Increased primary carnitine deficiency detection through second-tier newborn genetic screening

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Abstract

Background

Newborn screening for (NBS) for primary carnitine deficiency (PCD) is widely implemented worldwide, however, with poor sensitivity. This study aimed to evaluate the feasibility of improving the screening using second-tier genetic assay.

Methods

An Agena iPLEX assay was developed to identify 17 *SLC22A5* mutations in the Chinese populations, then this assay was applied in NBS for second-tier mutation screening of newborns with low free carnitine (C0) levels.

Results

The Agena iPLEX assay was successfully established and performed in second-tier genetic screening. A total of316 (0.15%) residual NBS-positive specimens were subjected to second-tier genetic screening. Twenty screen-positive newborns harbored biallelic mutations in *SLC22A5* gene, 99 screen-carriers with one mutation, and 197 screen-negative newborns with no mutation identified. Among 99 carriers, four newborns were found with a second disease-causing *SLC22A5*mutation by further genetic analysis. Four newborns were found with persistently low C0 levels among 197 screen-negatives, further genetic analysis revealed that one newborn with two novel *SLC22A5* pathogenic variants. In total, 25 newborns were diagnosed with PCD, for a positive predictive value of 7.91% (25/316). The incidence of PCD in Quanzhou was estimated at 1:8191.

Conclusion

Data from this study revealed that 24% (6/25) of PCD cases would have been missed by conventional NBS. The high throughput iPLEX assay is a powerful tool for PCD genotyping. The incorporation of second-tier genetic screening into NBS program could increase PCD detection, however, further studies are needed to optimize the workflow of new screening algorithm.

1. Background

Primary carnitine deficiency (PCD, OMIM #212140) is an autosomal recessive disorder of fatty acid oxidation caused by biallelic mutations of the *SLC22A5* gene encoding for the organic cation transporter type 2 (OCTN2) [1–3].This defect leads to urinary carnitine wasting, low serum carnitine levels and decreased intracellular carnitine accumulation. The clinical presentation of PCD is high variable, ranging from hypoketotic hypoglycemia and hepatic encephalopathy early in life, progressive hypertrophic

cardiomyopathy later in life, sudden death from cardiac arrhythmia, or no clinical symptoms at all [4–6]. Although some patients appear to be asymptomatic, PCD is a potentially lethal disease and recent studies have shown that untreated PCD is associated to episodes of sudden death [7, 8].Fortunately, the long-term prognosis is favourable with timely treatment as most symptoms are reversible [9].

Given the potential severity of PCD and the availability of effective treatment, PCD has been included in many newborn screening (NBS) programs worldwide [10–12].Tandem mass spectrometry (MS/MS) is mainly used in NBS for PCD, affected newborns can be detected by measuring free carnitine (C0) levels in dried blood spot. However, current MS/MS-based PCD NBS with poor sensitivity, leading to some neonates pass NBS but then present with cardiac failure and dilated cardiomyopathy [13–17]. Therefore, additional or second-tier testing are required to improve the performance of PCD NBS.

The Agena iPLEX assay is an effective and reliable approach for mutation screening, and our previous work has demonstrated the feasibility of incorporating genetic screening into the current NBS program [18, 19]. Here, an Agena iPLEX PCD assay was developed to identify 17 *SLC22A5* mutations in the Chinese populations, then this assay was applied as a second-tier testing to improve the performance of PCD NBS. This study is to present the results of the implementation of new screening algorithm.

2. Materials And Methods

2.1. Study cohort

A total of 20DBSspecimens from confirmed PCD patients were used to assess the robustness of the iPLEX assay. From January 2017 to December 2018, a total of 204,777 newborns were screened via MS/MS at Quanzhou Maternity and Children's Hospital, and the newborns with low C0 levels (C0 < 8μmol/L) were recruited for this study. This study was approved by the Ethical Committee of Quanzhou Maternity and Children's Hospital. Written informed consent was obtained from the parents of all infants for the collection of DBS samples and publication of medical data.

2.2. Multiplex PCD assay design and genotyping

PCR and iPLEX extension primers were designed for a total of 17 *SLC22A5* mutations, using MassArray Assay Design 3.1 software (Agena, San Diego, CA)with 80 < amplicon length (bp) < 120 and 4,500 < Mass Range (Da) < 9,000. These primers were run through BLAT and modified where necessary to avoid pseudogene amplification. Targeted primer design covers almost all the important *SLC22A5* gene mutation sites based mainly on the mutation frequencies reported in previous studies [5, 9, 20–23] and local mutation database(Supplementary file 1: Table S1). Genotyping using the MassARRAY platform was performed per the manufacturer’s instructions.

