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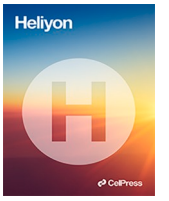

Research article

A capillary electrophoresis-based assay for carrier screening of the hotspot mutations in the *CYP21A2* gene

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ABSTRACT

Molecular genetic analysis of the cytochrome P450 family 21 subfamily A member 2 (*CYP21A2*) gene is challenging owing to the highly homologous with its pseudogene. A reliable approach for the large-scale population screening of *CYP21A2* is required. This study aimed to establish and evaluate a capillary electrophoresis-based assay for hotspot mutation carrier screening of the *CYP21A2* gene. A total of 22 different variants in the *CYP21A2* gene were detected by a capillary electrophoresis-based assay consisting of single nucleotide primer extension (SNaPshot) and high-throughput ligation-dependent probe amplification (HLPA) in the Chinese population, and the results were validated by alternative methods. Among the 5376 subjects, 1.51 % (81/5376) individuals were identified as *CYP21A2* pathogenic variant carriers, with a carrier rate of 1/66. A total of 11 different variants were identified, of which c.293-13A/C > G (33.33 %) was the most common variant, followed by c.844C > T (19.75 %), c.518T > A (19.75 %), and Del/Con (16.05 %). There was a 100 % concordance between capillary electrophoresis and alternative method results. Furthermore, a total of 63 individuals (1.17 %, 63/5376) carried the c.955C > T (p.Q319*) variant, among which 61 (61/63, 96.83 %) had a duplicated *CYP21A2* gene and are therefore not carriers of a *CYP21A2* allele. In conclusion, the capillary electrophoresis-based assay is an accurate and effective approach for genotyping the *CYP21A2* gene and has the potential for the large-scale population screening of *CYP21A2*.

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1. Introduction

Congenital adrenal hyperplasia (CAH) is a group of autosomal recessive disorders caused by single gene defects in various enzymes for cortisol biosynthesis [1]. 21 hydroxylase deficiency (21-OHD, [OMIM] 201910), due to mutations in the cytochrome P450 family 21 subfamily A member 2 (*CYP21A2*) gene, is an autosomal recessive disorder accounting for approximately 90 % ~ 95 % of CAH [2]. The incidence of 21-OHD is 1/14,000–1/18,000 newborns worldwide, and the main clinical symptoms of 21-OHD are adrenal dysfunction and androgen hypersecretion [3]. 21-OHD is classified into 3 sub-types according to clinical symptoms, including classic salt wasting (SW), classic simple virilizing (SV), and non-classic CAH (NCCAH) [2]. If timely and appropriate therapy are not administered, SW-CAH is life-threatening because of severe electrolyte imbalance in couple with dehydration and hypotension during the neonatal period and infancy [4]. Owing to the relative high incidence, the definitive genotype-phenotype association, and the severity of disease, carrier screening of *CYP21A2* for couples of reproductive ages has been recommended [5,6].

The *CYP21A2* gene is consisted of 10 exons and 9 introns, which located 30 kb apart in the HLA class III region in the MHC locus on the short arm of chromosome 6 (band 6p21.3) [7]. Molecular analysis of the *CYP21A2* gene is challenging owing to the proximity and homology (98 % in exons and 96 % in introns) of a pseudogene, *CYP21A1P* [8]. Traditional assays for analyzing the *CYP21A2* gene include Southern blotting [9], DNA sequencing [10], allele specific polymerase chain reaction (AS-PCR) [11], multiplex ligation dependent probe amplification (MLPA) [12], denaturing high performance liquid chromatography (DHPLC) [13], and long-read sequencing [8,14]. However, these assays are not suitable for large-scale clinical application because of complicated operation, low throughput, high cost, or high requirement for laboratory personnel. A reliable approach for the large-scale population screening of *CYP21A2* is required.

