Screening and Diagnosis of Children with Primary Carnitine Deficiency in Zhejiang Province, China

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LL Yang, XW Huang, JB Yang, XL Zhou, XL Huang, RL Yang

Abstract

Primary carnitine deficiency (PCD) is a rare autosomal recessive disorder, caused by deficiency in the plasma membrane carnitine transporter. In this report, we aimed to describe the diagnosis and treatment of 12 PCD patients. Blood spots were collected from the children, and analysed using liquid chromatography-mass spectrometry (LC-MS). Newborns and children who had repeated lower dried blood spot free carnitine levels than the cut-off value will be recalled. Children who had positive recalled tests were subjected to confirmatory tests including urinary organic acid analysis with gas chromatography-mass spectrometry (GC-MS), urine ketone, glucose, ammonia, lactate, liver and kidney function, and molecular analysis, etc. Three children were diagnosed to have PCD clinically, and 9 by neonatal screening. One mother was also diagnosed to have PCD. Thirteen *SLC22A5* gene mutations were found in these patients. The 9 patients identified by neonatal screening were asymptomatic at diagnosis, and had normal growth and development during follow-up. Two of the three clinically diagnosed patients turned normal after treatment, and the other showed retarded growth compared to his normal peers. PCD incidence is not that rare in China. It is crucial to increase the coverage of newborn screening for early diagnosis of PCD.

Keyword : Inborn errors of metabolism; Mass spectrometry; Newborn screening; Primary carnitine deficiency

Introduction

Primary carnitine deficiency (PCD) (OMIM 212140), a rare autosomal recessive disorder, is caused by deficiency in the plasma membrane carnitine transporter in the muscle and kidney, with urinary carnitine wasting causing systemic carnitine depletion.[1,2] This disorder arises from a mutation in *SLC22A5*, a gene that encodes the Na^+/carnitine plasma membrane transporter OCTN2, resulting in urinary carnitine wasting, low serum carnitine levels, and decreased intracellular and tissue carnitine accumulation.[2,3] Carnitine takes pivotal role in the transfer of long-chain fatty acids across the inner mitochondrial membrane for subsequent b-oxidation. The lack of carnitine impairs the ability to use fat as fuel during periods of fasting or stress. Affected persons can have a wide spectrum of manifestations such as acute metabolic decompensation in early life; skeletal myopathy or cardiomyopathy in childhood;[4] or even end up as sudden death.

The diagnosis of PCD is made by demonstrating global decrease in all carnitine related species in plasma and low urine carnitine reabsorption. It is confirmed by skin biopsy demonstrating decreased fibroblast carnitine transport or by showing mutation of *SLC22A5* gene. In patients with PCD, serum free and acylated carnitine are markedly reduced (free carnitine <5 μmol/L, normal: 25-50 μmol/L). PCD can be identified in infants shortly after birth by expanded newborn screening using tandem mass spectrometry that can detect low levels of free carnitine (C0). Women can be identified with PCD after low carnitine levels are detected in their infants through newborn screening. Mothers with this condition are mostly asymptomatic, although evidences reveal that some feel better after initiation of carnitine therapy.[5]

Herewith, we report the diagnoses of PCD in 12 children, as well as the molecular analysis of their parents.

Subjects and Methods

Study Subjects

The Newborn Screening Center of Zhejiang Province, the largest screening center in China, implemented tandem mass spectrometry for screening PCD in high-risk children since 2008 and then expanded to newborn screening in 2009. High-risk children were those with symptoms suggesting inborn errors of metabolism including: metabolic acidosis, jaundice, hepatosplenomegly, recurrent vomiting, hypoglycaemia, hyperammonaemia, mental retardation of unknown reasons, language delay, seizures and unconsciousness. Samples from the symptomatic patients with suspected inborn errors of metabolism were referred and sent from throughout the province and neighboring provinces. This study was approved by the Ethical Committee of Children's Hospital, Zhejiang University School of Medicine. Written consents were obtained from the parents for retrieving clinical details of the affected infants.

PCD Screening

Dried blood spots (DBS) were collected on Scheicher and Schuell 903 filter paper for all patients. For newborn screening, blood spots were collected at days 3-6 of life. Blood spots were analysed using liquid chromatography-mass spectrometry (LC-MS) with electrospray ionization, and Waters Quattro API tandem mass spectrometer (Waters Quattro, USA) was used. All procedures of sample preparation and MS analysis were performed according to the protocol provided by the manufacturers.