2.3. New screening algorithm

Second-tier newborn genetic screening was performed, the analytical workflow is summarized in Fig. 1. Initial DBS specimens with low C0 levels from newborns were submitted to Hangzhou Genuine Clinical

Laboratory (Hangzhou, Zhejiang, China) for *SLC22A5* mutation screening, after the center had completed NBS. Newborns with two, one and no *SLC22A5*mutation detected were defined as screen-positives, screen-carriers and screen-negatives, respectively. All screen-positives and screen-carriers were recalled and confirmed by targeted next-generation sequencing (NGS) as previously described [24]. All screen-negatives were also recalled even no mutations were identified, only if newborns with low C0 levels on second screen were referred for further genetic testing. Confirmatory diagnosis is made based on the presence of two pathogenic mutations in *SLC22A5*, diagnosed patients were treated with L-carnitine supplementation (50–100 mg/kg d).

3. Results

3.1. Assay validation

To validate the robustness of iPLEX genotyping assay, a double-blind analysis of 20 positive DBS samples was conducted. The iPLEX assay correctly detected all *SLC22A5* mutations from 20 samples, indicating its high sensitivity and specificity (Table 1).

Table 1
Genotypes of 20 patients with PCD detected by the iPLEX assay

No.	Genotype	iPLEX NICCD assay	Targeted NGS
1	c.760C > T/c.1400C > G	6	6
2	c.760C > T/c.760C > T	3	3
3	c.51C > G/c.760C > T	2	2
4	c.51C > G/c.51C > G	2	2
5	c.760C > T/c.797C > T	2	2
6	c.695C > T/c.1139C > T	2	2
7	c.51C > G/c.338G > A	1	1
8	c.51C > G/c.1195C > T	1	1
9	c.517delC/c.797C > T	1	1
Total		20	20

3.2. Newborn genetic screening and diagnosis

Overall, 316 (0.15%) newborns had C0 low levels on first screen were received second-tier genetic screening. Twenty screen-positive newborns harbored biallelic mutations in *SLC22A5* gene, 99 screen-carriers with one mutation, and 197 screen-negative newborns with no mutation identified. Four of these 99 carriers were found with a second *SLC22A5* mutation by further genetic testing. All 197 screen-negative newborns were recalled and retested using MS/MS, four of them with persistently low C0 levels

on second screen were confirmed by NGS, and one newborn with two novel *SLC22A5* pathogenic variants were detected. In total, 25 newborns were diagnosed with PCD and confirmed by genetic analyses, for a positive predictive value of 7.91% (25/316) (Table 2). Therefore, during the study period the incidence of PCD in selected population was estimated at 1:8191.

Table 2
Biochemical and genetic characteristics of patients with PCD

No.	Gender	NC0 levels (μmol/L)	RC0 levels (μmol/L)	Screened by MassArray assay		Confirmed by NGS	
				Allele 1	Allele 2	Allele 1	Allele 2
1	Male	1.96	1.73	c.760C > T	c.51C > G	c.760C > T	c.51C > G
2	Female	2.40	1.44	c.760C > T	c.760C > T	c.760C > T	c.760C > T
3	Male	5.78	10.67	c.760C > T	c.797C > T	c.760C > T	c.797C > T
4	Male	5.95	8.64	c.695C > T	NF	c.695C > T	**c.1160A > G**
5	Female	7.27	6.66	c.760C > T	c.797C > T	c.760C > T	c.797C > T
6	Female	5.58	5.59	c.760C > T	c.1400C > G	c.760C > T	c.1400C > G
7	Female	5.34	6.02	c.797C > T	NF	c.797C > T	**c.394-1G > A**
8	Female	1.78	1.90	c.695C > T	c.1139C > T	c.695C > T	c.1139C > T
9	Male	4.34	4.45	c.51C > G	c.51C > G	c.51C > G	c.51C > G
10	Female	4.75	4.16	c.760C > T	NF	c.760C > T	c.845G > A
11	Female	3.45	5.24	c.760C > T	c.1400C > G	c.760C > T	c.1400C > G
12	Female	6.82	5.02	c.760C > T	c.1400C > G	c.760C > T	c.1400C > G
13	Male	2.19	2.12	NF	NF	**c.822G > A**	**c.782_799del**
14	Male	2.73	9.84	c.51C > G	NF	c.51C > G	**c.1144_1162del**

NC0: free carnitine detected at newborn screening, RC0: C0 retested at recall stage, cutoff value: 8–50 μmol/L.

