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Reproductive management through integration of PGD and MPS-based noninvasive prenatal screening/diagnosis for a family with *GJB2*-associated hearing impairment

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A couple with a proband child of *GJB2* (encoding the gap junction protein connexin 26)-associated hearing impairment and a previous pregnancy miscarriage sought for a reproductive solution to bear a healthy child. Our study aimed to develop a customized preconception-to-neonate care trajectory to fulfill this clinical demand by integrating preimplantation genetic diagnosis (PGD), noninvasive prenatal testing (NIPT), and noninvasive prenatal diagnosis (NIPD) into the strategy. Auditory and genetic diagnosis of the proband child was carried out to identify the disease causative mutations. The couple then received in-vitro-fertilization treatment, and eight embryos were obtained for day 5 biopsy. PGD was performed by short-tandem-repeat linkage analysis and Sanger sequencing of *GJB2* gene. Transfer of a *GJB2*c.235delC heterozygous embryo resulted in a singleton pregnancy. At the 13th week of gestation, genomic DNA (gDNA) from the trio family and cell-free DNA (cfDNA) from maternal plasma were obtained for assessment of fetal chromosomal aneuploidy and *GJB2* mutations. NIPT and NIPD showed the absence of chromosomal aneuploidy and *GJB2*-associated disease in the fetus, which was later confirmed by invasive procedures and postnatal genetic/auditory diagnosis. This strategy successfully prevented the transmission of hearing impairment in the newborn, thus providing a valuable experience in reproductive management of similar cases and potentially other monogenic disorders.

preimplantation genetic diagnosis (PGD), noninvasive prenatal testing (NIPT), noninvasive prenatal diagnosis (NIPD), *GJB2* (encoding the gap junction protein connexin 26), hearing impairment

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Hereditary hearing loss is a highly heterogeneous disease affecting approximately one in 500 newborns [1]. Prenatal diagnosis of hereditary hearing loss relies on genetic testing samples obtained from amniocentesis or chorionic villus sampling. These procedures involve minor risk of fetal loss, and cause mental stress to patients and their family [2,3]. New technologies such as pre-implantation genetic diagnosis (PGD) and non-invasive prenatal diagnosis (NIPD) can prevent the recurrence of the disease and avoid repeated invasive diagnosis, thus offering alternative reproduction choices to women with the risk of pregnancy with hereditary hearing loss. Since 2009, PGD for hearing impairment has been performed in Europe and Taiwan [4–6]. For instance, Altarescu et al. reported PGD for nonsyndromic deafness by Sanger sequencing and short tandem repeat (STR) linkage analysis of polar body and blastomere [7]. Recently, Meng et al. also demonstrated that by massively parallel sequencing (MPS) of genomic DNA (gDNA) from a family with autosomal recessive congenital deafness and maternal plasma DNA, the fetal condition of the deafness causative mutation can be accurately predicated by NIPD using single nucleotide polymorphism (SNP)-based chromosome phasing and haplotype-assisted analysis [8]. Well-designed integration of such technologies can improve the reproductive care for patients and their families. In contrast, providing different tests, lacking in strategic integration and less-considered workflow, may pose a great challenge for both patient and clinicians to understand the technical backgrounds and testing results. In case of discrepant results between different tests, misunderstanding of the disease may hinder the timely treatment of disease.

In this study, we aimed to develop a preconception-to-neonate testing strategy for the couple who bore the child with the hearing loss. We provided auditory and genetic diagnosis for a family with *GJB2* (encoding the gap junction protein connexin 26)-associated non-syndromic hearing loss. More importantly, we coordinated services at different medical centers to provide various preconception and prenatal tests, including PGD, noninvasive prenatal testing (NIPT), NIPD, and postnatal confirmation.

1 Materials and Methods

1.1 Study overview

Auditory and genetic diagnosis was performed by the Otolaryngological Department of the PLA General Hospital. In vitro fertilization (IVF)-PGD and prenatal healthcare was collaborated by the PLA General Hospital and the Reproductive Hospital Affiliated to Shandong University. NIPT and NIPD were performed at the Clinical Laboratory of BGI. Post-test auditory and genetic confirmation was performed at the Chinese PLA General Hospital and Clinical Laboratory of BGI-Shenzhen. The entire process was coordinated and overall genetic counseling was provided by clinicians from the Otolaryngological Department of the Chinese PLA General Hospital. Specific pre-test counseling and opt-out choice was provided to the family before each individual test, and informed consent was obtained before each test. Ethical approvals were obtained from the Chinese PLA General Hospital Institution Review Board of the Ethics Committee, the Reproductive Hospital Affiliated to Shandong University Institutional Review Board, and the Institutional Review Board of BGI for this research.

1.2 Auditory and genetic diagnosis of *GJB2*-assocatied hearing loss

For auditory diagnosis of the proband child, physical examination, tympanometry, acoustic reflex, normal auditory brainstem response (ABR), distortion product otoacoustic emission (DPOAE), 40Hz auditory event related potential test (AERP) and auditory data analysis was carried out using the standard protocol described above [9]. For genetic diagnosis, gDNA was extracted from the father, the mother and the proband child. Primer pairs were synthesized to amplify the mitochondrial DNA (mtDNA) *12SrRNA*, the second exon of *GJB2*, and exon 8, 10, 15, 18 of *SLC26A4* including the flanking sequences [10]. PCR products were directly sequenced by ABI 3730 Sequencer (Applied Biosystems).

