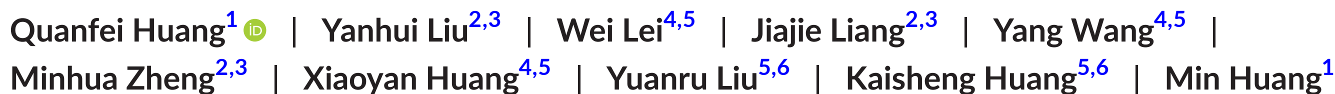
Received: 9 September 2022 | Revised: 11 December 2022 | Accepted: 18 December 2022

DOI: 10.1002/jcla.24827

RESEARCH ARTICLE

WILEY

Detecting mitochondrial mutations associated with aminoglycoside ototoxicity by noninvasive prenatal testing



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Funding information

The Dongguan Social Development Project, Grant/Award Number: 201950715007158; The National Key Research and Development Program, Grant/Award Number: 2017YFC0909303

Abstract

Objectives: Numerous diseases and disorders are associated with mitochondrial DNA (mtDNA) mutations, among which m.1555A > G and m.1494C > T mutations in the 12 S ribosomal RNA gene contribute to aminoglycoside-induced and nonsyndromic hearing loss worldwide.

Methods: A total of 76,842 qualified non-invasive prenatal (NIPT) samples were subjected to mtDNA mutation and haplogroup analysis.

Results: We detected 181 m.1555A > G and m.1494C > T mutations, 151 of which were subsequently sequenced for full-length mitochondrial genome verification. The positive predictive values for the m.1555A > G and m.1494C > T mutations were 90.78% and 90.00%, respectively, a performance comparable to that attained with newborn hearing screening. Furthermore, mitochondrial haplogroup analysis revealed that the *12 S rRNA* 1555A > G mutation was enriched in sub-haplotype D5[p = 0, OR = 4.6706(2.81–7.78)].

Conclusions: Our findings indicate that the non-invasive prenatal testing of cell-free DNA obtained from maternal plasma can successfully detect m.1555A > G and m.1494C > T mutations.

KEYWORDS

mitochondrial DNA (mtDNA), newborn hearing screening (NBHS), noninvasive prenatal testing (NIPT), PPV, secondary finding

1 | INTRODUCTION

Mitochondria are double-membraned organelles that play essential roles in a series of signaling and metabolic pathways, including the tricarboxylic acid cycle, β-oxidation of fatty acids, calcium regulation,[1] intrinsic apoptosis[2,3] and cell cycle regulation.[4] It has been established that numerous diseases and disorders are associated with mitochondrial DNA (mtDNA) mutations, and most maternal mtDNA diseases can be transmitted to the offspring due to the matrilineal inheritance of mtDNA.[5] In mammalian, mitochondrial

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mtDNA is inherited exclusively from the mother and is characterized by a high mutation rate, with 250 pathogenic mtDNA mutations having been reported.[6] A variety of complex human traits, including metabolic diseases,[7–9] neurodegenerative diseases,[10] longevity,[11,12] and renal functions,[13] have been confirmed to be associated with mtDNA variants.

The mitochondrial *12S rRNA* gene has been found to be particularly prone to mutation, among which are those associated with aminoglycoside-induced and non-syndromic hearing loss. In particular, 1555A>G and 1494C>T mutations in a highly conserved region of the *12S rRNA* gene have frequently been identified in patients with hearing loss.[14,15] The A-to-G transition at position 1555 (m.1555A>G) and C-to-T transition at position 1494 (m.1494C>T) are predicted to generate binding sites for aminoglycosides. The clinical symptoms of heteroplasmic pathogenic mtDNA mutations are closely related to the relative proportion of wild-type and mutant-type mtDNA (heteroplasmy level) in different tissues, and it has been established that among individuals carrying these mutations, those with a level of heteroplasmy greater more than 50% manifest varying degrees of hearing loss, with the risk of deafness tending to increase with an increase in heteroplasmy level.[16] In China, the proportions of the population suspected to be carriers of 1555A>G and 1494C>T have been reported to be 0.2% and 0.03%, respectively,[17–19] whereas mutation frequencies of 3.96% and 0.18% have been reported for 1555A>G and 1494C>T, respectively, in a cohort of 1742 hearing-impaired Chinese pediatric subjects.[20,21] Among individuals carrying the m.1555A>G mutation, values of 29.5% and 17.6% have been reported for the average penetrances of deafness when aminoglycoside-induced hearing loss was included or excluded, respectively,[21] thereby indicating that exposure to aminoglycosides can induce or exacerbate hearing loss in individuals carrying this mutation.[22]

