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Screening for Five Prevalent Mutations of *SLC25A13* Gene in Guangdong, China: A Molecular Epidemiologic Survey of Citrin Deficiency

Zhan-Hui Zhang,[1,2,*] Zhi-Gang Yang,[1,*] Feng-Ping Chen,[3] Atsuo Kikuchi,[4] Zhen-Huan Liu,[5] Li-Zhen Kuang,[6] Wei-Ming Li,[7] Yuan-Zong Song,[1] Shigeo Kure[4] and Takeyori Saheki[8]

[1]Department of Pediatrics, The First Affiliated Hospital, Jinan University, Guangzhou, Guangdong, P.R. China
[2]Central Laboratory, The First Affiliated Hospital, Jinan University, Guangzhou, Guangdong, P.R. China
[3]Department of Laboratory Science, The First Affiliated Hospital, Jinan University, Guangzhou, Guangdong, P.R. China
[4]Department of Pediatrics, Tohoku University Graduate School of Medicine, Sendai, Miyagi, Japan
[5]Department of Pediatric Neurorehabilitation, Nanhai Maternity and Child Care Hospital, Guangzhou University of Chinese Medicine, Foshan, Guangdong, P.R. China
[6]Department of Laboratory Science, Nanhai Maternity and Child Care Hospital, Guangzhou University of Chinese Medicine, Foshan, Guangdong, P.R. China
[7]Department of Pediatrics, Maternal and Child Health Hospital of Qingyuan City, Qingyuan, Guangdong, P.R. China
[8]Institute of Resource Development and Analysis, Kumamoto University, Kumamoto, Kumamoto, Japan

Citrin is the liver-type aspartate/glutamate carrier isoform 2 (AGC2) encoded by *SLC25A13* gene, playing important roles in the urea cycle and the malate-aspartate shuttle. Citrin deficiency (CD) has proven a disease entity with high prevalence in south China, including Guangdong with the largest population, but CD epidemiology in this province has not been well characterized. This study aims to screen for five prevalent *SLC25A13* mutations, c.851_854del (p.R284fs286X), c.1638_1660dup (p.A554fs570X), c.615+5G>A (p.A206fs212X), IVS16ins3kb (p.A584fs585X) and c.1399C>T (p.R467X), to calculate the mutation carrier rate in Guangdong. A total of 2,428 used blood samples for health examination were collected as the research subjects, including 1,558 from 5 cities in the Pearl River Delta area and the remaining 870 from 4 peripheral cities, and the 5 mutations screened using High Resolution Melting Assay and HybProbe assay. A total of 52 carriers were detected, including 2 carriers of a novel c.1420G>A (p.V474M) mutation that impairs citrin function, as judged by the functional analysis in the yeast system. The carrier rate was higher in Pearl River Delta area than that in the peripheral cities (26/1,558 vs. 26/870, with $\chi^2 = 4.639$ and $P < 0.05$). The carrier rate was around 1/47 (52/2,428), theoretically with the CD morbidity of 1/8,800 and the number of CD patients over 11,800 in Guangdong population. This study has provided primary epidemiologic data for the evaluation of CD effect in Guangdong province. Moreover, the newly identified c.1420G>A mutation that impairs AGC2 function has enriched the mutation spectrum of human *SLC25A13* gene.

Keywords: citrin deficiency; high-frequency mutations; melting curve analysis; mutation; *SLC25A13* gene
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Introduction

Human *SLC25A13* gene encodes citrin, the liver-type aspartate/glutamate carrier isoform 2 (AGC2) (Kobayashi et al. 1999; Palmieri et al. 2013, 2014). In hepatocytes, citrin functions to export mitochondrial aspartate from the mitochondrial matrix in exchange for cytosolic glutamate and H^+, playing important roles in the urea cycle and malate-aspartate shuttle (Begum et al. 2002; Saheki et al. 2004, 2005; Palmieri et al. 2013, 2014). Biallelic *SLC25A13* mutations result in citrin deficiency (CD) (Kobayashi et al. 1999), and currently three age-dependent clinical phenotypes of CD have been described, i.e. Neonatal Intrahepatic Cholestasis caused by Citrin