1.3 IVF-PGD treatment

After completing the necessary physical examination and biochemical and hormone test, the couple received treatment of GnRH (gonadotropin-releasing hormone) agonist and recombinant FSH (Gonal-F). Follicles were monitored by ultrasound. Fourteen mature oocytes were retrieved for intracytoplasmic sperm injection (ICSI) treatment and eight embryos were obtained. Blastocysts' biopsies were conducted on day 5 or 6.

Preimplantation diagnosis of *GJB2*c.235delC/c.299-300delAT was based on multiplex nested PCR and short tandem repeat (STR)-based linkage analysis. Seven STR markers (*GJB2*-AT2, D13S1830, D13S633, D13S250, D13S1275, D13S232, and D13S292) and associated forward/reverse primers were selected from a previously reported study [7] for linkage analysis (Supplemental Material). Size discrimination of the STR makers was achieved by capillary electrophoresis analysis (3500 Genetic Analyzer Capillary Array, 36 cm). gDNA of the parents and proband was extracted from their peripheral blood (QIAgene) following the manufacturer's instruction. Blastocysts' analysis was carried out by a two-step multiplx PCR method (Supplementary Method and Table S1 in Supporting Information). PCR products were diluted and run on an ABI Prim 3500 Avant automated sequencer, and analyzed using GeneMapper software using the default setting and follow-

ing the manufacturer's instructions. To directly show the *GJB2* genotype of the embryos, primers were also used to amplify the second exon of *GJB2* (Table S2 in Supporting Information) for direct sequencing.

1.4 NIPT for fetal aneuploidy

At the 13th gestational week, 5 ml peripheral blood of the parents and 2 ml of the proband were collected into tubes containing EDTA. For NIPT, 2.5 ml of maternal blood was ice-centrifuged twice to extract plasma as described above [11], while the remaining samples were used for NIPD. Plasma was frozen and delivered to the BGI Clinical Laboratories where it was prepared for cell-free DNA (cfDNA) extraction, library construction, quality control, and pooling. Low-coverage whole-genome sequencing was performed with the BGISEQ-100 platform. This platform has been approved by the China Food and Drug Administration for NIPT. A barcode tracking system was employed during sample preparation. Eleven bases of sequencing reads were trimmed and aligned to a universal unique read set incised from the human reference genome (hg18, NCBI build 36). At least three million unique "after-alignment" sequencing reads were used for bioinformatics analysis. A binary hypothesis *t*-test and a logarithmic likelihood ratio L-score between the two *t*-tests were used to classify fetal autosomal aneuploidy of trisomy 21 (T21), trisomy 18 (T18), and trisomy 13 (T13), as described earlier [12].

1.5 NIPD of *GJB2*-associated hearing loss

gDNA of both parents and the proband was extracted from peripheral blood (TIANamp Blood DNA Kit, TIANGEN) and fragmented by sonication (Covaris), yielding fragments with an average size of 200–250 bp. A sequencing library of gDNA was prepared by four cycles of PCR. This was performed with index primers following procedures of end-repair, "A"-overhanging and adapter-ligation. cfDNA extracted from maternal blood was used to prepare the library with the KAPA library preparation kit (KAPA Biosystems) according to the manufacturer's protocol. After the ligation of the adapter, eight cycles of PCR were performed with index primers.

Enrichment of the *GJB2* target region was performed by a customized NimbleGen SeqCap EZ array (4 M region covering exons of *GJB2* and highly heterozygous SNPs located 1 Mb upstream and downstream of *GJB2*) following the manufacturer's instructions. Post-capture libraries were amplified by a 14-cycle PCR, and then sequenced using a HiSeq2500 platform (Illumina) with 90-bp paired-end sequencing.

BWA software (0.7.12) was used to map the paired-end reads to the human reference genome (hg19, build 37) with the default parameters. Then the SNPs calling were performed using the GATK software and the filter criteria of depth $\geqslant$40 and quality value>20. SNPs, homozygous in both parents, but with different genotypes, were used for calculating fetal DNA fraction using the formula: fetal DNA fraction=$2d_{\text{father}}/(d_{\text{mother}}+d_{\text{father}})$, where d_{mother} and d_{father} stand for the allele count of the special base of the mother and the father respectively. A strategy of trios analysis based on Mendel′s law was used to deduce the parental haplotype, and a Hidden Markov Model and Viterbi algorithm were used to calculate the inherited status of maternal haplotype or paternal haplotype respectively [8,13,14].

Amniocentesis was performed at the 19th week of gestation and DNA from 20 ml amniotic fluid was extracted. PCR and Sanger sequencing were applied to analyze the *GJB2* gene mutations, using the primers and reaction conditions in Supplement Materials.