The novel *SLC22A5* variants identified by our team are in boldface type.

NF: Not found.

No.	Gender	NC0 levels (μmol/L)	RC0 levels (μmol/L)	Screened by MassArray assay		Confirmed by NGS	
				Allele 1	Allele 2	Allele 1	Allele 2
15	Male	3.00	10.81	c.51C > G	c.1400C > G	c.51C > G	c.1400C > G
16	Male	6.46	5.10	c.695C > T	c.1400C > G	c.695C > T	c.1400C > G
17	Male	3.02	1.77	c.760C > T	c.760C > T	c.760C > T	c.760C > T
18	Female	6.77	10.05	c.1400C > G	c.1400C > G	c.1400C > G	c.1400C > G
19	Female	2.36	1.75	c.760C > T	c.760C > T	c.760C > T	c.760C > T
20	Female	3.12	2.88	c.760C > T	c.51C > G	c.760C > T	c.51C > G
21	Male	3.64	3.80	c.695C > T	c.1139C > T	c.695C > T	c.1139C > T
22	Female	3.56	4.31	c.760C > T	c.1139C > T	c.760C > T	c.1139C > T
23	Female	6.27	3.43	c.695C > T	c.1139C > T	c.695C > T	c.1139C > T
24	Female	2.70	3.46	c.760C > T	c.51C > G	c.760C > T	c.51C > G
25	Male	7.35	14.27	c.338G > A	c.338G > A	c.338G > A	c.338G > A
NC0: free carnitine detected at newborn screening, RC0: C0 retested at recall stage, cutoff value: 8–50 μmol/L.							
The novel *SLC22A5* variants identified by our team are in boldface type.							
NF: Not found.							

Thirteen distinct *SLC22A5* mutations were identified, seven of which were included in the designed panel, one previously reported mutation c.845G > A was not included in the designed panel, the remaining five were newly identified pathogenic variants and were recently reported by our team[25].The most common mutation was c.760C > T with an allelic frequency of 32% (16/50), followed by c.1400C > G (7/50, 14%) and c.51C > G (7/50, 14%). Besides, c.695C > T, c.1139C > T, c.338G > A, and c.797C > T were also relatively common mutations. These seven mutations accounted for 88% (44/50) of the mutant alleles (Table 3).

Table 3
Detected *SLC22A5* mutations and their frequencies

No.	Mutaions	alleles	Frequencies (%)
1	760C > T	16	32
2	c.1400C > G	7	14
3	c.51C > G	7	14
4	c.695C > T	5	10
5	c.1139C > T	4	8
6	c.797C > T	3	6
7	c.338G > A	2	4
8	c.845G > A	1	2
9	**c.394-1G > A**	1	2
10	**c.782_799del**	1	2
11	**c.822G > A**	1	2
12	**c.1144_1162del**	1	2
13	**c.1160A > G**	1	2
The novel *SLC22A5* variants identified by our team are in boldface type.			

3.3. Biochemical characteristics

The mean C0 concentration at NBS in this cohort was 4.34 ± 1.81 μmol/L. After recalled, the mean C0 concentration was increased to 5.40 ± 2.94 μmol/L on second screen. Of note, six patients with low C0levels (2.73–7.35 μmol/L) on first screen, but the C0 levels (8.64–14.27 μmol/L) were within the normal cut-off value on second screen (Table 2).

4. Discussion

The sensitivity of NBS for PCD has been unsatisfactory since a small proportion of PCD patients are missed by current MS/MS-based screening approach. Our findings revealed that an overall proportion of missed cases of 24% (6/25) at conventional NBS, physicians should note thata normal C0 level during the recall stage does not necessarily rule out PCD. Incorporating second-tier method into the current NBS program may introduce an opportunity for missed PCD cases. The high throughput iPLEX genotyping assay we used has proven to be robust and reliable in the validation. Six PCD patients who would have been missed were successfully identified when we applied the iPLEX assay as a second-tier screening method. Therefore, we have clearly demonstrated that incorporating the second-tier molecular genetic testing into current NBS program could increase PCD detection.