Capillary electrophoresis, as a well-established genotyping assay, has been successfully applied to DNA sequencing analysis owing to short turnround time and high multiplexing ability without additional expensive equipment [15]. In this study, we established a multiple PCR assay based on capillary electrophoresis, consisting of single nucleotide primer extension (SNaPshot) and high-throughput ligation-dependent probe amplification (HLPA), for analyzing the hotspot mutations of the *CYP21A2* gene and used for carrier screening in the reproductive age population. This assay is accurate, simple to use, and cost-effective, providing an effective platform for molecular analysis of the *CYP21A2* gene.

2. Materials and methods

2.1. Subjects

Between 2017 and 2019, a total of 5376 subjects (3132 females and 2244 males) who underwent carrier screening for spinal muscular atrophy were included in this study. Among them, 3930 were overlapped with those in our previous study [16], and 1446 new participants were included. All these participants provided an informed consent for the analysis. This study was approved by the

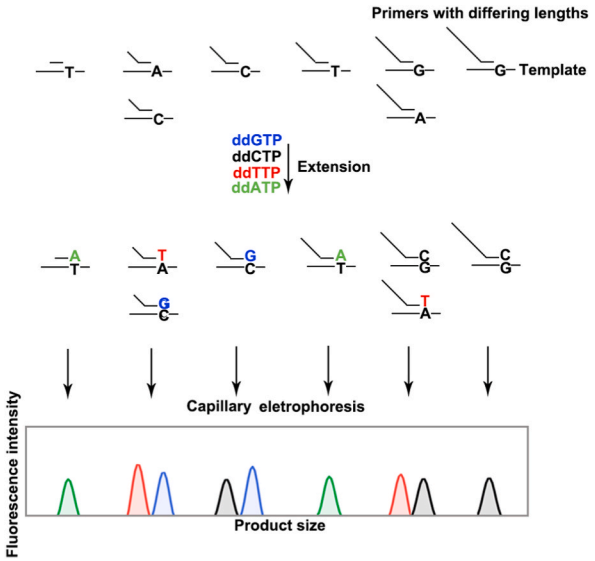

Fig. 1. Graphical representation of general principles for the SNaPshot assay. SNaPshot primers were designed one base upstream of each defined variant and synthesized with varying length of tails to allow separation of PCR products on the basis of size. When the primer anneals to the specific DNA template, the polymerase catalyzes single-base extension of the primers by incorporating fluorescently labelled dideoxynucleotides (ddNTPs) corresponding to each variant. The length of the resulting products was the size of the specific primers with the additional ddNTP that terminates the extension of the primers. Subsequently, the products were separated by capillary electrophoresis, and variants are genotyped based on product length and the color of the fluorescence.

Institutional Review Board of Nanjing Women and Children's Healthcare Hospital (No. 2020KY-057).

2.2. DNA extraction

DNA was extracted from peripheral blood using an automated nucleic acid extractor (Concert Bioscience, Xiamen, China). DNA concentration and purity were determined using a nanodrop 2000 ultra micro spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA).

2.3. Capillary electrophoresis-based assay

SNaPshot and HLPA, two assays based on capillary electrophoresis, were used to detect 22 pathogenic variants in the *CYP21A2* gene (NM_000500.9), including gene deletion, gene rearrangements, conversion, c.92C > T, c.274A > G, c.292+1G > A, c.293-13A/C > G, c.332_339del, c.449G > C, c.518T > A, c.844G > T, c.923dupT, c.949C > T, c.955C > T, c.1069C > T, c.1225C > T, c.1226G > T, c.1450dupC, c.1452G > C, c.1455delG, c.1279C > T, and [c.710T > A; c.713T > A; c.719T > A]. All variants are based on literature review and local databases combined with databases such as ClinVar (http://www.ncbi.nlm.nih.gov/clinvar/), and Human Gene