Predicting the individual risk of ototoxicity can contribute to reducing the incidence of hearing loss, and in this regard, newborn hearing screening (NBHS) programs have been globally adopted since the 1990s. These programs serve as a primary approach to detecting hearing loss in neonates and can thereby facilitate early interventions. In China, two representative examples of large NBHS program have been described by Wang et al.[18] and Dai et al.[19] wherein 1,172,234 and 180,496 newborns were screened, respectively, with reported of m.1555A>G and m.1494C>T frequency of to be 0.1934% and 0.0125%, and 0.212% and 0.015%, respectively. Rodney Howell, an expert from the American College of Medical Genetics and Genomics, has similarly emphasized the importance of screening for neonatal deafness.[23]

In 1997, Lo et al. confirmed that cell-free fetal DNA circulates with in maternal plasma early in gestation,[24] and since then, there has been a relatively rapid development and clinical implementation of related tests. In China, noninvasive prenatal testing (NIPT) using cell-free DNA (cfDNA) for aneuploidy screening has been widely applied. In China, the number of infants born in 2020 was approximately 12 million (official data from the National Bureau of Statistics), approximately 40% of whom were screened using NIPT. Given that mtDNA is present in the maternal plasma,[25] this plasma is also considered a potential source of material that can be used to test for diseases associated with mtDNA mutations, NIPT has the potential to identify individuals susceptible to aminoglycosides during pregnancy, and hence in addition to undertaking screening for newborn hearing, we can also focus on genetic screening for susceptible individuals.

In this study, we selected the m.1555A>G and m.1494C>T mutations of mtDNA *12S rRNA* as representative mutations to assess the feasibility of applying NIPT for the detection of mtDNA mutations. To evaluate the performance of NIPT, we performed full-length mitochondrial sequencing to verify NIPT-positive cases and performed similar evaluations using the data from the two aforementioned national studies.[18,19] In addition, we also analyzed the haplogroups based on NIPT and discussed their correlations with the *12S rRNA* 1555A>G mutation.

2 | MATERIALS AND METHODS

2.1 | Patients and NIPT

In this study, we processed 77,328 samples from participants subjected to NIPT analysis at the Dongguan Maternal and Child Health Hospital. The study was approved by the Ethics Committee of the Dongguan Maternal and Child Health Hospital. Prior to blood sampling, a signed consent form was obtained from each participant. Participants were at least 18 years old with a singleton or twin pregnancy. After excluding the samples lacking province of residence information, 76,842 samples were included in the subsequent analyses. With regard to the gestational stage of pregnant women, there were 10,893 women in the first trimester (9~13 weeks), 65,553 in the second trimester (14~27 weeks) and 416 in the third trimester (≥28 weeks). Whole blood samples were collected in two types of cfDNA tubes (Vangenes, China; and Guangdong Ardent BioMed, China) and processed within 72 h according to the following procedures.

Cell-free DNA (cfDNA) extraction, library construction, quality control, and pooling were performed according to the instructions of at JingXin Fetal Chromosome Aneuploidy (T21, T18, T13) Testing Kit (CFDA registration permit No. 20153400300). Details of the processing methods have been described in previous studies.[26–29] Briefly, the blood samples were initially centrifuged at 1600 × g for 10 min at 4°C using refrigerated centrifuge (Eppendorf). The resulting plasma fraction was carefully transferred to a polypropylene tube and subjected to centrifugation at 16,000 × g for 10 min at 4°C using a refrigerated centrifuge (Eppendorf). CfDNA was extracted from the maternal plasma using a TIANamp Micro DNA Kit (Tiangen) following the manufacturer's protocol. Library construction was performed using an Ion Plus Fragment Library Kit (Thermo Fisher), which entailed end-repairing, adaptor ligation and PCR amplification. The purification and selection of DNA fragments after each step were performed using AMPure XP beads (Beckman