The iPLEX assay has several advantages, our previous work have demonstrated that it is a powerful tool for population-based genetic screening [18, 19]. In this study, the iPLEX assay was applied in NBS for second-tier mutation screening of suspected PCD patients, this provides rapid diagnose of most PCD patients and helps shorten the time of disease diagnosis. The application of second-tier testing lead to early identification of all six PCD patients, which enables them to receive timely treatment and prevent the occurrence of adverse symptoms in these patients. These six PCD patients would be excluded from conventional NBS based on the normal second screen results, which means that all patients would have escaped detection if the new screening algorithm not been implemented, indicating the important value of second-tier genetic testing for discovering latent PCD patients in the NBS program. However, it is noteworthy that the improvement of sensitivity comes at the expense of increased carrier identification. A previous study in Taiwan used second-tier molecular test to screen c.760C > T in 206 newborns with low C0 levels and found 10 carriers, these carriers were directly excluded due to the normal C0 levels on second screen [26]. In contrast, our study found that 4% (4/99) of carriers had a second *SLC22A5*mutation/variant were true patients rather than carriers, especially two of them have normal C0 levels on second screen. The challenge is how best to use this assay to increase the detection and minimize the detection of unaffected carriers that require further genetic analysis. Further large-scale studies are needed to optimize the workflow of second-tier genetic screening. Notably, despite the designed panel with 17 hotspots represents the majority of *SLC22A5* mutations in China, one patient with extremely low C0 levels was missed by our second-tier genetic testing. Nevertheless, all predefined panels are faced with this deficiency because the known mutations targeted for a specific population have been studied far less. Due to the extremely low C0 levels on second screen, NGS was performed and two novel *SLC22A5* pathogenic variants were identified in this patient. Therefore, further genetic analysis is also required when newborns with persistently low C0 levels but no mutation was found in the second-tier genetic screening.

The incidence of PCD in selected population was estimated at 1:8191 by incorporating second-tier genetic screening into NBS programs. The incidence is higher than that reported in most region of China [9, 27, 28]. Of course there may be regional differences, but our findings suggest that the true incidence of PCD in China might be much underestimated. Consistent with most previous studies [22, 29], c.760C > T (p.R254*) was the most frequently occurring mutation in this cohort. The three mutationsc.760C > T, c.1400C > G and c.51C > G together had a relative frequency of 60%, confirming that these are hotspot mutations among Chinese populations [22, 23].

One limitation of this study is our selection of subjects based solely on the population with abnormal NBS results. It is well known that the C0 levels in newborns can be affected by the mother because C0 is transported to the fetus via the placenta [6, 12, 30]. If PCD patients with falsely normal C0 levels during NBS, the second-tier *SLC22A5* mutation analysis would not have been done. It is therefore possible that some PCD cases missed during NBS may not come to our attention. Utilizing a higher C0 cut-off value could reduce the number of missed cases during NBS, but was expected to produce an unacceptable percent of false positives, balancing these two metrics remains challenging.

To summarize, the incidence of PCD is relatively high in Quanzhou, China. The high throughput iPLEX assay is a powerful tool for PCD genotyping. The incorporation of second-tier genetic screening into current NBS program could increase PCD detection.PCD NBS continues to be a challenge, newborns with persistently low C0 levels would require combined genetic analyses, and further studies are needed to optimize the workflow of new screening algorithm.

Abbreviations

PCD: primary carnitine deficiency; OCTN2: organic cation transporter type 2; MS/MS: tandem mass spectrometry; NBS: newborn screening; C0: free carnitine; DBS: dried blood spot; NGS: next-generation sequencing.

Declarations

Ethics approval and consent to participate

This study was approved by the Ethical Committee of Quanzhou Maternity and Children's Hospital and was performed in accordance with the Declaration of Helsinki. Written informed consent was obtained from the parents of all infants for collection of DBS samples and publication of medical data.

Consent for publication

Consent was obtained from the parents of all patients for publication.

Availability of data and materials

The datasets used and/or analysed during the current study can be obtained from the corresponding author upon a reasonable request.

Conflict of Interest

The authors declare that they have no conflicts of interest.