The cut-off for free carnitine level in our laboratory was 17-90 μmol/L. Newborns and symptomatic children who had repeated lower DBS free carnitine levels than the cut-off value at the newborn screening were recalled. Children who were positive for the recall or rescreening DBS were subjected to confirmatory tests, including urinary organic acid analysis with gas chromatography-mass spectrometry (GC-MS), urine ketone, blood gas, glucose, ammonia, lactose, liver and kidney function, electrocardiography and molecular analysis. DBS free carnitine measurements and molecular analyses were also done for their parents after obtaining informed consents. All the confirmed cases were given L-carnitine supplementation (50-200 mg/kg/day), and animal-based diet is recommended. Plasma carnitine levels were monitored every 2-3 months for patients during treatment. L-carnitine dosage was adjusted based on the blood free carnitine level.

Deoxyribonucleic Acid (DNA) Analysis

Molecular analysis was done at Genetic Diagnostic Laboratory at Zhejiang University. DNA sequencing of the entire coding regions of the *SLC22A5* gene was performed. DNA for sequencing was extracted with extraction kit (TIANGEN BIOTECH, Beijing). Primers were synthesized by Sangon Biotech (Shanghai, China). Sequence-specific oligonucleotide primers for the *SLC22A5* gene were designed to amplify the 10 coding extrons. Primers are shown in Table 1. The sequencing results were compared with the GenBank *SLC22A5* sequence (NT_003060.2) by using the Mutation Surveyor Version 3.0.

Table 1 The primers used in sequencing *SLC22A5* gene

	Primers		**Product length, bp**
	Forward	**Reverse**	
SLC22A5-extron1	GCAAAGCCCGCCGCGTT	CAGCGTCCAGTGCGCATCTG	550
SLC22A5-extron2	GAGCGTGTGGGGATGGCAGG	GGCAAGCCAGGCTACTGCAGA	500
SLC22A5-extron3	CGAGACTGTCCCTGGCAGCC	GGGACCCCAGGCTGGTCTCA	489

SLC22A5-extron4	GGCCTGAGAGGCCACAGGGA	GGATTCATGGGTTGTTGCTGCCCT	459
SLC22A5-extron5	GAGGTGGCCAGCCAGCATGG	TTCAGGGCTGGGTGCTGCTG	439
SLC22A5-extron6	TCCCCGACGCTGAGATGCAGA	TCCCACAACAGCCTCCACAATTGT	439
SLC22A5-extron7	CGCAGGGTTACAGTTACTGCTGCC	GCAAGGGAGCTGTGATGGGCT	470
SLC22A5-extron8	GCTGCAGGTCCCAGCCTCC	ACACGCAACCTCCAGCTCACA	457
SLC22A5-extron9	CCCCTTCCAGAGTCCTGGGAGC	CCAGTGCGGCTACTGCCATGG	457
SLC22A5-extron10	CCATGGCAGTAGCCGCACTGG	TGGGTGCCCATCCAGGAGCA	402

Table 2 *SLC22A5* gene sequencing results of primary carnitine deficiency in symptomatic patients

Cases	Gender	Age (months)	Symptoms	Concentration of free carnitine at diagnosis (mmol/L)	Variation range in follow-up(mmol/L)	Allele1	Allele2
1	Female	1.7	Convulsion	1.37	4.19-11.12	Extron 4 c.680G>A (p.R227H)	Extron 5 c.825G>T (p.W275C)
2	Male	88	Skeletal and cardiacmyopathy	0.87	4.92-24.04	Extron 4 c.760C>T (p.R254X)	
3	Female	1.2	Convulsion	4.56	4.56-10.32	Extron 4 c.680G>A (p.R227H)	Extron 5c.825G>T (p.W275C)

Table 3 SLC22A5 gene sequencing results of primary carnitine deficiency in newborn screening