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*These two authors contributed equally to this study.
Correspondence: Yuan-Zong Song, M.D., Ph.D., Department of Pediatrics, The First Affiliated Hospital, Jinan University, No. 613 Huangpu Dadao Xi, Tianhe district, Guangzhou, Guangdong 510630, P.R. China.
e-mail: songyuanzong@vip.tom.com

Deficiency (NICCD, OMIM 605814) in infants, adult-onset citrullinemia type II (CTLN2, OMIM 603471) in adolescents/adults, and Failure to Thrive and Dyslipidemia caused by Citrin Deficiency (FTTDCD) between the two aforementioned CD stages (Saheki et al. 2011; Song et al. 2011, 2013; Kobayashi et al. 2012). As a worldwide distributed disease entity, CD is relatively more common among East Asian population (Fu et al. 2011; Kobayashi et al. 2012; Song et al. 2013; Vitoria et al. 2013; Wongkittichote et al. 2013a). The estimated carrier rate of *SLC25A13* mutations had been documented to be 1/63 in China, and particularly 1/48 in its south area (Lu et al. 2005; Kobayashi et al. 2008). Over 80 *SLC25A13* mutations/variations have been reported so far, and among the 26 mutations detected in Chinese CD patients, the 5 mutations c.851_854del (p.R284fs286X), c.1638_1660dup (p.A554fs570X), c.615+5G>A (p.A206fs212X), IVS16ins3kb (p.A584fs585X) and c.1399C>T (p.R467X) account for 85.40% of all mutated alleles (Song et al. 2013), constituting the prevalent *SLC25A13* mutations among Chinese population.

The definite diagnosis of CD requires *SLC25A13* gene analysis with such tools as Polymerase Chain Reaction (PCR), PCR-Restriction Fragment Length Polymorphism (RFLP) and direct sequencing (Lu et al. 2005; Song et al. 2007, 2008, 2009; Tabata et al. 2008; Zhang et al. 2012). Nonetheless, these tools are laborious and costly. Recently, melting curve analysis, including High Resolution Melting Assay (HRMA) and HybProbe Assay, has been developed as convenient, accurate and rapid method for the screening of *SLC25A13* mutations (Lin et al. 2011; Kikuchi et al. 2012; Wongkittichote et al. 2013a). Guangdong is a province in south China, and its permanent resident population has reached 104 million according to the latest official data (http://www.gdstats.gov.cn/tjgb/t20110511_83329.htm). However, the epidemiology of CD in this region remains to be characterized. This study aimed to screen for the prevalent *SLC25A13* mutations and then to calculate the mutation carrier rate and the theoretical CD morbidity in Guangdong population, thereby providing direct molecular evidence for the epidemiological evaluation of CD in this province.

Materials and Methods

Subjects

Used blood samples for health examination from the medical centers in different cities of Guangdong province (Fig. 1) were collected from April 2011 to March 2013. The sample size was calculated based on the carrier rates of 2% which is less than the estimated carrier rate (1/48) of *SLC25A13* mutations from previous studies (Lu et al. 2005; Kobayashi et al. 2008). Using the Sample Size Calculator (http://www.nss.gov.au/nss/home.nsf/pages/Sample+size+calculator) with the confidence level of 95%, the population size of 100 million, the estimated proportion of 0.02 and the confidence interval of 0.01, the target sample size to achieve statistical significance was calculated to be 753. In order to increase the screening reliability, the actual sample size was added up to 2,428,