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Author contributions

YM Lin performed the data analysis, drafted and revised the manuscript; WF Zhang collected the clinical data, drafted and revised the manuscript; CG Huang carried out the genetic tests, mutation analysis and paper editing; CM Lin, WH Lin, and WL Peng followed the patients and collected the clinical data; QL Fu

assisted with data collection, reviewed and revised the manuscript; DM Chen designed and supervised the research study. All authors contributed to the data analysis, revising and approving the final manuscript to be published.

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Figures

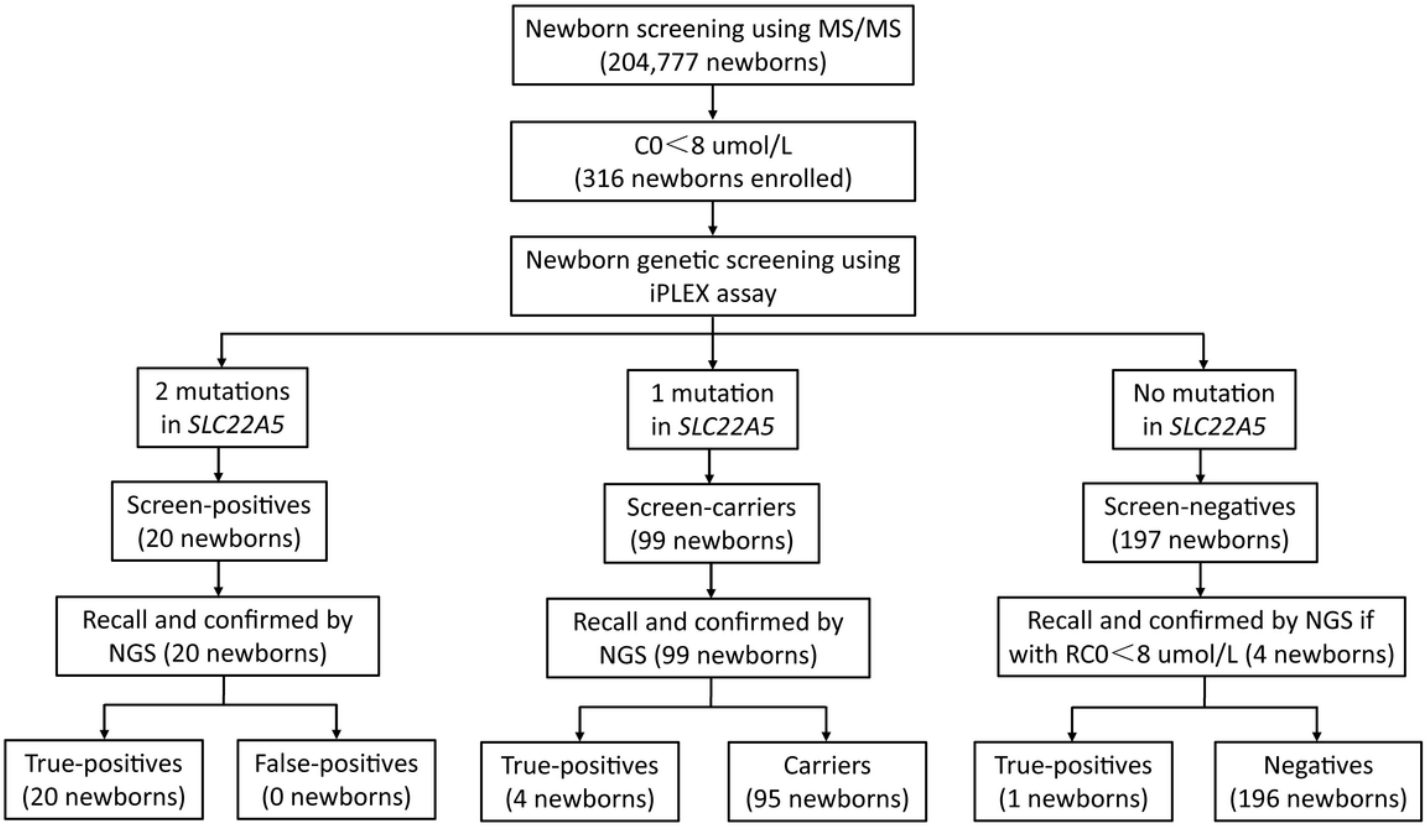

Figure 1

The analytical workflow of newborn second-tier genetic screening for primary carnitine deficiency (PCD). MS/MS indicates tandem mass spectrometry; NGS: next-generation sequencing; C0: free carnitine; RC0: retested C0 during the recall stage.

Supplementary Files

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