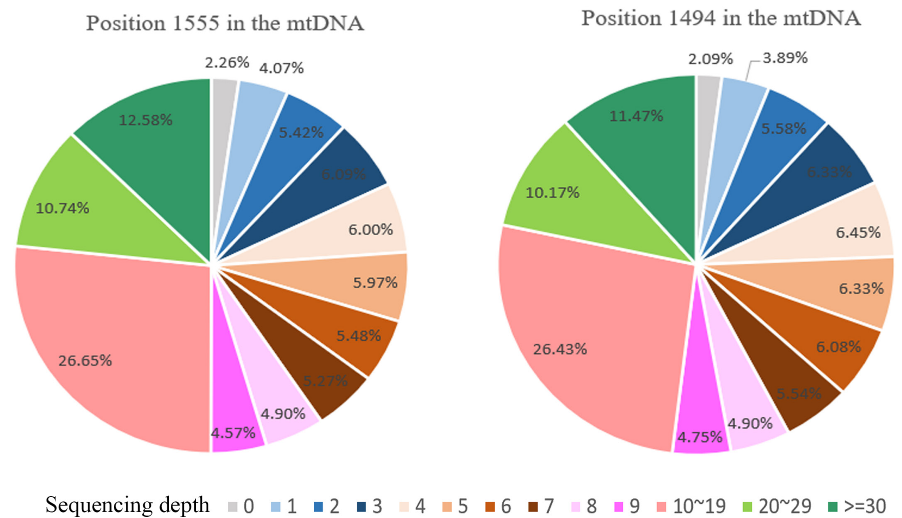


FIGURE 1 Sample proportions at different sequencing depths. The numbers 0–30 represent different sequencing depths and are represented by different colors. The numbers on the pie chart indicate the proportions corresponding to different depths.

Coulter). Having constructed libraries, these were qPCR quantified using a Step One Plus real-time PCR system (Thermo Fisher). For DNA sequencing, 15–20 qualified libraries were pooled in equal proportions and approximately 200-bp reads were sequenced using a JingXin BioelectronSeq 4000 System (CFDA registration permit No. 20153400309) semiconductor sequencer. Sequencing reads were filtered and aligned to the human reference genome (hg19), following the same procedures reported in previous study.[26–29]

2.2 | Data analysis

Combined GC correction and Z score testing methods were used to identify fetal autosomal aneuploidies, as reported in our previous studies.[26,27] The unmapped reads in the detection process were compared with the mitochondrial and human genome sequences using the Genome Analysis Toolkit (GATK),[30] retaining the best alignment result for each read. The reads aligned to the mitochondrial genome were selected and used to calculate the alignment depth of each base of the mitochondrial *12 S rRNA* gene.

2.3 | NIPT for the m.1555A > G and m.1494C > T mutation of mtDNA 12 S rRNA

The criteria for positivity were either ≥2 reads supporting the mutation with a mutation allele ratio > 0.1 or 1 read supporting the mutation with a mutation allele ratio ≥ 0.3.

2.4 | NIPT for the mitochondrial haplogroups

For each sample, the data were filtered, and the samples with an average mitochondrial sequencing depth ≥ 5× and coverage ≥ 95% were retained. The Genome Analysis Toolkit was used to mitochondrial whole genome variants, and the qualified samples were assigned to mitochondrial haplogroups based on the PhyloTree database (http://www.phylotree.org).[5]

2.5 | Full-length mitochondrial sequencing and mutation detection

The entire mitochondrial genome was amplified using the following two pairs of primers: Primer pair 1: 5′-AAATCTTACCCCGC CTGTTT-3′, 5′-AATTAGGCTGTGGGTGGTTG-3′; and Primer pair 2: 5′-GCCATACTAGTCTTTGCCGC-3′, 5′-GGCAGGTCAATTTCACT GGT-3′. The amplified products were fragmented and sequenced using a JingXin BioelectronSeq 4000 System (CFDA registration permit No. 20153400309) semiconductor sequencer with an average sequencing depth of over 1000 ×.

2.6 | Genetic screening for newborns

Genetic screening for newborns was performed as described in our previous study.[31] Briefly, genomic DNA was extracted from dried blood spot samples collected from infants within 3 days after birth, using which 101 hot spot mutation alleles and their neighboring sequence regions were analyzed using a deafness diagnostic screening panel.

2.7 | Statistical analysis

In the context of the present study, the positive predictive value (PPV) was the proportion of positive samples in the full-length mitochondrial genomes sequenced. We used the chi-squared test (χ^2) to compare the detection rate of a variant between the two cohorts, and a *p* Value of less than 0.05 was considered to be statistically significant. Statistical analysis was performed using IBM SPSS Statistics, version 24 (SPSS).