1.6 Auditory examinations of newborns

Twenty-four hours after delivery, the newborn child received neonatal hearing screening by AABR and DPOAE [15]. At two months after delivery, the child received auditory diagnosis by ABR and 40Hz following the standard protocol described above [9].

2 Results

2.1 Case presentation

The parents of of a four-year-old child with delayed lingual development and hearing impairment was referred to our department in July 2010 (Figure 1). At the time of visit, the proband child was wearing a hearing aid and had severe recurrent otitis externa. The proband was born by natural pregnancy with no known family history of genetic disease except a cousin with Down syndrome (T21). Auditory diagnosis (ABR, DPOAE, 40Hz AERP, auditory steady-state response (ASSR), and visual reinforcement audiometry) of the proband indicated severe bilateral sensorineural hearing impairment. The proband then received temporal high resolution computed tomography (HRCT) and brain magnetic resonance imaging, and was suggested to receive cochlear implantation, which was performed in July 2011. Meanwhile, the parents and proband were tested for *GJB2, SLC26A4,* and *12SrRNA* to identify the disease-causative mutation. Sanger sequencing of these genes confirmed that the proband contained compound heterozygous mutations of c.235delC/c.299-300delAT of the *GJB2* gene, while both parents were carriers of the disease (Figure 2). After genetic counseling, we organized a whole trajectory of reproductive management for this couple, including IVF-PGD, NIPT/NIPD, and postnatal confirmation.

2.2 IVF and PGD

The couple received reproductive counseling and treatment

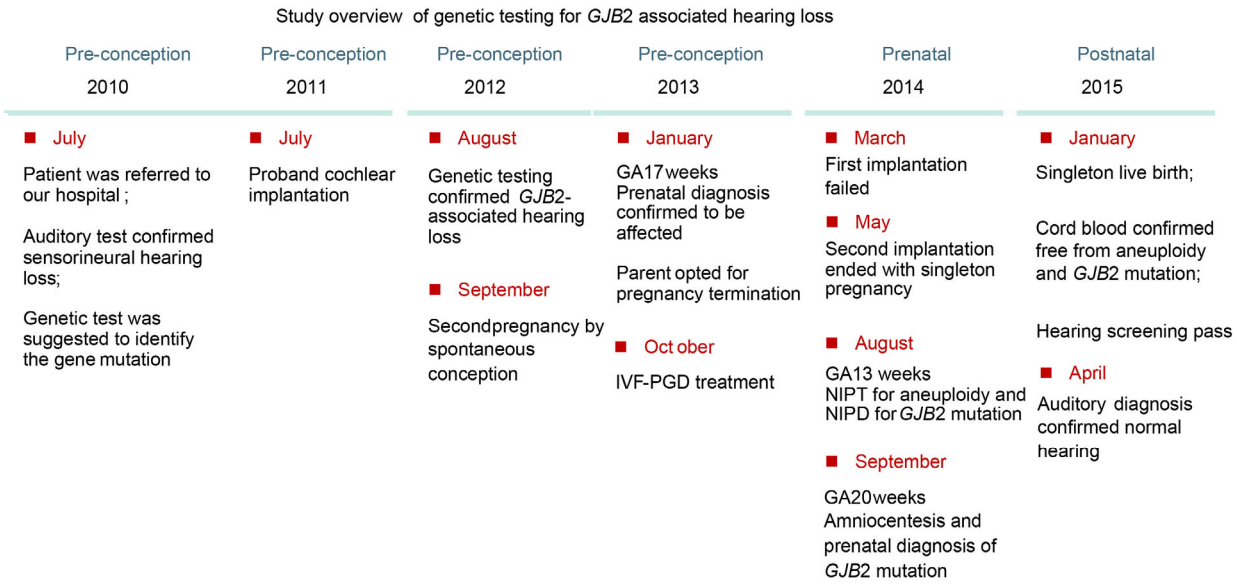

Figure 1 Timescale of the reproductive management of *GJB2*-associated hearing loss in a family desired to have a hearing normal child. GA, gestational age.

of IVF and PGD in Oct 2013. One IVF cycle was performed by a standard IVF protocol, and 14 oocytes were retrieved through ICSI treatment. Eight embryos were obtained for day 5 biopsy. DNA was extracted from the trophectoderm cells of each embryo and analyzed for *GJB2* and STR makers by Sanger sequencing and multiplex nested PCR. Both DNA sequencing and linkage analysis showed that five among eight embryos were carriers of the *GJB2*-associated hearing loss, while the remaining three embryos contained compound heterozygous mutations (Figure 2). After genetic counseling, the couple decided to accept embryo transfer of a carrier embryo. Thus single embryo transfer using the embryo #13 was performed in May 2013, resulting in a successful singleton pregnancy confirmed by hCG (human chorionic gonadotropin) 12 days after transfer and by ultrasound examination 35 days after transfer.