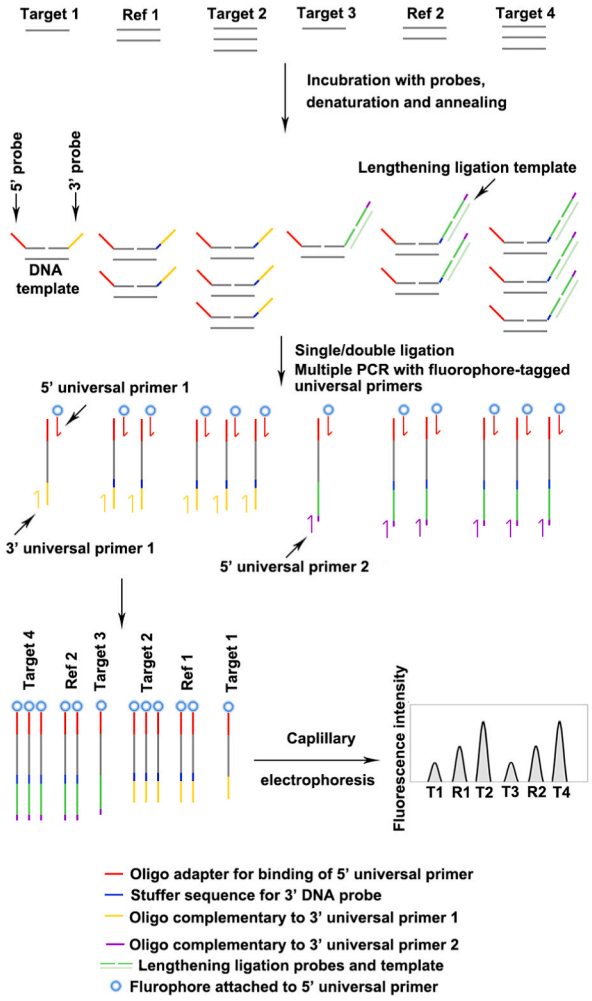

Fig. 2. Overview of the HLPA strategy. Two specific probes, including upstream (5′) and downstream (3′), are designed for each target sequence (grey). The 5′ end of the upstream probe contains a universal adapter sequence that anneals to the fluorophore-labelled forward universal PCR primer (red), and the 3′ end of the downstream probe contains a universal adapter sequence complementary to the reverse universal PCR primers (yellow and magenta). The downstream probe also contains different length of stuffer sequences to lengthen the final products, thereby allowing separation of PCR products on the basis of size in a single test. In addition, three probes (upstream, middle and downstream) and an oligonucleotide template (light green) are synthesized for the double ligation system. A contiguous DNA fragment is generated by two template-dependent ligations. After the single/double-ligation, the ligated probes were amplified by 5′ universal primer 1 and 2 and 3′ universal primer 1 to obtain PCR products in different size. The products were separated by capillary electrophoresis, and the copy number of the target sequences was calculated based on the fluorescence intensity.

Mutation Database (HGMD) (http://www.hgmd.cf.ac.uk). Primer sequences used in this study are listed in Supplementary Table 1 and Supplementary Table 2.