3 | RESULTS

3.1 | NIPT performance in detecting the m.1555A > G and m.1494C > T mutation

To assess the performance of NIPT in detecting mtDNA mutations, we selected m.1555A > G and m.1494C > T as representative mutations. A total of 76,842 samples were included in the subsequent analyses, and by analyzing the sequencing depth, we found that approximately 98% of the samples covered the 1555 and 1494 sites of the mitochondrial genome (Figure 1). We accordingly detected a total of 181 positive cases, (165 m.1555A > G and 16 m.1494C > T cases). Among these positive samples, the full-length mitochondrial genome was sequenced for 151 (141 m.1555A > G and 10 m.1494C > T cases), which confirmed 137 true positives and 14 false positives, attaining an overall PPV of 90.73% (90.78% for m.1555A > G and 90.00% for m.1494C > T) (Figure 2).

3.2 | Performance evaluation of NIPT for detecting the m.1555A > G and m.1494C > T mutation

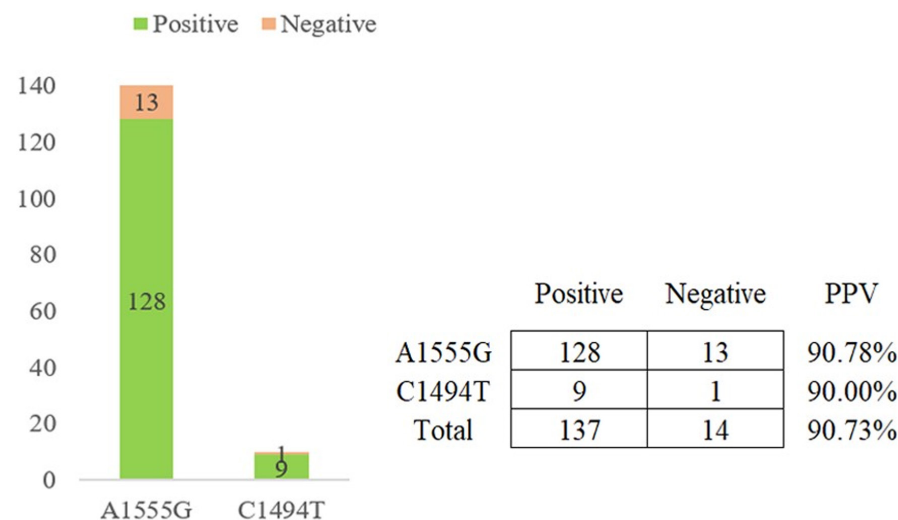
For the samples assessed in the present study, we obtained rates of detection of 0.215% and 0.021% for m.1555A > G and m.1494C > T, respectively. Comparatively, using data obtained from two representative studies based on the national NBHS in China, detection rates of 0.21% and 0.015% were obtained for m.1555A > G and m.1494C > T, respectively, in NBHS1,[19] which did not differ significantly from the values obtained in the present study (p = 0.88, p = 0.29). Similarly, comparison with NBHS2 study[18] also revealed no significant differences (p = 0.21, p = 0.07) (Figure 3A). In addition, we used PPV to correct the detection rates in the present study (detection rate × PPV) and compared these values with those obtained in NBHS1 and NBHS2 studies. The corrected detection rates for m.1555A > G and m.1494C > T were 0.195% and 0.018%, respectively, neither of which differed significantly from those obtained in the NBHS1 (p = 0.40, p = 0.55) and NBHS2 (p = 0.95, p = 0.23) (Figure 3B).

3.3 | Genetic screening results for newborns

For 327 cases, we collected paired data both maternal NIPT and genetic testing data for neonatal deafness after birth. Among these 327 cases, there were three positive cases (one m.1494C > T, and two m.1555A > G) and 324 negative cases predicted by NIPT. Among the three positive cases, there were two positive and one negative finding using the newborn deafness genetic test. In the latter case, the mutation frequency of the NIPT test result was only 0.15, which indicated a heterogeneous mutation with low frequency. All 324 negative cases, as determined by NIPT, also showed negative results for newborn deafness genetic testing.

3.4 | Regional distributions of mtDNA haplogroups in China

It has previously been established that there is a general south–north genetic divergence among the Han Chinese with respect to mitochondrial haplogroups.[32] In the present study, we analyzed mitochondrial haplogroups according to case geographical location. Haplogroup classification was performed based on the province information provided with the samples, with subsequent clustering. Haplogroup D4 was identified as the most frequent haplogroup, followed by M7, F1, and R. Moreover, we found that the frequencies of these dominant haplogroups varied to different extents among different regional populations. For example, whereas haplogroup D4 was identified as being the most frequent in the northern and northeastern provinces, including Shanxi, Inner Mongolia, Liaoning, and Heilongjiang, M7 was mainly detected among populations in southern provinces, particularly in Hainai, Guangxi, and Guangdong (Figure 4).