2.3 Prenatal testing for fetal aneuploidy and *GJB2* associated hearing loss

The pregnancy was classified as high-risk owing to the family history of hearing loss, IVF treatment, and a T21 family member in the pedigree. At the 13th week, 5 ml of maternal blood was collected with informed consent, and plasma DNA was tested for fetal aneuploidy and *GJB2*-associated hearing loss by NIPT and NIPD respectively. Low risk of T21 (t=–0.98), T18 (t=0.54), and T13 (t=–0.04) was received by NIPT, and no other pathogenic chromosome deletion or duplication was observed (Figure 3 and Figure 4).

To test for *GJB2*-associated mutation by NIPD, approximately 164 kb target region covering the exons of *GJB2* sequence and flanking SNPs was sequenced in the mother, the father, the proband and the maternal plasma, using a customized Nimble Gen SeqCap EZ array. SNPs obtained from the parents and proband were used to construct the parental haplotype, while the successfully phased SNPs in the maternal plasma cfDNA were used for fetal haplotype deduction. The fetal haplotype was deduced with a Hidden Markov Model via two steps: (i) predicting the paternal transmitting haplotype by SNPs heterozygous in father, while homozygous in mother; (ii) predicting the maternal transmitting haplotype by SNPs heterozygous in mother, while homozygous in father.

Table 1 shows the statistics of target sequencing after removing duplication. A mean sequencing depth of 130.91x, 116.38x, and 176.04x was respectively obtained in the *GJB2* gene from the mother, father, and proband, resulting in 436, 443 and 423 SNPs for haplotype analysis. One hundred and sixty four SNPs were successfully phased, of which 73 SNPs were used to deduce paternal inherited haplotype and 91 SNPs were used to deduce maternal inherited haplotype. A total of 3.94 Gb raw data was obtained from sequencing maternal plasma DNA. After filter of duplication, a mean depth of 188.4x was obtained in the *GJB2* gene, covering 99.56% of the target region. The fraction of fetal cfDNA in the maternal plasma was estimated as 15%. Prediction of fetal haplotype showed that the fetus inherited the *GJB2*c.235delC allele from the mother, and the non-pathogenic allele from the father; thus indicating a carrier status (Figure 5A). To confirm the results, amniocentesis was performed at the 20th week, and prenatal diagnosis of fetal *GJB2* gene by Sanger sequencing was applied. These showed that the fetus was, as expected, a carrier of *GJB2*c.235delC (Figure 5B).