2.4. SNaPshot assay

The principle of the SNaPshot assay is illustrated in Fig. 1. The SNaPshot assay was used to analyze 19 single nucleotide variations (SNV) in the *CYP21A2* gene. Primers were designed on Primer3 software v.0.2 (http://bioinfo.ut.ee/primer3-0.4.0/). To generate the template for the SNaPshot extension reaction, multiplex PCR reactions were carried out in a volume of 10 μl containing 1 × Takara PCR buffer, 1.5 mM $MgCl_2$, 0.3 mM dNTPs, 0.7 μM of each primer, 0.035 U Takara Taq polymerase (Takara, Dalian, China), and 20 ng of template. Thermal cycler conditions were: 95 °C for 2 min, 11 cycles (94 °C for 20 s, 65°C–0.5 °C/cycle for 40 s, 72 °C for 90 s), 35 cycles (94 °C for 20 s, 59 °C for 30 s, 72 °C for 90 s), and 2 min at 72 °C. The final PCR products were treated with 3 U of shrimp alkaline phosphatase (SAP) and 2 U of exonuclease I at 37 °C for 60 min to eliminate excess deoxyribonucleotide triphosphates (dNTPs) and primers, respectively. SNaPshot assay was carried out using an Applied Biosystems SNaPshot Multiplex Kit. Reactions were conducted in a volume of 6 μl containing 0.8 μl of SNaPshot Ready Multiplex Ready Reaction Mix (Applied Biosystems, Foster City, CA, USA), 0.6 μl 5 × Sequencing buffer, 1 μl of probe mixture and 1 μl of multiplex PCR product. Single-based extension reactions were run on a thermal cycler under the following cycling conditions: 96 °C for 1 min, 28 cycles (96 °C for 10 s, 52 °C for 5 s, 60 °C for 30 s). Extension reaction products were then treated with SAP to remove recessive ddNTPs. Subsequently, the mixture containing 1 μL extension product, 8.5 μL Hi-Di and 0.5 μL GeneScan Liz 120 was denatured for 5 min at 95 °C, and was sequenced on Genetic Analyzer ABI 3730XL (Applied Biosystems). The results were analyzed by GeneMapper Software v.5.0 (Applied Biosystems).

2.5. HLPA assay

The detailed graphical illustration of the HLPA assay is shown in Fig. 2 as previously described [17]. The HLPA assay was used to analyze deletion, gene rearrangements, and conversion in the *CYP21A2* gene. In brief, 100–200 ng of genomic DNA was denatured at 98 °C for 5 min and then added into 11 μl of ligation mixture containing 1.2 μl of 10 × Taq Ligase buffer, 0.4 μl of Taq ligase (50U/μl), 1 μl of 20 × Probe Mix, and 8.4 μl of ddH_2O. The ligation reaction was performed under the following cycling conditions: 98 °C for 6 min, 5 cycles (96 °C for 20 s, 60 °C for 3 h), 94 °C for 2 min. The ligation reaction was terminated by 12 μl of ethylene diamine tetraacetic acid (EDTA, 20 mM). Subsequently, PCR reaction was carried out in a volume of 20 μl containing 2 μl of 10 × Takara PCR buffer, 2.4 μl of dNTP (2.5 mM), 0.8 μl of $MgCl_2$ (25 mM), 1 μl of Fluorescence Primer Mix, 0.1 μl of Taq (5U/μl), and 1 μl of ligation reaction mixture. Thermal cycler conditions were: 95 °C for 2 min, 5 cycles (94 °C for 20 s, 62°C–1°C/cycle for 40 s, 72 °C for 90 s), 27 cycles (94 °C for 20 s, 57 °C for 40 s, 72 °C for 90 s), and 60 min at 68 °C. Finally, 1 μL of the diluted PCR product was mixed with 0.1 μL of Liz500 SIZE STANDARD and 8.5 μL of Hi-Di. After denatured at 95 °C for 5 min, the mixture was analyzed by ABI-3730XL sequencer