	Positive	Negative	PPV
A1555G	128	13	90.78%
C1494T	9	1	90.00%
Total	137	14	90.73%

FIGURE 2 Non-invasive prenatal testing (NIPT) performance in detecting m.1555A > G and m.1494C > T mutations. The histogram on the left represents the number of cases for which full-length mitochondrial validation was performed, and the positive and negative full-length validations are shown in green and orange, respectively. The graph on the right represents the NIPT performance in detecting m.1555A > G and m.1494C > T mtations, including the positive predictive values.

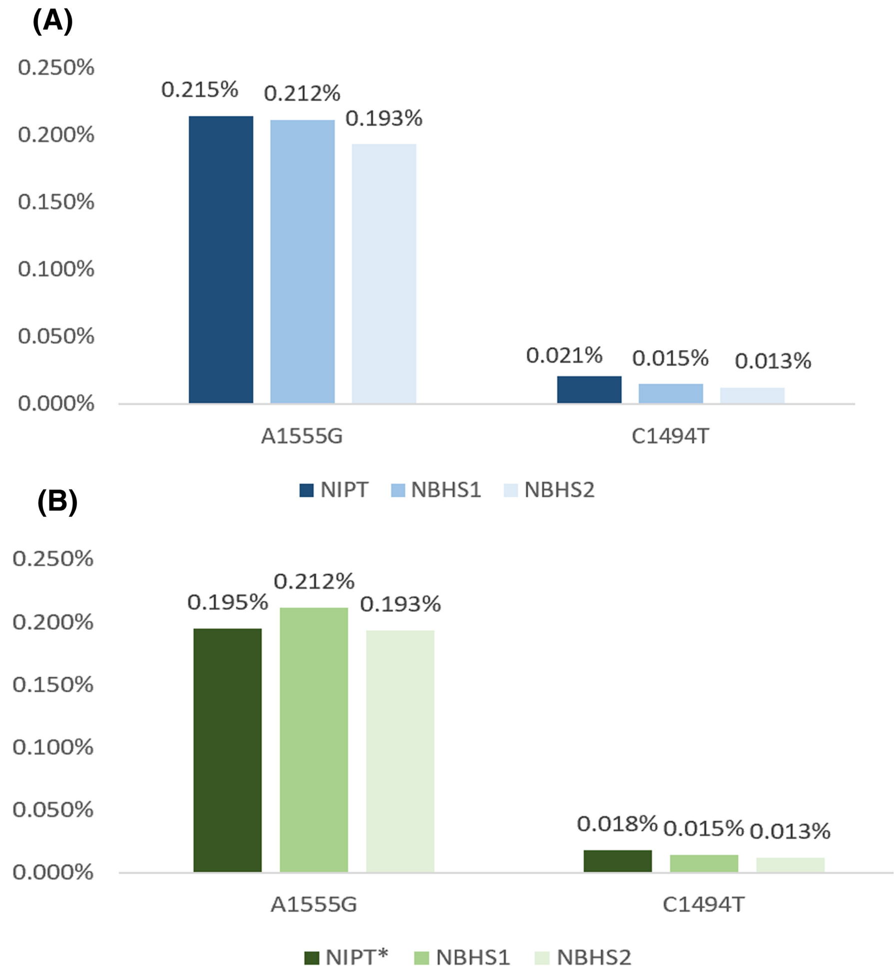


FIGURE 3 Evaluation of the performance of non-invasive prenatal testing (NIPT) in detecting the m.1555A > G and m.1494C > T mutations. Panel A shows the detection rates for m.1555A > G and m.1494C > T mutations in three screening programs. NIPT, NBS1, and NBS2 indicate values obtained for data from the present study, the study of Dai et al.[19] and the study of Wang et al.[18] Panel B shows the corrected detection rates for m.1555A > G and m.1494C > T in the present study, and the studies of Dai et al.[19] (NBS1) and Wang et al.[18] (NBS2).

3.5 | Distribution of m.1555A > G in the mtDNA haplogroups

The mitochondrial *12S rRNA* 1555A > G mutation is among the important causes of aminoglycoside-induced and nonsyndromic deafness. Haplogroup analysis was performed on samples, and cluster analysis was performed based on the participants' province. We accordingly identified this mutation among 10 common haplogroups and found that haplogroup D accounted for nearly 50% of the patients' mtDNA samples and that the *12S rRNA* 1555A > G mutation was enriched in sub-haplotype D5 [$p = 0$, OR = 4.6706 (2.81–7.78)] (Table 1).