2.4 Auditory and genetic examinations after birth

In January of 2015 a male baby weighing 3,150 g was de-

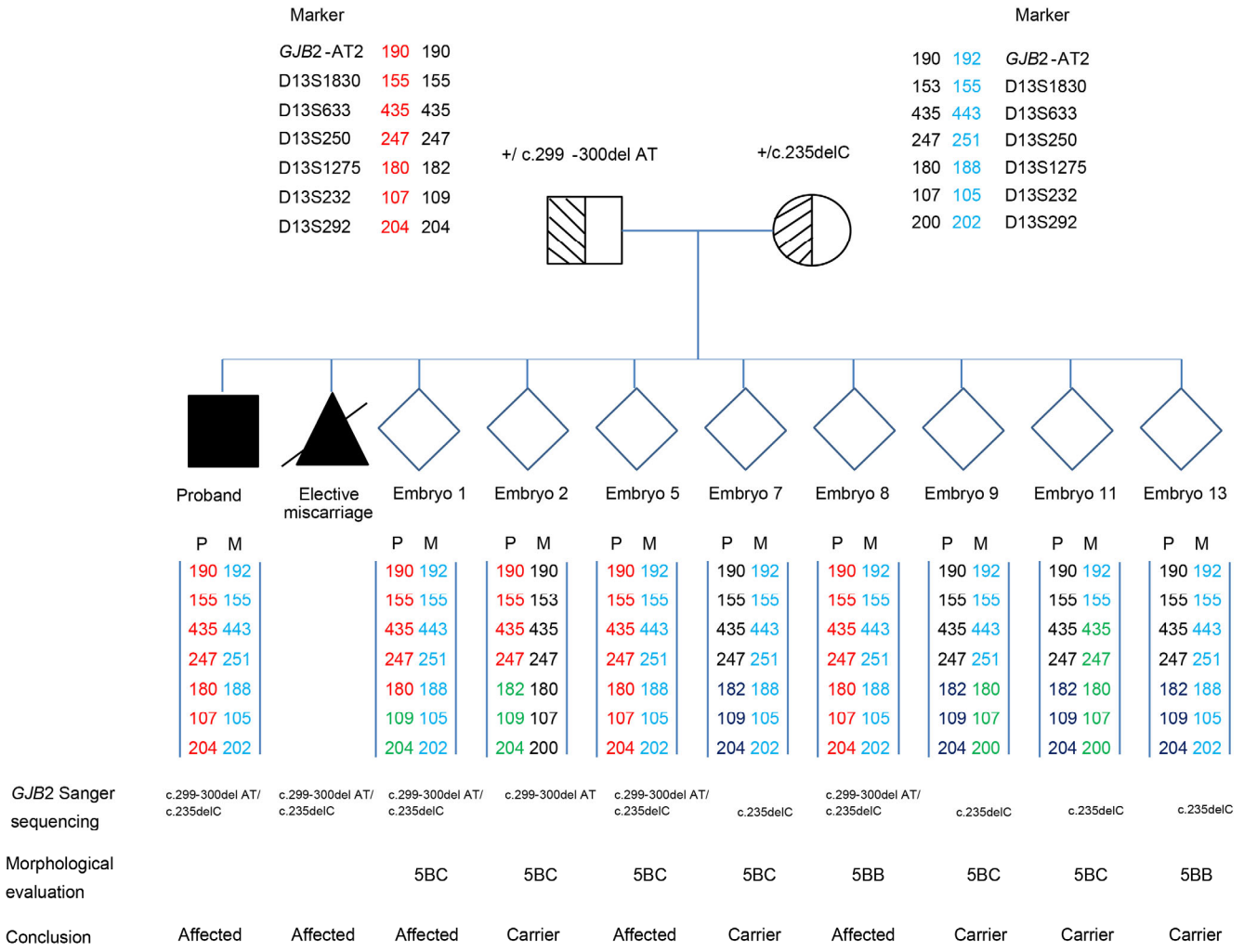

Figure 2 Genetic diagnosis of the hereditary hearing loss in the family and PGD of the *GJB2* mutation in eight embryos after IVF treatment. Sanger sequencing of *GJB2* showed that the parents were carriers of c.299-300delAT and c.235delC respectively, whereas the proband and the first fetus inherited both alleles from the parents. STR analysis using seven STR markers showed that five embryos were carriers of the disease (#2, #7, #9, #11, #13), and three embryos (#1, #5, #8) were compound heterozygous of *GJB2*c.299-300delAT/c.235delC. Development of embryos was morphologically evaluated on day 5 or day 6. The sizes of PCR products for STR markers are shown for the couple, the proband and embryos. The alleles in red and blue color indicated mutated alleles from father and mother, respectively. The green alleles indicate cross-over events.

Table 1 Sequencing characteristics of NIPD of *GJB2*-associated hearing loss in the family

	Data (Gb)	Total SNP markers	*GJB2* region for targeted sequencing				
			Reads mapped to target region	Mean depth	Depth≥20x (%)	No. of SNP markers	Phased SNPs
Mother	2.36	11251	0.33M	130.91x	99.11%	436	164
Father	1.93	12320	0.29M	116.38x	98.66%	443	164
Proband child	2.91	12019	0.44M	176.04x	99.24%	423	164
Plasma	3.94	11670	0.77M	188.40x	99.56%	441	164

livered at the 39th week of gestation with apparently normal phenotypes. The newborn baby passed the neonatal auditory screening by DPOAE and AABR at 24 hours after delivery. Therefore, PGD and NIPT/NIPD results were verified by neonatal phenotypes and genetic confirmation. Further diagnostic audiology tests at two months after birth confirmed that the newborn is with normal hearing (Figure S1 in Supporting Information).

3 Discussion

We report the successful birth of a child free of *GJB2*-associated hereditary hearing loss through a preconception-

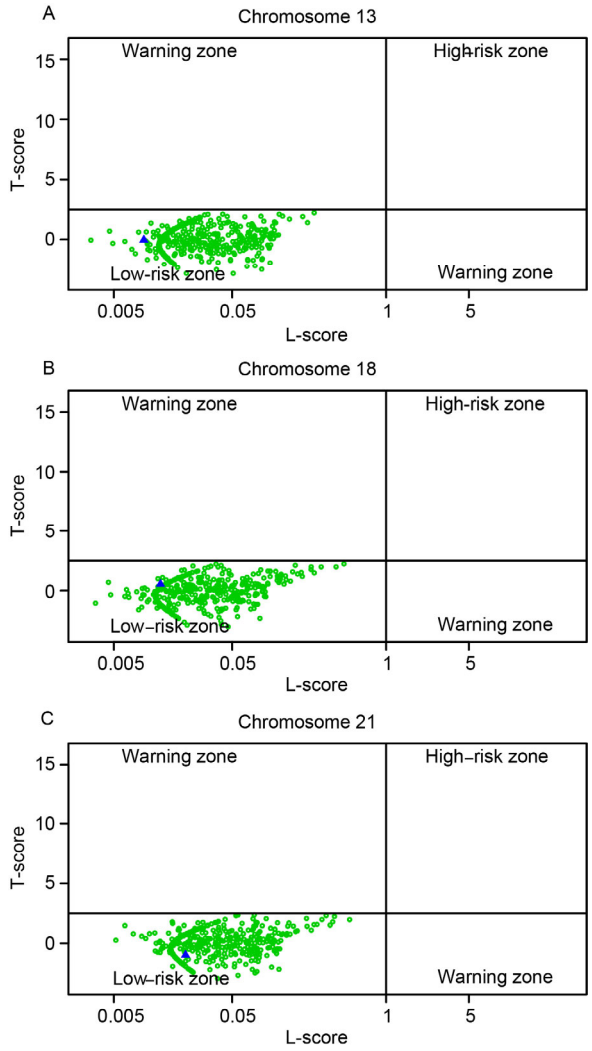

Figure 3 NIPT results at 13th gestation week for testing T21 (A), T18 (B), T21 (C). The tested sample representing by the blue triangle had the T-score <0.25 and L-score <1 of all three chromosomes, suggesting the low risk of aneuploidy of these three chromosomes. Green circles stands for control sets with known euploid karyotypes.