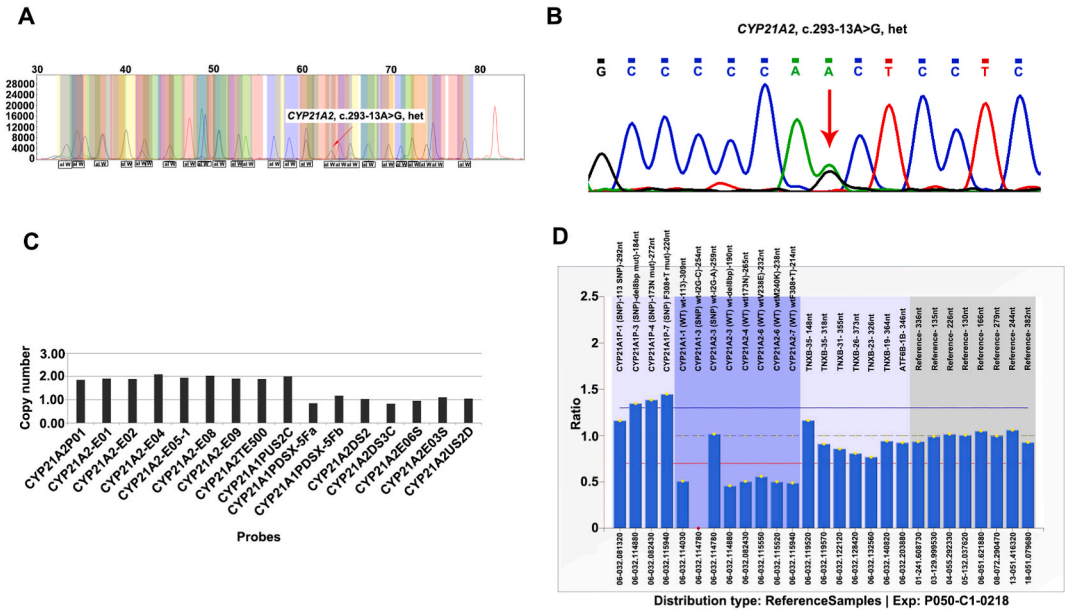

Fig. 3. The representative images of carrier screening results analyzed by capillary electrophoresis and confirmed by alternative methods. (A) Representative electropherograms for SNaPshot. The x axis is size in bases, and the y axis is fluorescence intensity. The variant *CYP21A2*, c.293-13A > G is denoted by a red arrow. het, heterozygous. (B) The variant *CYP21A2*, c.293-13A > G was confirmed by Sanger sequence. (C) The large deletion of *CYP21A2* was detected by HLPA. Columns represent the copy numbers for each probe. (D) The large deletion of *CYP21A2* was confirmed by MLPA.

(Applied Biosystems). Original data were analyzed by GeneMapper 5.0 software (Applied Biosystems). Copy number of the exported data were analyzed by CNV read1.0 software.

2.6. Confirmation of detected variants by alternative methods

SNVs in the *CYP21A2* gene were confirmed by PCR and Sanger sequencing according to our previous study [10]. Deletions, rearrangements, and copy number variations were validated using a multiplex ligation dependent probe amplification (MLPA) kit (Probemix P050 CAH, MRC Holland, Amsterdam, The Netherlands) according to the manufacture's protocol.

3. Results

Herein, we established a rapid and two-tubes capillary electrophoresis method consisting of SNaPshot and HLPA to simultaneously analyze 22 hotspot variants in the *CYP21A2* gene. Then, we successfully used this approach as a carrier screening test to investigate the mutational spectrum of *CYP21A2* in 5376 reproductive-aged individuals. All observed variants were validated by alternative approaches, including Sanger sequencing and MLPA. The representative results from capillary electrophoresis assays and validation assays are shown in Fig. 3A–D. As a result, there was a 100 % concordance between the capillary electrophoresis and alternative method results. Furthermore, 1.51 % (81/5376) individuals were identified as carriers of *CYP21A2* with a carrier rate of 1/66, and a total of 11 different variants in the *CYP21A2* gene were identified in 5376 reproductive-aged individuals, including one large deletion, one complex variation, and nine SNVs (Table 1). The most common variant was c.293-13A/C > G (33.33 %), followed by c.844C > T (19.75 %), c.518T > A (19.75 %), deletion and large fragment conversion (16.05 %), c.332_ 339del (3.70 %), c.955C > T (2.47 %), c.292+1G > A (2.47 %), c.92C > T (1.23 %), c.923dupT (1.23 %), and c.710/713/719T/A (1.23 %).