4 | DISCUSSION

In 2011, NIPT was initially introduced in clinical practice in China and Hong Kong, and thereafter was rapidly adopted worldwide. With the development of whole-genome sequencing and an increase in the frequency of NIPT, many unexpected results have emerged.[33] Moreover, using such testing there is potential for the recognizing and reporting incidental or secondary findings unrelated to the indication, thereby providing a rationale for undertaking sequencing of medical relevance for patient care. In this regard, an American College of Medical Genetics and Genomics expert has emphasized that it is important to disclose such incidental/secondary findings in clinical testing and report the results.[34,35]

To date, however, there has been comparatively little research on the screening of maternal plasma for detecting diseases associated with mtDNA mutations. The mitochondrial *12S rRNA* mutations 1555A > G and 1494C > T can alter binding affinity for aminoglycosides, and individuals bearing these mutations are more prone to aminoglycoside-induced ototoxicity. The Clinical Pharmacogenetics Implementation guideline for aminoglycosides and *MT-RNR1* state that individuals with the m.1494C > T or m.1555A > G variant have an increased risk of aminoglycoside-induced hearing loss, and consequently, screening individuals at risk of ototoxicity is deemed important for safe aminoglycoside treatment. In China, the *12S rRNA* m.1555A > G and m.1494C > T mutations are among the targeted variants assessed in genetic screening programs for deafness.[15,21]

Our findings in this study showed revealed mtDNA mutations can be detected in the cell-free fetal DNA circulating in maternal plasma. The PPVs of m.1555A > G and m.1494C > T detection were comparable to that of fetal aneuploidy T21,[36] and we detected m.1555A > G and m.1494C > T at specificities higher than 99.9%. Screening plasma derived cfDNA collected from pregnant women for detection of the m.1555A > G or m.1494C > T mutations is of considerable great significance, for both the women themselves and