to-newborn care solution integrating IVF-PGD and next-generation sequencing (NGS)-based NIPT/NIPD. In this clinical case, the family was facing a difficult situation involving a proband child with *GJB2*-related hearing loss, a sibling with Down syndrome, and a singleton pregnancy conceived by IVF treatment. For the patient's best benefits, a coordinated solution covering disease diagnosis, preimplantation testing, prenatal testing, and neonatal confirmation was customized for this family. These efforts effectively reduced the patient's difficulty in selecting proper clinical care. Owing to this integrated management, precise genetic counseling was quickly provided to the patient, collectively contributing to a good clinical outcome.

In this study, PGD was performed using both STR-based linkage analysis and direct sequencing of the *GJB2* mutation. This approach reduced such technical risks as allelic drop out, amplification failure, and recombination event [7]. Thus the accuracy of this PGD method can be guaranteed. A day 5 blastocyst, rather than a day 3 blastocyst, was used for biopsy in order to have developmentally more competent embryos for diagnosis. Such an approach has been suggested to have technical advantages and an improved pregnancy rate [16,17]. Nonetheless, misdiagnosis can occur during PGD, and several types of errors causing adverse pregnancy outcomes have been reported [18]. Recently the haplotype-assisted NIPD was successfully applied in the prenatal diagnosis of maple syrup urine disease [13]. Several other monogenic diseases have also been validated with this haplotype-assisted method. This approach sequenced the entire exons of the target gene and the flanking heterozygous SNPs, thus providing over 99% accuracy when constructing fetal haplotype [8,19–22]. In this study, the the father and mother had a low tolerance for invasive testing due to a tragic experience in a previous pregnancy. Thus the haplotype-assisted NIPD was adopted and it was offered as an option in addition to the standard procedures. After counseling with regard to the benefit and limitation of the technology, the couple decided to receive NIPD for earlier anxiety relief. The NIPD result was in concordance with the PGD result and invasive confirmation which confirmed the high accuracy and reliability of the technology. At the end of the management, clinical outcomes were evaluated by newborn hearing screening and final auditory diagnosis. The results of these provided the definitive evidence needed to safely discharge the case from the hospital.

At the time of sample collection for NIPD at the 13th gestational week, a maternal blood sample was also used to evaluate fetal chromosome aneuploidy by NIPT. This is important because IVF pregnancy has a high probability of chromosomal abnormality in embryos [6,23–24]. Moreover, the family reported that a relative having Down syndrome was in the pedigree. Thus the current pregnancy was classified as high-risk. Owing to the integration of NIPT and NIPD at an early prenatal stage, chromosomal aneuploidy and *GJB2* mutation were both evaluated in the fetus with one-step of sample collection. This provided the most comprehensive results for relief of the patient's anxiety.

The successful PGD in this study required extensive effort in optimizing appropriate STR loci and PCR conditions. This process was time-consuming and laborious, leading to prolonged turnaround time and elevated mental stress for the patient. Furthermore, the current PGD protocol cannot be used to identify chromosome aneuploidy of candidate embryos after IVF treatments. Recent application of single-cell whole-genome amplification and NGS in preimplantation testing showed the detection of chromosomal aneuploidy, copy number variant, and even single-base mutation in embryos [25–28]. Thus it is possible to simultaneously test for chromosome aneuploidy and single gene disease using NGS-based PGD and preimplantation genetic