Cases	Gender	Concentration of free carnitine at newborn screening (μmol/L)	Follow-up period (months)	Variation range infollow-up (μmol/L)	SLC22A5 gene sequencing	
					Allele1	Allele2
NBS 1	Female	8.30	18	1.31-23.84	Extron 4 c.760C>T, p.R254X	Extron 8 c.1433C>T, p.P478L
NBS 2	Male	4.01	10	6.73-10.58	Extron 3 c.505C>T, p.R169W	Extron 4 c.760C>T, p.R254X
NBS 3	Male	7.22	22	1.98-16.10	Extron 4 c.760C>T, p.R254X	
NBS 4	Male	8.21	21	4.21-9.56	Exton 8 c.1400C>G,p.S467C	
NBS 5	Female	4.00	16	3.21-20.71	Extron 10 p.F17L	
NBS 6	Male	9.71	8	6.01-9.91	Extron 1 c.338G>A, p.C113Y	Extron 8 c.1400C>G, p.S467C

NBS 7	Female	13.3	7	9.55-7.87	Extron 10 p.F17L	Extron 10 p.S467C
NBS 8	Male	11.04	5	5.19-6.43	Extron 10 p.F17L	
NBS 9	Female	6.58	3	3.78-6.52	Extron 10 p.F17L	Extron 10 c.497+1G>T

NBS: Newborn screening

Table 4 *SLC22A5* gene sequencing results of PCD in the five mothers

Cases, n	Age at diagnosis (years)	Clinical manifestation	Concentration of free carnitine in newborn screening (μmol/L)	Variation range in follow up (μmol/L)	SLC22A5 gene sequencing	
					Allele1	Allele2
1	28	No	4.71	2.18-7.58	Extron 8 c.1400C>G, p.S467C	-
2	26	No	8.06	8.67-9.12	Extron 8 c.1400C>G, p.S467C	-
3*	31	Fatigue	5.01	4.28-7.69	Extron 4 c.760C>T, p.R254X	-
4	27	No	3.87	3.97-5.29	Extron 10 p.G462D	Extron 10 p.S467C
5	29	No	2.33	3.73-6.66	Extron 3 c.505C>T, p.R169W	Extron 4 c.760C>T, p.R254X

-: no mutations; *: Mother of NBS-3

Results

From 2008 to 2011, 17,124 symptomatic patients (9958 boys, 7166 girls) with a median age of 17.8 months (range: 0.05-123.2 months) were screened. Among the symptomatic patients, three were diagnosed with PCD with a detection rate of 1/5,708 (Table 2). One was male and the other two were females (SPS 1-3). From 2009 to 2011, 460,000 newborns were screened. Totally 78 newborns with reduced free carnitine levels, and their parents were recalled for confirmatory testing. Nine of them were diagnosed with PCD at a prevalence of 1/51,111 (NBS 1-9) (Table 3). Six mothers had persistent low carnitine level, and five were diagnosed with PCD. One of the five mothers was the mother of a newborn case (NBS 3) (Table 4). The mother showed fatigue at diagnosis, and symptoms improved after supplementation with L-carnitine. All of the other mothers were recommended to take oral L-carnitine and animal-based diet.

All the newborns with confirmed PCD were asymptomatic at diagnosis. They were born term with normal birth weight (5 males and 4 females). Their DBS free carnitine level at screening ranged from 4.01 μmol/L to 13.30 μmol/L. Some cases showed reduced free carnitine level during the follow-up. One infant had free carnitine level of 8.31 μmol/L at screening, which reduced to 1.31 μmol/L during the follow-up and increased to normal at 18 months

of age. Most cases had free carnitine level increased to above 10.0 μmol/L after treatment. No cardiac or other clinical symptoms were found in these children. Echocardiography or myocardial enzymes profiles should be done for these children in the further follow-up. All these newborn cases showed normal growth and development during the follow-up (Table 2).

All three PCD children diagnosed by high-risk screening were symptomatic at diagnosis (Table 2). Two of these three patients presented with convulsion and epilepsy when diagnosed at the age of 1.7 and 1.2 months. Both had no family history. They showed hypoglycaemia, anaemia and hyperlactacidaemia at diagnosis. All symptoms disappeared after supplementation with oral carnitine. The third patient was otherwise healthy until four years of age when he presented with weakness and decreased exercise tolerance. Growth parameters and development were lower than age-matched control. He presented with cardiomyopathy and skeletal muscle disability at age of 6 years. He has been misdiagnosed until 7 years old and was found to have wasting (weight, 18 kg) and anaemia (Hb, 86 g/L). Blood creatine kinase-MB was high at 8.56 ng/mL (reference: <4.94 ng/mL) and cTnT<0.003 ng/mL at diagnosis. Chest X-ray and echocardiography revealed a dilated left heart and low ejection fraction of 43%. Coronary computed tomography angiography (CTA) showed cardiomyopathy and cranial magnetic resonance imaging revealed patch-like abnormal signals at bilateral frontal lobes. Clinical symptoms quickly improved after he was given intravenous carnitine (200 mg/kg/d). Oral carnitine was prescribed upon discharge. His cardiac function returned to normal after one month, and he did not have any metabolic disorders recurrence. The free carnitine was still low at <5 μmol/L after one year of treatment (Table 2). His physical growth remained retarded compared to his peers at latest follow-up.