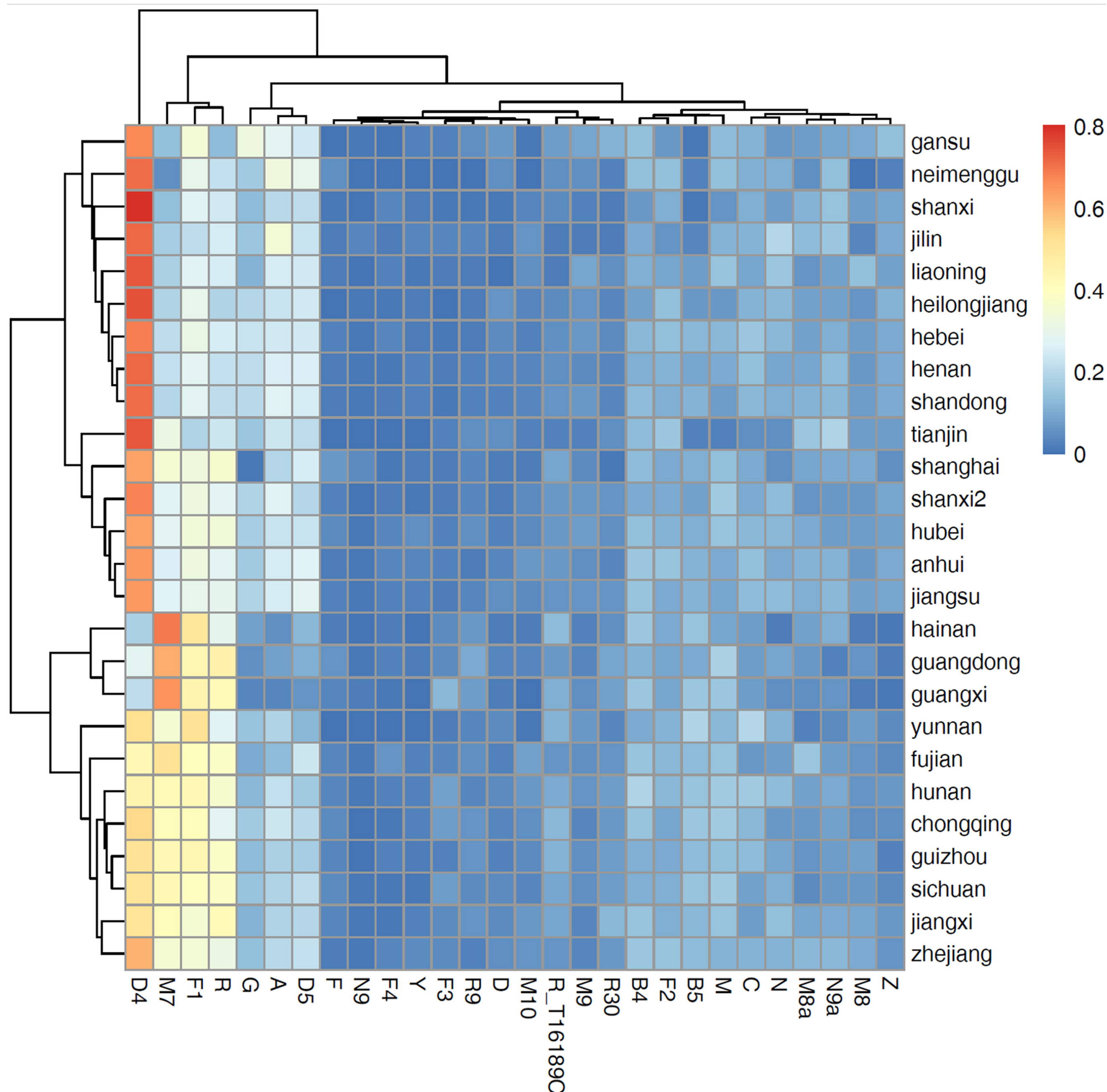


FIGURE 4 Haplogroup classification and province clustering information. The horizontal axis represents different haplogroups, and the vertical axis represents different provinces.

their offspring. For pregnant women, if a mutation is detected, this can provide information as a secondary finding. In general, the use of aminoglycoside antibiotics should be avoided, unless the high risk of permanent hearing loss is outweighed by the severity of the infection and a lack of safe or effective alternative therapies.[37] From the perspective of newborns, given that maternal mtDNA is transmitted to the offspring, neonatal genetic testing should be performed to confirm the presence of mtDNA mutation.

In this study, we obtained detection rates of 0.215% and 0.021% for the m.1555A > G and m.1494C > T mutations, respectively, which is consistent with the previously reported NBHS program results.[18,19] Similarly, the findings of a large-scale genetic screening for deafness conducted for 142,417 neonates in Wuhan, China, showed no significant differences from those obtained in the present study.[38] When NIPT predicts that a fetus might inherit m.1555A > G or m.1494C > T mutation in the *12 S rRNA* gene, hearing screening should be recommended after birth. Indeed, as Dai et al.[16] have reported, the transmission rate of mtDNA mutations was greater than 98% in five pedigrees. Currently, however, there is limited number of cases for which paired data between NIPT and newborn deafness genetic test data are available, and the relatively small sample size of positive cases in the present

TABLE 1 Correlations between m.1555A > G and mtDNA haplogroups.

Type	Number_of_ A1555G($N = 87$)	Number_of_No_ A1555G($N = 58,320$)	p-Value	OR(95% CI)
A	3	3310	0.4896	0.5935(0.19–1.88)
B	3	3815	0.3800	0.5103(0.16–1.61)
C	3	1739	0.7461	1.1620(0.37–3.68)
D	41	12,746	0.0000	3.1869(2.09–4.86)
D4	18	8766	0.1353	1.4747(0.88–2.48)
D5	19	3292	0.0000	4.6706(2.81–7.78)
F	9	8355	0.3581	0.6900(0.35–1.38)
G	2	2366	0.5876	0.5565(0.14–2.26)
M	16	12,441	0.6005	0.8310(0.48–1.43)
N	3	3416	0.4907	0.5740(0.18–1.82)
R	5	8101	0.0283	0.3780(0.15–0.93)
Z	2	1114	0.6836	1.2083(0.30–4.92)

study is insufficient to reach definite conclusions. Thus, on the basis of the findings of the present study and those presented in the literature, it can be deduced that if mtDNA mutations are detected in the plasma of pregnant women, there is high probability that their offspring will inherit those mutation. Overall, our findings highlight that NIPT detection of mtDNA mutations may occur as an incidental or secondary finding, and in such cases, genetic counselors should inform expectant mothers of their test results and the significance of the findings and emphasize the imperative of post-natal verification after birth.