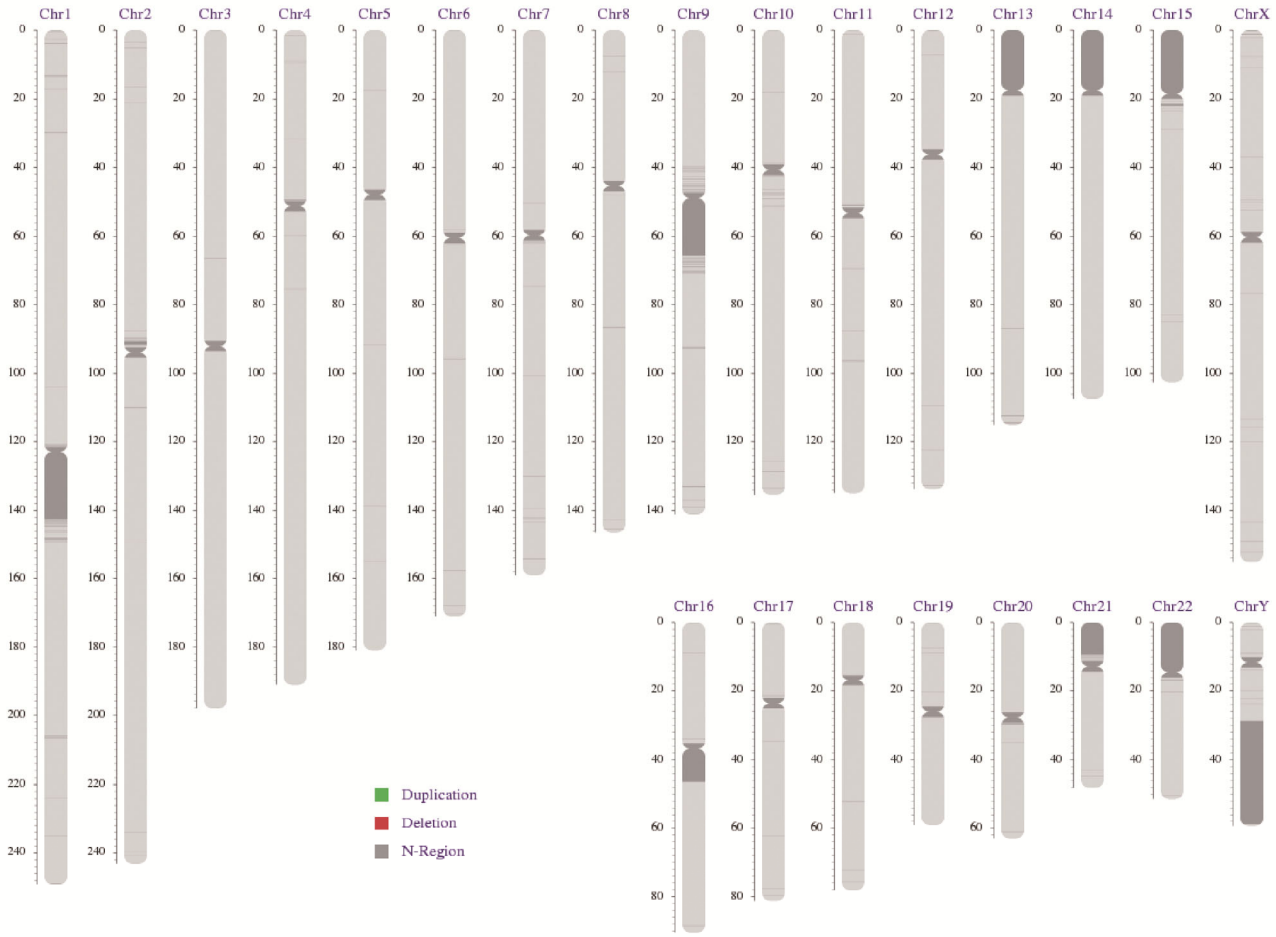

Figure 4 NIPT results at 13th gestation week for testing chromosome copy number variants. No. >10 Mb duplication marked by green events or deletion marked by red events were not detected.

screening (PGS). For future development, NGS-based pre-conception-to-neonate tests may add extra benefits to the clinical utility by reducing technical barriers between different tests.

The cost of this management strategy is expensive, which can be a major obstacle for clinical application. However, economically this may still be a better choice compared to the expense of cochlear implantation and lifelong rehabilitation, which is the only treatment for congenital profound hearing impairment. Moreover, as the sequencing cost rapidly reduces, future application of this trajectory could be more cost-effective, especially if NGS-based tests are used to cover different tests from preconception to postnatal stage.

As a validation study of the pre-conception-to-neonate care strategy, the accuracy of the genetic methods (i.e. PGD, NIPT, and NIPD) was verified by several confirmatory tests. With accumulated evidence and proven efficacy, some of these confirmatory tests can be omitted in the future application to reduce the patient's burden. Currently, such invasive procedures as chorionic villus sampling (CVS) and amniocentesis remain the golden standard of prenatal diagnosis, and NIPD cannot substitute for conventional invasive procedures. However, the result in this study, as well as several previous validation studies, [8,13–14,19–22] showed that NIPD has the feasibility of providing accurate and reliable diagnosis just as invasive tests. This can be particularly important to patients receiving IVF-PGD treatment or with conditions inappropriate for invasive procedures. Further studies are still required to evaluate the potential of replacing invasive testing in this subgroup of pregnancy by NIPD.

Supported by the NGS technology, the benefit of the testing strategy presented in this study is obvious. Nevertheless, the strategy also raised challenges of integrating new technologies into clinical practice, especially when the clinical utility is not fully evaluated. Further study is still required to validate the strategy and standardize the clinical workflow. In conclusion, the presented case demonstrates an integrated strategy of reproductive management of