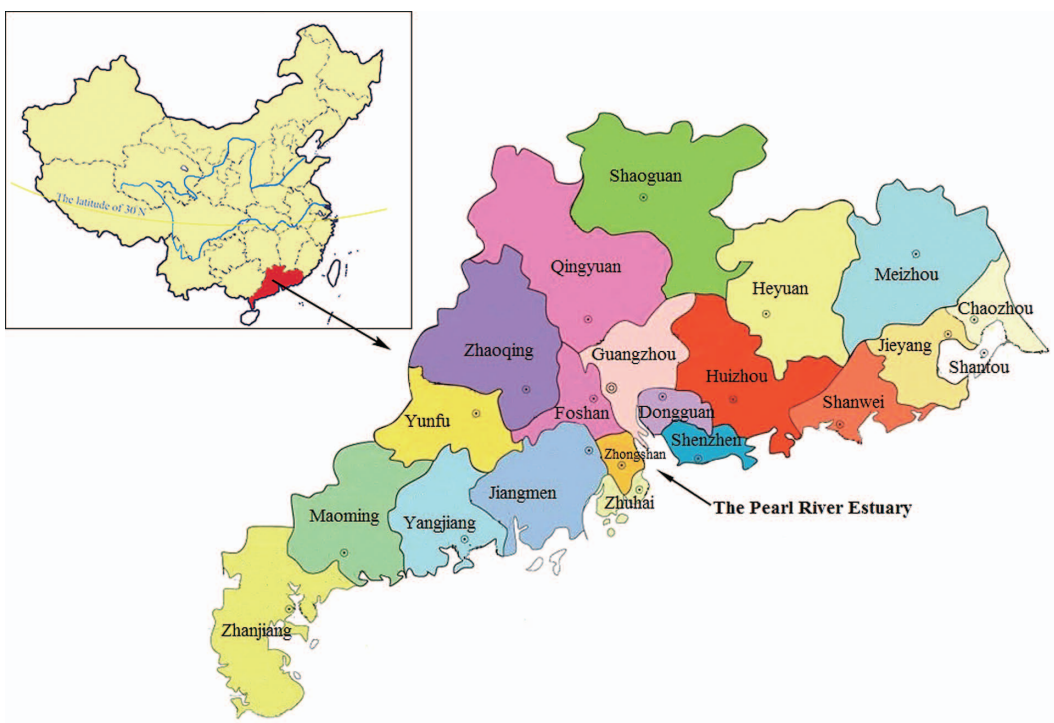

Fig. 1. The administrative cities in Guangdong Province, China.
In this figure, mainland China and the administrattive cities in Guangdong province were illustrated in the upper left and the lower right, respectively. The Pearl River Delta area comprised the cities of Guangzhou, Dongguan, Shenzhen, Foshan, Zhongshan and Zhuhai.

and every sample number in corresponding city was greater than the calculated product of the target sample size multiplied by the population proportion of the corresponding city in Guangdong Province. This research was approved by the Committee for Medical Ethics, the First Affiliated Hospital, Jinan University, adhering to the World Medical Association Declaration of Helsinki (WMADH 2008), which was adopted by the 59th WMA General Assembly, Seoul, in October 2008.

Screening of the mutations c.851_854del, c.1638_1660dup and IVS16ins3kb by HybProbe assay

Three mutations c.851_854del, c.1638_1660dup and IVS16ins3kb were screened by HybProbe assay, as in the previous publication by some co-authors (Kikuchi et al. 2012). Three pairs of HybProbe probes were designed to hybridize adjacently to their target sites in the amplified DNA fragments corresponding to different mutations. The donor and acceptor probes were labeled with the dyes fluorescein isothiocyanate (FITC) and LC Red640, respectively. When a probe pair hybridizes to the amplicon, the dyes produce fluorescence resonance energy transfer (FRET) which emits light that can be quantified by real-time PCR, and the mutant amplicons could be reflected by signal peaks distinct to the wild-type ones. In this study, PCR and the following melting curve analyses were performed in a closed tube on a LightCycler 1.5 (Roche Diagnostics, Germany). All detected mutations were further confirmed by direct DNA sequencing.