With respect to sampling procedures, it has previously study reported that different blood collection tubes might affect the mtDNA content and thus the analysis results.[39] In the present study, we assessed the effect using two types of blood collection tube (Ardent and Vangenes tubes) for the preservation of samples, for which we compared the detection ratios and PPVs of samples using chi-squared or Fisher exact tests, as appropriate. Using the Ardent and Vangenes tube, we obtained detection rates of 0.195% (24/12,283) and 0.218% (141/64,559) for m.1555A > G, which did not differ significantly ($p = 0.61$). Similarly, in the case of the m.1494C > T mutation, we detected a non-significant difference between the detection rates of 0.033% (4/12,283) and 0.019% (12/64,559) for the Ardent and Vangenes tubes, respectively ($p = 0.52$). Consistently, with respect to PPV, we detected no statistically significant differences ($p = 0.41$) between Ardent (95.5%, 21/22) and Vangenes (89.9%, 107/119) tubes for m.1555A > G mutation. Although for the m.1494C > T mutation, the PPV value obtained using the Ardent tubes (66.7%, 2/3) was notably lower than that obtained using the Vangenes tubes (100%, 7/7), the difference fell short of the level of significance ($p = 0.3$). However, given the very low frequency of the m.1494C > T mutation and the small number of positive cases, further studies based on larger sample sizes are needed for confirmation.

For the present study, we selected only two *12 S rRNA* mutations (m.1555A > G and m.1494C > T) for analysis. However, unlike NBHS, which covers only a few specific mutations, NIPT can be used to analyze the entire mitochondrial genome for mutations, and thus it would be presumed that considerably more information regarding mtDNA mutations could be obtained. Indeed, over 250 pathogenic mtDNA mutations have been reported to date.[6] Moreover, in addition to hearing impairment, mtDNA mutations are associated with a range of other human diseases, including diabetes, Alzheimer's disease, Parkinson's disease and disorders, and cancer. Thus, NIPT has potential applications in the screening of a broad spectrum of diseases, thereby providing more clues for the early diagnosis and treatment of diseases associated with mtDNA mutations.

Studies have reported that the m.1555A > G mutation is associated with certain haplotypes that may modulate its phenotypic manifestations. Hearing impairment could also be caused by a combined inheritance of genomic and mitochondrial DNA mutations.[40] Guan et al. have reported that mitochondrial haplotypes can modulate the phenotypic manifestation of the deafness-associated *12 S rRNA* 1555A > G mutation,[21] and the *12 S rRNA* A1555G mutation in the mitochondrial haplogroup D5a has been shown to be linked with maternally inherited hypertension and hearing loss.[41] NIPT can be used to fully sequence the mtDNA, and thus, we analyzed mitochondrial haplogroups and verified a correlation between m.1555A > G and m.1494C > T and the D haplogroup, particularly sub-haplogroup D5. Typically, genetic screening for neonatal deafness focuses exclusively on these two loci, which represents the major difference between neonatal deafness and NIPT for detecting mtDNA mutations.

In conclusion, we established that NIPT can be used detect m.1555A > G and m.1494C > T mutations in the mtDNA, with a performance comparable to that of newborn hearing screening. This unexpected discovery could be considered as a secondary finding of NIPT. Mothers carrying fetuses bearing either the m.1555A > G or m.1494C > T mutation predicted by NIPT should be encouraged to arrange hearing screening for their infants after birth. NIPT analysis of mitochondrial haplogroups showed that the m.1555A > G mutation was enriched in the sub-haplotype D5.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: Quanfei Huang, Yanhui Liu and Min Huang. Data analysis: Quanfei Huang, Yanhui Liu, Wei Lei and Yang Wang. Clinical data collection and experimental verification: Yanhui Liu, Jiajie Liang, Minhua Zheng, Xiaoyan Huang, Yuanru Liu and Kaisheng Huang. Drafted and revised the manuscript: Quanfei Hunag, Yanhui Liu, Wei Lei and Min Huang. All authors provided important feedback on the analysis of the results and the revision of the paper.

ACKNOWLEDGMENTS

None.

FUNDING INFORMATION

This work was supported by the National Key Research and Development Program (No. 2017YFC0909303) and the Dongguan Social Development Project (No. 201950715007158).

CONFLICT OF INTEREST

Authors Wei Lei, Yang Wang, and Xiaoyan Huang are affiliated with CapitalBio Genomics Co., Ltd. and CapitalBio Technology Co., Ltd. Authors Yuanru Liu and Kaisheng Huang are affiliated with Guangdong CapitalBio Medical Laboratory and CapitalBio Technology Co., Ltd. The other authors declare that they have no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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How to cite this article: Huang Q, Liu Y, Lei W, et al. Detecting mitochondrial mutations associated with aminoglycoside ototoxicity by noninvasive prenatal testing. *J Clin Lab Anal*. 2023;37:e24827. doi:10.1002/jcla.24827