The results of molecular analysis of these patients and their parents are shown in Tables 2-4. Thirteen *SLC22A5* gene mutations were found in the study. Three of them were novel mutations including p.C113Y, p.R227H and p.W275C (Figures 1 and 2). p.R254X and p.S467C were the most common mutations in these newborns. Mutations of the five mothers are shown in Table 4.

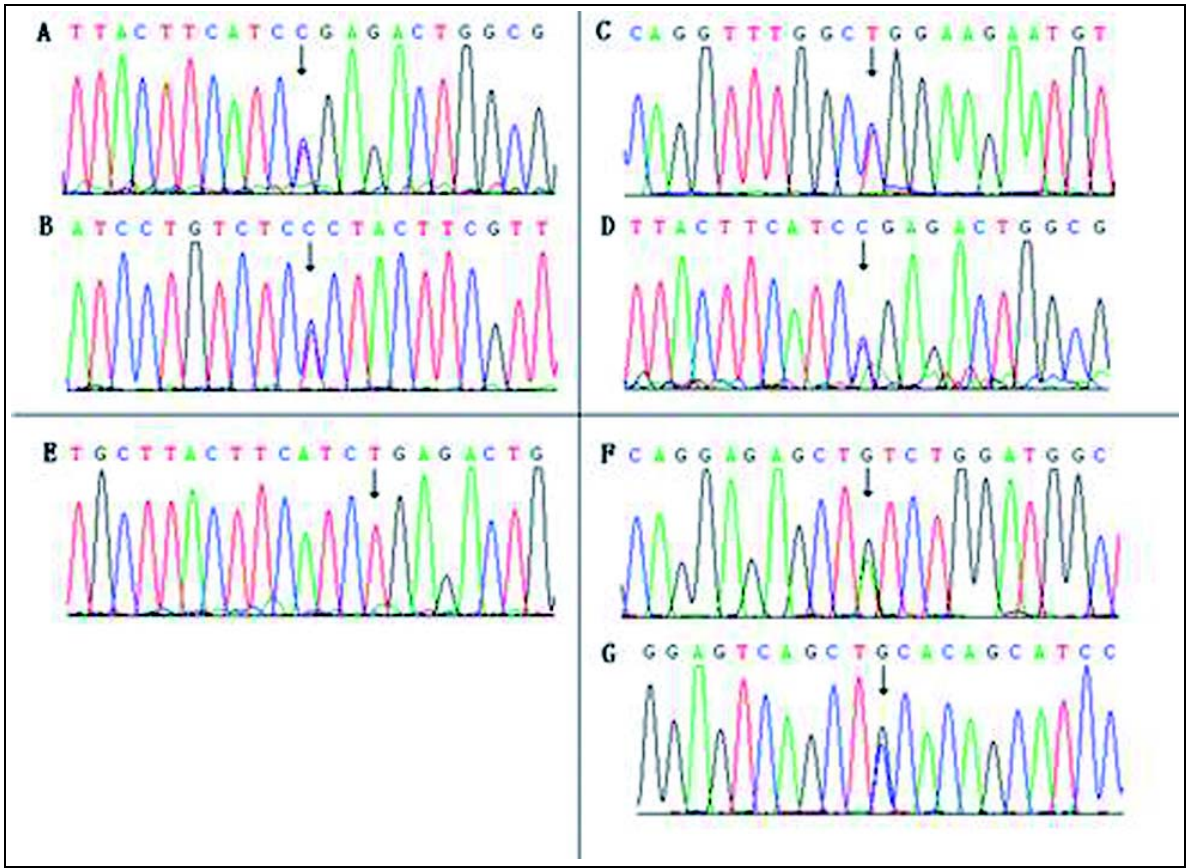

Figure 1 *SLC22A5* gene sequences in the newborn primary carnitine deficiency cases.