HRMA screening of the mutations c.615+5G>A and c.1399C>T

Mutations c.615+5G>A and c.1399C>T were screened by PCR amplification followed by HRMA in closed tubes on Real-Time PCR Detection System (CFX96, Bio-Rad). The primer sequences in PCR reaction for c.615+5G>A analysis are 5′-CTTGACTCCTTTTGT AGAAGAATG-3′ (forward) and 5′-CCACTTCATTAGGGC AAGTTAG-3′ (reverse), and for c.1399C>T, 5′-TCCGTTTGCAAG TGGCAGGAGAA-3′ (forward) and 5′-GATCCCAAAAAACCC CAGGTCCC-3′ (reverse), respectively. The PCR mixtures consisted of 2 μl of genomic DNA (10-30 ng), 4 μl of forward primer (15 μM), 4 μl of reverse primer (15 μM), and 10 μl of Eva Green reagent (Bio-Rad, The USA) in a total volume of 20 μl. PCR thermal profile included an initial denaturation 95°C for 2 min followed by 40 amplification cycles of 95°C for 5 s and 60°C for 60 s. After the amplification, the samples were held at 95°C for 1 min, and then cooled to 37°C for 1 min, followed by the HRMA at a ramp rate of 0.2°C/s extending to 95°C with continuous fluorescence acquisition. Mutants were identified through the melting curve differences, and were further confirmed by direct sequencing.

Calculation of the carrier rate and CD theoretical morbidity

The carrier rate of *SLC25A13* mutations was represented as the proportion of the number of confirmed carriers in all the research subjects, and then the theoretical CD morbidity was calculated according to Hardy-Weinberg principle, i.e. for a single gene locus with two alleles A (wild type) and a (Mutant), the expected genotype frequencies are $f(AA) = p^2$ for AA homozygotes (Healthy individuals), $f(aa) = q^2$ for aa homozygotes (Patients), and $f(Aa) = 2pq$ for heterozygotes (Carrier), and the sum of these three frequencies equals to 100%. Moreover, the carrier rates of *SLC25A13* mutations in different cities were compared using Chi square tests, with $P < 0.05$ as the significant criterion.

Functional analysis

The pathogenicity of a novel missense mutation identified during screening analysis was explored with the *agc1*Δ yeast (*Saccharomyces cerevisiae* BY4741) model system, as in previous publications (Wongkittichote et al. 2013b; Zhang et al. 2014). Shortly, the *SLC25A13* cDNA harboring the mutation was constructed by overlap-extension PCR and recombined with the constitutive expression plasmid pYX212, and then the recombinant plasmid was transformed into *agc1*Δ yeast strain, constituting the group named as pYX212-mutant. And in this study, the two control groups refer to BY*agc1*Δ strains transformed with empty vector pYX212 (Vector) and citrin-encoding recombinant plasmid (Citrin), respectively. To evaluate the functional effect of the novel mutation, the growth abilities of the three groups were tested by measuring OD_{600nm} after 96 hours of culture in SA medium with acetate as the unique carbon source. The results were means ± S.D. of six repeated experiments, and the data were analyzed by means of one-way ANOVA followed by the Bonferroni method to compare the differences in the mean values among the different groups above, with $P < 0.05$ as the significance criterion.

Results

Mutation screening by HybProbe assay and HRMA

The detection of mutations c.851_854del, IVS16ins3kb and c.1638_1660dup by HybProbe assay was shown in Fig. 2A-C, respectively, and all mutations led to generating of signal peaks distinct to wild-type alleles. HRMA analysis for mutations c.1399C>T and c.615+5G>A was shown in Fig. 2D and E. The heterozygous WT/c.615+5G>A and WT/c.1399C>T mutants had distinct melting curves, when compared with their corresponding wild types. On the screening for the mutation c.1399C>T, a blue curve distinct to heterozygous c.1399C>T was revealed (Fig. 2E), and further direct DNA sequencing proved it due to a novel variation c.1420G>A (Fig. 2F).

