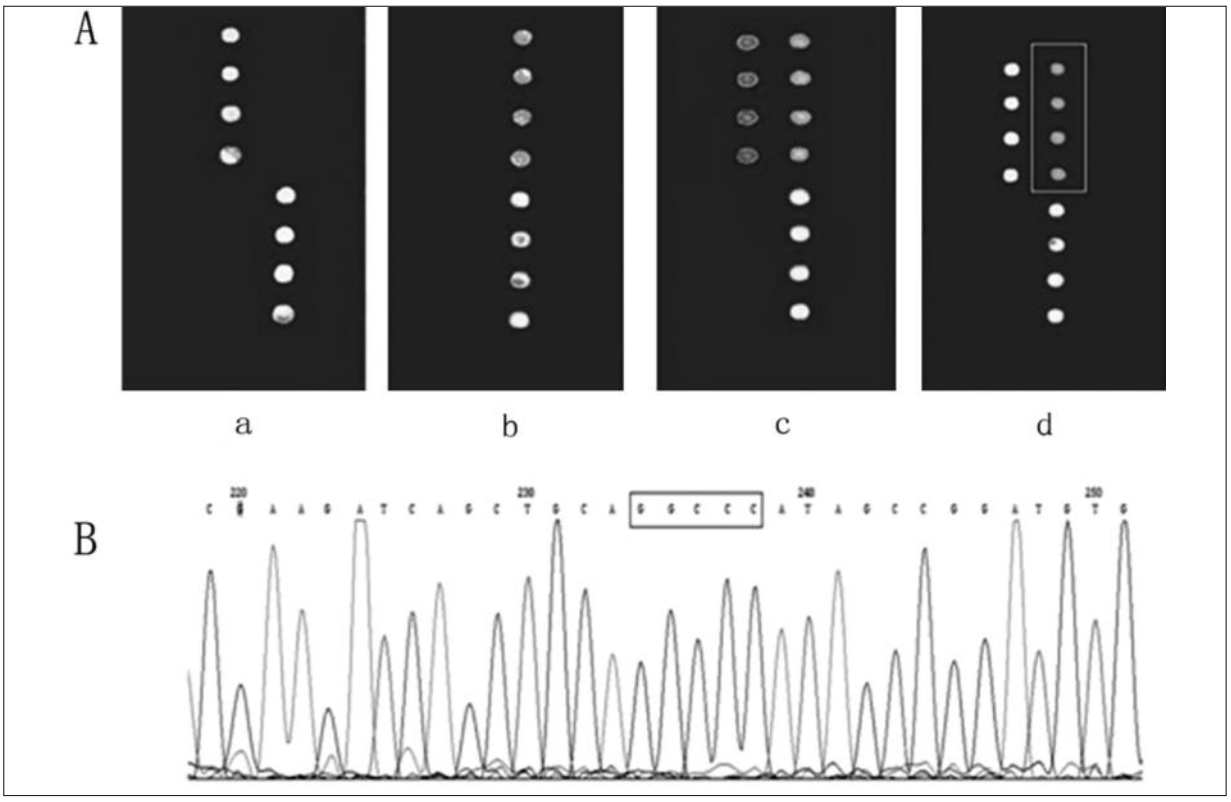

Figure 1. Characteristics evaluation of the LDR microarray procedure. ***A,*** In each picture, the four spots in the bottom right are for positive control, and the bottom left for negative control. The four spots in the upper right are for the mutant, and the upper left for the wild. ***a,*** Both the mother and the fetus are mutant-type homozygote. ***b,*** Both the mother and the fetus are wild-type homozygote. ***c,*** Both the mother and the fetus are heterozygote. d. The fetus carries the wild allele, while the mother is the mutant-type homozygote. In this situation, the fetus is heterozygote and can avoid the invasive prenatal diagnosis. ***B,*** The genotyping result comparisons between the microarrray and the sequencing method. The marked part of the picture is the genotyping result of the GJB2 235delC locus of the amniotic fluid (reverse sequencing primers).

tect different specified sites based on the same array and avoids the need to optimize operation conditions each time[5]. Also, it has the advantage of increasing the detection of more mutations without having to refabricate the entire array that means the whole nine hotspots of deafness can be added into this platform for routine diagnosis.

Compared with other approach to prenatal diagnosis of autosomal recessive diseases through detection of a paternally inherited fetal mutation in maternal plasma, the procedure only need maternal blood, which is much easier for clinical protocol.

However, there are two limitations of the platform. First, it would not provide information on whether the fetus has inherited the maternal mutation, and second, it could not be used when both the father and mother carry the same mutation. So when the mother is heterozygous, it is better to identify the paternal genotype, if the genotype is homozygous wild, the fetus would be safe. In fact, we find that there are minute changes of the W/M ratio according to the different fetal genotype in our study, which is in accordance with the literature reported[9,16]. It is implied there may be a cutoff W/M ratio to identify the fetal genotype; however, it needs huge data accumulation. There are also reports to distinguish fetal genotype by screening the informative SNPs linking to the mutation[17,18], but it is individualized and hard to carry out in clinics.

Conclusions

This study estimated the role of LDR microarray in noninvasive prenatal diagnosis of deafness in a small scale samples. The microarray can detect the difference of the deletion between the pregnant women and the fetus by cell-free DNA in the maternal plasma. The validity of the DNA microarray was showed 100% agreement with di-

rect sequencing. With an assay time within one day, the LDR microarray may be a potential way to screen for congenital deafness based on maternal plasma DNA. However, more data is needed for evidence.

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Conflict of Interest

The Authors declare no conflict of interest.

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