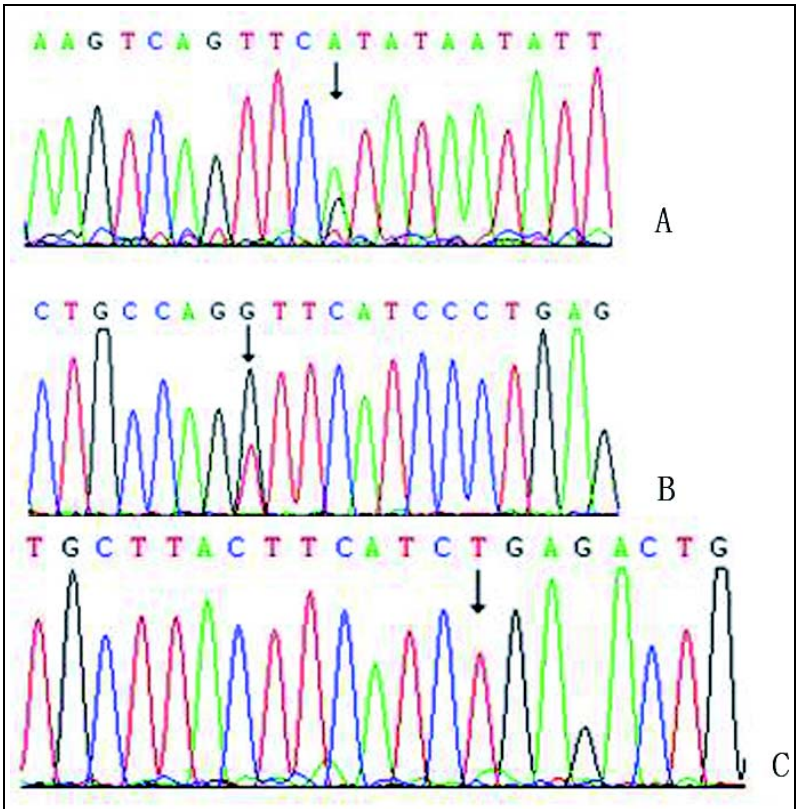

Figure 2 *SLC22A5* sequences. (A) Mutations in the *SLC22A5*-extron 4: c.680G>A, p.R227H; (B) Mutations in the *SLC22A5*-extron 5: c.825G>T, p.W275C; (C) Mutations in the *SLC22A5*-extron 4 c.760C>T, p.R254X.

Discussion

The incidence of PCD is about 1/50,000 based on NBS studies in USA. The incidence is below 1 in 100,000 newborns in many European countries; however, the incidence of PCD is very high in Faroe Island with the carrier frequency of about 1:20, and estimated disease prevalence of 1:1300.[6] For Asian countries, the incidence is 1 in 40,000 newborns in Japan and 1 in 67,000 newborns in Taiwan.[7-9] The incidence of PCD in China remains unclear now. We report the results of PCD screening from a single province in China. During the three-year pilot study, a total of 12 PCD cases were identified, and nine were found by newborn screening with an incidence of 1 in 35,000 newborns. The incidence in the present study is similar to that of Japan but higher than that of Taiwan. The incidence of PCD in southern China was reported to be higher due to a founder mutation (p.R254X) of the *SLC22A5* gene.[10] PCD is one of the most common fatty acid oxidation disorders (FAOD) in our children cohort. Whereas in most European countries and the United States, medium-chain acyl-CoA dehydrogenase (MCAD) deficiency is the most prevalent FAOD. It indicates that PCD incidence has geographical and ethnic variation.

The incidence of maternal PCD has been proposed to be higher than that in newborns.[5,11,12] Carnitine can be transported through placenta, therefore low blood carnitine concentration detected in the newborn should be followed by investigations of the mother's blood carnitine level.[12] Our study measured blood carnitine level in 74 mothers whose newborn infants had a reduced carnitine level at screening, and five mothers were diagnosed with PCD. Previous reports found that the prevalence of mothers with PCD is 1 in 33,000 in Taiwan, and 40,000 in Minnesota.[12] There are other reports describing the detection of mothers with PCD through newborn screening

programs.[5,11,13] We couldn't conduct population study in the mothers because majority of parents declined the test. Investigation of mothers whose infants had low carnitine level after birth is effective and efficient in diagnosing maternal PCD.