Mutation carrier rate and CD morbidity

A total of 52 mutation carriers were detected in this study, including 2 carriers of a novel c.1420G>A (p. V474M) mutation with a frequency less than 1% (2/2,428), as shown in Table 1. The mutation carrier rate was around 1/47 (52/2,428) with the theoretical CD morbidity about 1/8,800 in Guangdong population. The carrier rate and theoretical morbidity were about 1/60 (26/1,558) and 1/14,400 in Pearl River Delta area, whilst about 1/34 (26/870) and 1/4,600 in the peripheral area, respectively. Based on the screening results in Table 1, the theoretical CD morbidities in Guangzhou, Shenzhen, Foshan, Huizhou, Zhongshan, Meizhou, Heyuan Zhanjiang and Qingyuan were calculated with Hardy-Weinberg principle to be about 1/12,100, 1/15,400, 1/12,500, 1/36,100, 1/14,400, 1/45,800, 1/1,600, 1/4,400, and 1/4,600, respectively.

The carrier rate difference between Pearl River Delta area and the peripheral area was significant statistically ($\chi^2 = 4.639$, $P < 0.05$). In particular, the carrier rate in Heyuan was much higher than that in most of the cities in Pearl River Delta area (when compared with Guangzhou, $\chi^2 =$

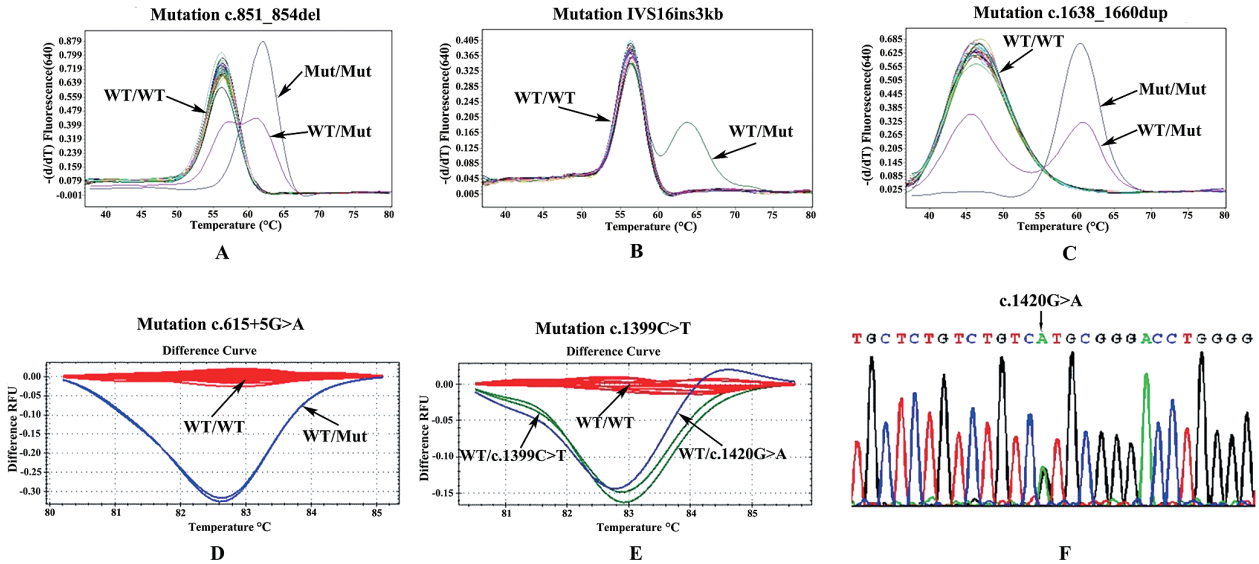

Fig. 2. Screening for the prevalent mutations by melting curve analysis.
The mutations c.851_854del, IVS16ins3kb and c.1638_1660dup were detected by HybProbe assay (A, B and C) and c.615+5G>A and c.1399C>T by HRMA (D and E), respectively. The curves with different shapes in A to E refer to distinct *SLC25A13* genotypes. In 2E, the red curves indicate wild-type (WT/WT) while the green ones represent heterozygote of the c.1399C>T mutation (WT/c.1399C>T). Note the blue curve suggestive of the heterozygous status of a novel c.1420G>A variation, which is further confirmed by direct DNA sequencing as in 2F. WT, wild type allele; Mut, mutant allele.