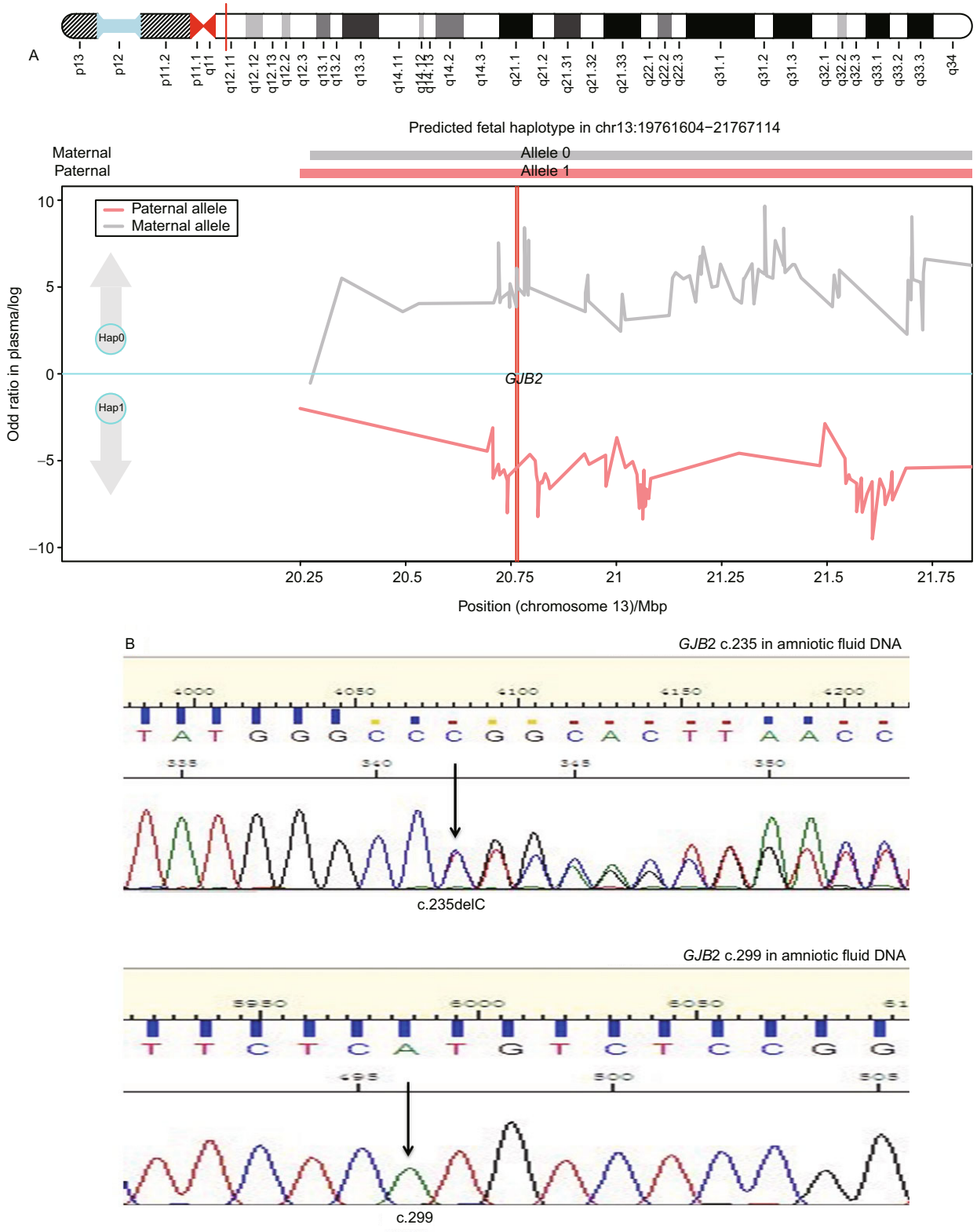

Figure 5 NIPD and invasive confirmation results of *GJB2*-associated hearing loss in the fetus. A, the deduced fetal haplotype of chromosome 13 where *GJB2* locates. X-Axis represents the loci on chromosome 13, and Y-axis represents the logarithmic values of the odd ratios in different combinations of the fetal haplotype. The paternal-originated haplotype and maternal-originated haplotype was shown by the grey and red lines respectively. The maternal haplotype 0 was the pathogenic haplotype which carries the c.235delC mutation, while maternal haplotype 1 was nonpathogenic; paternal haplotype 0 was the pathogenic haplotype which carries the c.229-300delAT mutation, and paternal haplotype 1 was nonpathogenic. NIPD result showed that the fetus carries the maternal haplotype 0 and paternal haplotype 1, thus the fetus carries the heterozygousc.235delC mutation. B, Sanger sequencing using amniotic fluid DNA confirmed the NIPD results, showing the heterozygous c.235delC mutation and wild type of c.229-300delAT.

GJB2-associated hearing loss. This approach can effectively stop the transmission of the hearing-related disease to the next generation, and has a potential to be used for other genetic disorders.

The authors declare that they have no conflict of interest.

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Supporting Information

Materials and methods

Figure S1 Audiology tests by ABR and 40Hz AERP at two months after birth confirmed the newborn is with normal hearing. A, ABR result of left ear at threshold of 20dBnHL. B, ABR result of right ear at threshold of 20dBnHL. C, 40Hz AERP result of left ear at threshold of 30dBnHL. D, 40Hz AERP result of right ear at threshold of 30dBnHL.

Table S1 Primer sequences for multiplex nested PCR analysis

Table S2 Primer sequences for confirmation of *GJB2* mutation by Sanger sequencing using the DNA extracted from amniotic fluid

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