Surprisingly, our SNaPshot assay identified a total of 63 individuals (1.17 %, 63/5376) carrying the c.955C > T (p. Q319*) variant, which is unexpectedly higher than those reported in the Chinese population [18]. We hypothesized that the high rate of this variant results from the Q319* variant in the gene-duplicated allele, which has been reported in other populations [19–21]. To confirm this, the 63 carriers with the Q319* variant were analyzed for the copy number variation of the *CYP21A2* gene by MLPA. The results showed that the majority (96.8 %, 61/63) of these individuals carried the Q319* mutation on a duplicated *CYP21A2* gene (Fig. 4A–D) and are therefore not carriers of the *CYP21A2* gene. In two (3.2 %) of the 63 individuals, the Q319* variant was not associated with a duplicated *CYP21A2* gene and thus identified as a carrier of *CYP21A2* (Table 1).

4. Discussion

CAH is a group of genetic autosomal recessive disorders, which is characterized by adrenal dysfunction and androgen hypersecretion. Newborns with SW-CAH are at risk of life-threatening if not diagnosis and treatment timely [4]. However, molecular genetic analysis of the *CYP21A2* gene is still challenging owing to its highly homologous with its pseudogene (*CYP21A1P*) [8]. Currently, there are multiple approaches for genetic analyses of *CYP21A2*, such as Sanger sequencing, MLPA, Southern blot analysis, and long-read sequencing [8,9,14,22,23]. Nonetheless, the main limitations encountered with these methods include complex operation, high requirement for DNA quality, expensive or time-consuming, which preclude the clinical utility for implementation in large-scale population screening. Studies have shown that the vast majority of CAH in a specific population is caused by a number of hotspot mutations [7,24,25]. Therefore, hotpot mutation-based screening is an ideal strategy for large-scale population screening. Furthermore, we used PCR combined with Sanger sequencing and MLPA to validate the detected variants, and the results showed that capillary electrophoresis-based method accurately genotyped variants in the *CYP21A2* gene. Compared with traditional approaches, this method has the advantages of high throughput, short turnaround time, simple operation, and low cost [15]. Capillary electrophoresis-based method is a reliable assay for detecting the *CYP21A2* gene variants with great potential for clinical application.

Table 1
Mutational spectrum of the *CYP21A2* gene in 5376 individuals.

Variants	Mutation on DNA level	Mutation on protein level	Alleles (n)	Ratio (%)
Large deletion or rearrangements	Del		5	6.17
	Chimera		8	9.88
Micro-conversion or SNV	c.293-13A/C > G	12G	27	33.33
	c.844G > T	p. V282L	16	19.75
	c.518T > A	p. I173N	16	19.75
	c.332_339del	E3del8bp (p. G111Vfs*21)	3	3.70
	c.955C > T[a]	p.Q319*	2	2.47
	c.292+1G > A		1	1.23
	c.710/713/719T/A	E6 cluster (p.I236N; p.V237E; p.M239K)	1	1.23
	c.92C > T	p. P31L	1	1.23
	c.923dupT	p. L308Ffs*6	1	1.23
Total			81	100

[a] Individuals who carried the haplotype of a duplicated *CYP21A2* gene with the p. Q319* mutation on one of the genes were excluded. NCBI Reference Sequence: NM_000500.9.