Table 1. *SLC25A13* mutations detected in Guangdong population.

Area	City	Prevalent Mutations of *SLC25A13* gene					Carrier rate (mutations/ samples)
		c.851_854del	c.1638_1660dup	c.615+5G>A	IVS16ins3kb	c.1420G>T	
Pearl River Delta	Guangzhou	10		1			11/599
	Shenzhen	3			1	1	5/306
	Foshan	3				1	4/223
	Huizhou	2					2/190
	Zhongshan	3	1				4/240
Peripheral cities	Meizhou	1		1			2/213
	Heyuan	9			1		10/197
	Zhanjiang	4	1	3			8/260
	Qingyuan	6					6/200
In total		41	2	5	2	2	52/2,428

The samples were collected from different cities in the two areas within Guangdong Province, i.e. Pearl River Delta areas within Guangdong Province, i.e. Pearl River Delta area and Peripheral cities outside of Pearl River Delta area. There was a significant difference between the carrier rates of the two areas (26/1,558 vs. 26/870, $\chi^2 = 4.639$, $P < 0.05$).

6.058, $P < 0.05$; Shenzhen, $\chi^2 = 4.908$, $P < 0.05$; Huizhou, $\chi^2 = 5.211$, $P < 0.05$; Zhongshan, $\chi^2 = 4.056$, $P < 0.05$). In the peripheral cities, the carrier rate in Heyuan was higher than in Meizhou ($\chi^2 = 6.166$, $P < 0.05$), but no significant difference was observed when compared with that in Zhanjiang or Qingyuan (with Zhanjiang, $\chi^2 = 1.184$, $P > 0.05$, and Qingyuan: $\chi^2 = 1.106$, $P > 0.05$). There were no significant differences among the carrier rates in different cities within Pearl River Delta area.

Functional analysis of the novel g.1420G>A (p.V474M) mutation

The yeast strain BY*agc1*Δ transformed with empty vector pYX212 (Vector), normal control pYX212-CITRIN (Citrin), and the mutant variant pYX212-V474M (p.V474M) were tested, respectively, for their growth abilities with acetate as the unique carbon source, as illustrated in Fig. 3. After growth for 96 hours, the cell densities of the mutant strain (p.V474M) was significantly higher than that of the empty control (Vector), but significantly lower than that of the citrin control. The mutation p.V474M resulted

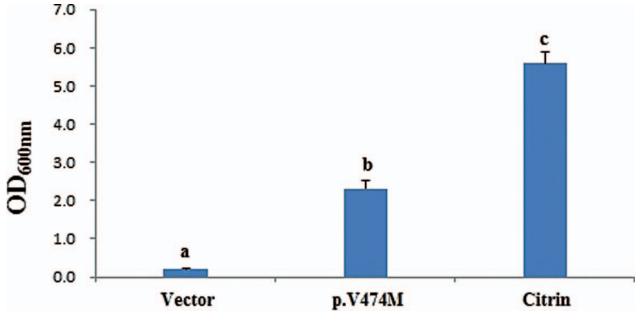

Fig. 3. Functional analysis of the novel c.1420G>T (p.V474M) mutation.
The BY*agc1*Δ strains transformed with empty plasmid pYX212 (Vector), wild type pYX212-CITRIN (Citrin) and mutant pYX212-V474M (p.V474M), respectively, were cultured in SA medium and the cell growth for 96 hours was monitored by measuring OD_{600nm}. Different letters above the bars indicated statistically significant difference from each other, and the p.V474M mutation led to a 58.5% loss of the AGC2 function of citrin protein.

in a considerable reduction of AGC2 function of citrin protein, and therefore could be considered as a deleterious mutation of *SLC25A13* gene.