PCD phenotypes are heterogeneous. PCD can present in children of different ages with hypoketotic hypoglycaemia, cardiomyopathy, and/or skeletal myopathy.[14] The initial symptoms in most patients are cardiomyopathy or hypoketotic hypoglycaemia, which appear in infancy. Some unconventional symptoms such as growth retardation, oesophageal reflux and hypotonia may appear.[15] All our newborns were asymptomatic at screening. For high-risk children, PCD manifested as acute encephalopathy during infancy as in two of our patients; one patient presented with skeletal and cardiomyopathy at four years of age.[12] Mothers with primary carnitine deficiency are usually asymptomatic, but at risk of sudden death from arrhythmia.[5] Previously asymptomatic women with primary carnitine deficiency can present with symptoms during pregnancy due to a reduction in carnitine levels and increased metabolic demand. Easy fatiguability seems to be the most common symptom in this group of women.[16] Two recent reports showed three of the six mothers had easy fatiguability after exercise and hunger intolerance.[9,11] In this study, four mothers were asymptomatic, and one complained of fatigue. Though with mild or no symptoms, PCD is a potential fatal condition like other fatty acid metabolism disorders, and it may cause sudden death under severe stress. Therefore, diet and medical intervention should be recommended to the PCD patients, though asymptomatic at diagnosis.

It is still unclear why some women remain asymptomatic while others with the same disease present early in life. Li et al identified 11 clinically asymptomatic mothers carrying two OCTN2 mutations through abnormal newborn screening in their infants.[17] Our study also found two asymptomatic mothers with two OCTN2 mutations. The other three mothers carried one mutation, and one of them presented with fatigue. Nine newborn cases were also asymptomatic, and three clinically diagnosed cases were symptomatic due to late diagnosis and treatment. Two symptomatic patients had the same OCTN2 mutations, and convulsion was the initiated symptom for them. The other one only had one OCTN2 mutation, but with skeletal and cardiomyopathy. Previous reports revealed no clear relationship between genotype and clinical phenotype, in particular among symptomatic subjects suspected to have PCD.[18] Therefore, phenotypes variation may be affected by environmental factors.

Treatment of PCD consists of dietary supplementation with carnitine and medium-chain fatty acids, minimising dietary intake of LCFAs and avoidance of fasting or prolonged exercise. Standard treatment can correct metabolic abnormalities and prevent debilitating or fatal complications. Cardiomyopathy associated with primary carnitine deficiency may be reversible after several months of treatment; carnitine supplementation can also relieve or prevent the chronic symptoms.[19,20] Free carnitine in the blood and tissue will drop continuously due to the persistent loss of carnitine in the urine.[21] We found that blood carnitine level can increase remarkably after treatment in most patients during the follow-up. However, some patients remained to have a low level of blood carnitine due to negative balance due to excessively urine carnitine excetion. The differences in presentations for different patients may be due to variation in phenotype-genotype correlation. All the symptomatic patients recovered well after medication and diet intervention. All of our newborns had a normal growth and development due to early diagnosis and treatment. Blood free carinitine also increased in the mothers after diet intervention. Therefore, in addition to carnitine supplementation, diet can also provide 75% of the daily carnitine requirements. However, it is unclear whether supplemental carnitine treatment also reduces the risk of sudden death in patients who experience life-threatening arrhythmias. Diet intervention is important in the treatment of PCD.

There are limitations of this study. Firstly, low blood carnitine is not specific to PCD; it can also be secondary to other conditions such as maternal carnitine deficiency, drugs, malnutrition, various organic acidemias and fatty acid oxidation defects. Urine organic acids do not show any consistent abnormalities in PCD as well; and a global decrease of all carnitine species in plasma and low urine carnitine reabsorption would be more useful biochemical markers for PCD. However, we cannot perform urine carnitine reabsorption analysis in our laboratory yet. Secondly,

for better diagnosis on PCD, we developed the molecular method in the laboratory. However, this method also has limitation. It may miss certain mutations or gene defects. Microarray may be useful in screening for the missing gene defect. Functional assay in fibroblasts remains the best confirmatory test.

In conclusion, PCD is not rare in our locality and it is a potentially fatal but highly treatable disease. It is crucial to increase the coverage of newborn screening for early diagnosis of PCD. Many PCD patients may present with either mild or no symptoms; screening will be valuable in relieving or preventing symptoms. The management of those asymptomatic cases, especially in adults, confers some controversies in the current management strategies.

Conflict of Interest

We declare that we have no conflict of interest.

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