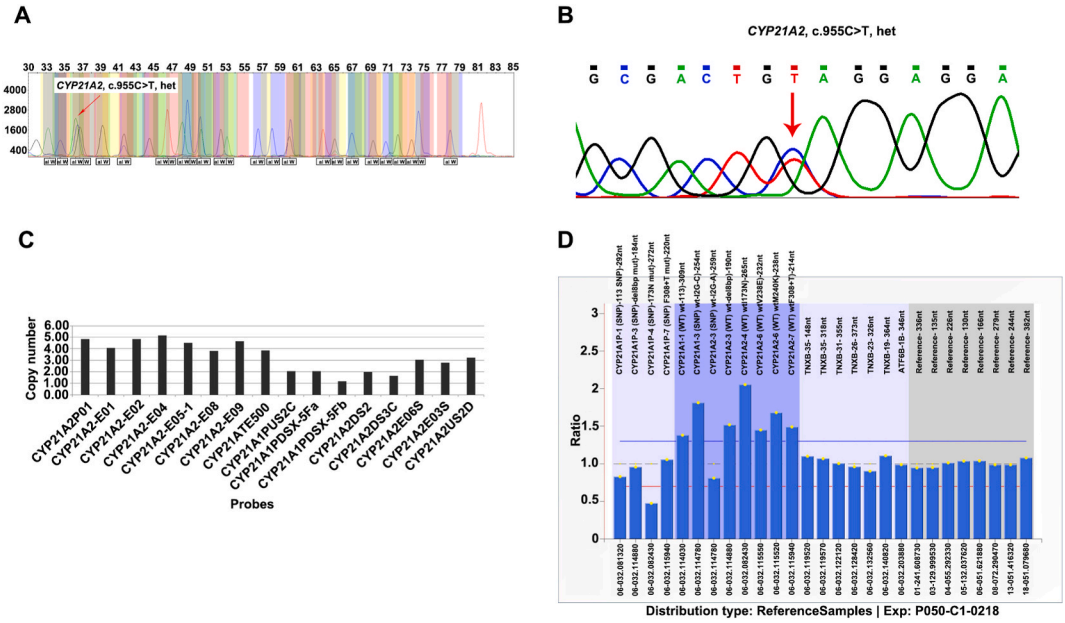

Fig. 4. The representative images of the haplotype with Q319* mutation and duplicated *CYP21A2*. (A) Representative electropherograms for SNaPshot. The x axis is size in bases, and the y axis is fluorescence intensity. The variant *CYP21A2*, c.955C > T is denoted by a red arrow. het, heterozygous. (B) The variant *CYP21A2*, c.955C > T was confirmed by Sanger sequence. (C) The duplication of *CYP21A2* was detected by HLPA. Columns represent the copy numbers for each probe. (D) MLPA showed an altered value around 1.5, suggesting a duplication event in heterozygous state.

In this study, 1.51 % (81/5376) individuals were identified as carriers of *CYP21A2* with a carrier rate of 1/66 and a presumed theoretical incidence of 1/17424. According to the reported neonatal screening statistics, the worldwide incidence of 21-OHD ranges from 1:14,000 to 18,000 live births, with significant racial and regional differences [2,26]. The incidence in North American and European countries ranges from 1/10,000 to 1/23,000 [27], 1/20,000 in Japan [28], 1/27,000 in New Zealand [29], and 1/6000 in India [30]. A recent meta-analysis of neonatal screening for 21-OHD in China showed that the incidence of CAH was 1/23,024, which was lower than most countries in Europe and America and was similar to Asia-Pacific countries such as Japan and Singapore [31]. This discrepancy in incidence can be explained by the following reasons. First, 21-OHD is autosomal recessive monogenic disease with inevitable ethnic aggregation effects, such as India and the Eastern Mediterranean region. Second, different diagnostic and statistical methods lead to differences in incidence. Finally, it is not excluded that a certain proportion of missed diagnosis and misdiagnosis due to the diversity of clinical phenotypes and the difficulty of gene diagnosis of 21-OHD.

In addition to racial and regional differences in 21-OHD incidence, hotspot mutations also differ across populations [24,26]. Del/Con had the highest mutation frequency among Native Americans [32], and c.293-13C/A > G had a high mutation frequency of 47.9 % and 30.3 % in Mexican and German populations, respectively [33,34]. In the Indian population, the most common mutation was c.293-13C/A > G (33 %), followed by deletion (24 %), and c.955C > T (21 %) [35]. Xia et al. summarized c.293-13A/C > G (36.2 %), large deletion or conversion (20.7 %) and c.518T > A (17.8 %) were the common mutations of the *CYP21A2* gene in 1442 Chinese patients, whereas the carrier rate of c.844G > T was very low [25]. However, our study showed that the most common mutation was c.293-13A/C > G (33.33 %), followed by c.844G > T (19.75 %), c.518T > A (19.75 %), and Del/Conv (16.05 %).