Discussion

In this paper, 5 prevalent *SLC25A13* mutations were screened in 2,428 used blood samples collected from different cities in Guangdong province, China. The discovery of the 52 carriers indicated a carrier rate of around 1/47, and suggested a theoretical CD morbidity of 1/8,800 in this specific population. According to this finding and the latest population data in Guangdong, it was speculated that there are at least 11,800 CD patients in this province currently. In addition, the mutation carried rate in the peripheral cities (1/34) was significantly higher than that in Pearl River Delta area (1/60), which might be partially attributed to the population migration from north to south China in the past decades, which has changed the local population composition in Pearl River Delta, an area with more developed economy in south China (You et al. 2013). As a relatively-detailed molecular survey on CD epidemiology in Guangdong, our findings in this study provided primary but important laboratory evidences for CD effect evaluation in this specific Chinese population.

HRMA and HybProbe Assay have been reported to be suitable for the screening of *SLC25A13* mutations (Lin et al. 2011; Kikuchi et al. 2012; Wongkittichote et al. 2013a). These 2 closed-tube methods did not need post-PCR handling of the samples, both demonstrating convenient, accurate and rapid features which were suitable for mutation screening in common population. The HybProbe assay was based on FRET phenomenon (Clegg 1995; Dietrich et al. 2002; Breunig et al. 2006), and genotyping is easily done according to the differences of the melting curves in the denaturation process. However, due to the high cost of probe synthesis, the HybProbe assay is more expensive than HRMA, and consequently HRMA was more widely applied in recent years (Vossen et al. 2009; Hill et al. 2010; Whittall et al. 2010; Yan et al. 2010). In this paper, the c.851_854del, c.1638_1660dup and IVS16ins3kb mutations were screened with the HybProbe Assay, while c.615+5G>A and c.1399C>T, with HRMA. Any base changes in the amplicon, reported or novel, could affect the melting curve and thus be reflected on HRMA. As shown in Fig. 2, a novel variant c.1420G>A (p.V474M) was identified by HRMA along with further direct sequencing of the PCR products.

To the best of our knowledge, the c.1420G>A (p.V474M) mutation has never been reported in any other references. However, since this missense mutation was identified in healthy individuals but not CD patients, bioinformatic analysis such as co-segregation and phenotypic association could not be performed for the evaluation of its pathogenicity. The *agc1*Δ yeast function analysis system used in this paper was really of importance for the functional analysis of missense mutations in human *SLC25A13* gene (Cavero et al. 2003; Wongkittichote et al. 2013b; Zhang et al. 2014). The findings in Fig. 3 clearly indicated that this mutation impaired the AGC2 function of citrin protein, constituting direct eukaryotic evidence lending support to its pathogenicity. Although the two individuals harboring this variation did not demonstrate any CD phenotypic feature, this missense mutation might be deleterious when it occurs in specific cases along with another pathogenic mutation in another *SLC25A13* allele. The identification of this novel mutation, which caused partial loss of AGC2 function, enriched the mutation spectrum of human *SLC25A13* gene.

In conclusion, by using HRMA and HybProbe assay, 5 prevalent *SLC25A13* mutations were screened among 2,428 blood samples collected from different medical centers in Guangdong province, China. The carrier rate was 1/47 (52/2,428) and the theoretical CD morbidity was calculated to be 1/8,800 in this population. The carrier rate in the peripheral area proved significantly higher than that in Pearl River Delta area. This study has provided primary epidemiologic data for CD effect evaluation in Guangdong population, and the novel c.1420G>A mutation, which impairs AGC2 function, has expanded the variation spectrum of *SLC25A13* gene.

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Conflict of Interest

The authors declare no conflict of interest.

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