It has been reported that the majority of individuals presenting with the Q319* mutation comprise the mutation on one of three functional *CYP21A2* genes and are therefore not carriers of a *CYP21A2* allele [36]. This haplotype was first reported in 1994 by Wedell et al. in three Swedish patients [37]. Since then, a number of studies have documented that the frequency of this haplotype varies among different populations. For instance, Kharrat and colleagues analyzed *CYP21A2* gene copy number in 38 unrelated individuals and 11 family members carrying the Q319* mutation and observed that 84.2 % of these individuals had a duplication of the *CYP21A2* gene [19]. A study in the Tunisian population identified 17 heterozygous carriers of the Q319* variant from 136 healthy individuals, and found the presence of a duplicated *CYP21A2* gene in all 17 heterozygous Q319* [20]. However, the frequency of this haplotype in the Chinese population remains unknown. In the present study, we identified a total of 63 individuals (1.17 %, 63/5376) carrying the c.955C > T (p. Q319*) variant. Among them, 61 (61/63, 96.83 %) had a duplicated *CYP21A2* gene and are not carriers of *CYP21A2*. It has been suggested that when a Q319* mutation is detected in a healthy individual or in a patient, the copy number of *CYP21A2* should be analyzed to discriminate the severe Q319* mutation and the non-deficient variant in gene-duplicated alleles, thereby avoiding erroneous assignment of carrier status and providing correct genetic counseling [20,21]. Our capillary electrophoresis-based assay can simultaneously detect SNVs and copy number of *CYP21A2* and is a desirable method for large-scale population screening.

Our study has some strengths. First, this study reported for the first time that 96.83 % individuals carrying the c.955C > T (p. Q319*) had a duplicated CYP21A2 gene in the Chinese population and are therefore not carriers of a CYP21A2 allele. Second,

compared with our previous study [16], the present study had a relatively large sample size, which may result in more accurate estimation of the carrier frequency of the *CYP21A2* gene in the Chinese population.

However, some limitations of our study are worth noting. First, this capillary electrophoresis-based assay is a targeted genotyping approach that fails to identify some rare and novel variants in the *CYP21A2* gene, which may lead to underestimation of *CYP21A2* carrier frequency. Second, the majority of the participants in this study were Chinese Han ethnicity. The carrier frequency and mutational spectrum of the *CYP21A2* gene in other ethnicities remain unknown.

In conclusion, our study successfully established a capillary electrophoresis-based assay for genetic analysis of *CYP21A2*, which can accurately and effectively detect SNVs and copy number of the *CYP21A2* gene. Therefore, this approach may be suitable for the large-scale population screening of *CYP21A2*.

Ethics statement

This study was approved by the Institutional Review Board of Nanjing Women and Children's Healthcare Hospital (No. 2020KY-057). All these participants provided an informed consent for the analysis.

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Data availability statement

The authors declare that the main data supporting the findings of this study are available within the article and its Supplementary materials. Additional data and/or materials are available from the corresponding author upon reasonable request.

CRediT authorship contribution statement

Juan Tan: Writing – original draft, Methodology, Investigation, Data curation. **Shuping Jin:** Validation, Methodology, Investigation, Data curation. **Linxiang Huang:** Writing – original draft, Methodology, Investigation, Data curation. **Binbin Shao:** Methodology, Investigation. **Yan Wang:** Methodology. **Yuguo Wang:** Validation. **Jingjing Zhang:** Data curation. **Min Su:** Data curation. **Jianxin Tan:** Writing – review & editing, Supervision, Resources, Conceptualization. **Qing Cheng:** Supervision, Resources, Project administration, Conceptualization. **Zhengfeng Xu:** Writing – review & editing, Supervision, Resources, Project administration, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Zhengfeng Xu reports financial support was provided by National Key R&D Program of China and Jiangsu Province Capability Improvement Project through Science, Technology and Education Jiangsu Provincial Medical Key Discipline. Juan Tan reports financial support was provided by Lianyungang Health Science and Technology Project. Min Su reports financial support was provided by Nantong Science and Technology Project.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e38222.

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