Original Article

Molecular epidemiologic study of citrin deficiency by screening for four reported pathogenic SLC25A13 variants in the Shaanxi and Guangdong provinces, China

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Background: Citrin deficiency (CD) is an autosomal recessive disease resulting from biallelic mutations of the *SLC25A13* gene. This study aimed to investigate the molecular epidemiological features of CD in the Guangdong and Shaanxi provinces of China.

Methods: A total of 3,409 peripheral blood samples from Guangdong and 2,746 such samples from Shaanxi province were collected. Four prevalent *SLC25A13* mutations NG_012247.2 (NM_014251.3): c.852_855del, c.1638_1660dup, c.615+5G>A, and c.1751-5_1751-4ins(2684) were screened by using the conventional polymerase chain reaction (PCR)/PCR-restriction fragment length polymorphism and newly-developed multiplex PCR methods, respectively. The mutated *SLC25A13* allele frequencies, carrier frequencies, and CD morbidity rates were calculated and then compared with the Chi-square and Fisher's exact tests.

Results: The mutations were detected in 68 out of 6,818 *SLC25A13* alleles in Guangdong and 29 out of 5,492 alleles in the Shaanxi population. The carrier frequencies were subsequently calculated to be 1/51 and 1/95, while the CD morbidity rates were 1/10,053 and 1/35,865, in the 2 populations, respectively. When compared with the Shaanxi population, Guangdong exhibited a higher frequency of mutated *SLC25A13* allele (68/6,818 *vs.* 29/5,492, χ^2=8.570, P=0.003) in general, with higher c.852_855del (54/6,818 *vs.* 13/5,492,

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χ^2=17.328, P=0.000) but lower c.1751-5_1751 -4ins(2684) (2/6,818 *vs.* 9/5,492, P=0.015) allele frequencies. The distribution of c.615+5G>A and c.1638_1660dup between the 2 provinces, as well as all 4 prevalent mutations among different geographic regions within the 2 provinces, did not differed significantly.

Conclusions: Our findings depicted the CD molecular epidemiological features in Guangdong and Shaanxi populations, providing preliminary but significant laboratory evidences for the subsequent CD diagnosis and management in the 2 provinces of mainland China.

Keywords: Citrin deficiency; epidemiology; Shaanxi; Guangdong

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Introduction

Citrin deficiency (CD) is an autosomal recessive genetic metabolic disease arising from a functional defect of citrin, the aspartate/glutamate carrier isoform 2 in the mitochondrial inner membrane of the hepatocyte in humans (1,2). The causative gene *SLC25A13* is localized at chromosome 7q21.3 and spans about 200 kb in length with 18 exons and 17 introns (3). Citrin functions to exchange aspartate synthesized by the mitochondrial matrix with glutamate and a proton in the cytoplasm of the hepatocyte, playing distinct roles in the urea cycle, malate-aspartate shuttle and gluconeogenesis (4-7).

From July 2005 to August 2020, our team diagnosed 458 CD patients by *SLC25A13* gene analysis from 29 different provinces, municipalities, and autonomous regions of China, and the 4 mutations of NG_012247.2 (NM_014251.3): c.852_855del, c.1638_1660dup, c.615+5G>A, and c.1751-5_1751-4ins(2684) were at the top of the list, accounting for 82.9% of all mutated alleles [(8) partial data unpublished]. In 2014, we screened the above 4 prevalent mutations using HybProbe assay and High Resolution Melting Assay in 2,428 healthy subjects from 9 cities of Guangdong, a province in the southeast of mainland China (9); this revealed a carrier frequency of 1/47 and a theoretical morbidity of 1/8,800. However, 64.2% (1,558/2,428) of the research participants were from 5 cities of the Pearl River Delta area, which only accounted for about 36.5% (38/104 million) of permanent resident populations in Guangdong, while the imbalance of the sample sizes might have led to a bias in the results.

According to the findings of human genetic diversity, the Chinese population can be divided into 2 large groups from the north to the south along the boundary of the Yangtze River (10,11). The carrier frequency of *SLC25A13* mutations in the north (1/940) was found to be significantly lower than that in the south (1/48) (12); however, the prevalent mutation c.1751-5_1751-4ins(2684) was uncovered in 2008 (13), and thus was not included in the previous study. Notably, most research participants in previous studies were from the middle and coastal areas of China, and an epidemiologic study of CD in the northwest China has not yet been conducted.

Shaanxi Province is located in northwest China and adjoins the other 8 provinces. In this study, the 4 prevalent *SCL25A13* mutations were screened using 981 additional peripheral blood samples collected from 4 peripheral cities in Guangdong and 2,746 of these samples from different cities in Shaanxi. The carrier frequencies of the prevalent mutations and the morbidity rates of CD were then calculated and their distribution was compared.

We presented the following article in accordance with the STROBE reporting checklist (available at http://dx.doi.org/10.21037/tp-21-58).

Methods

Participants

According to the latest official data by the National Bureau of Statistics, China, the permanent resident populations of the provinces of Guangdong and Shaanxi at the time of this study were 104 and 37 million, respectively (http://www.stats.gov.cn/tjsj/tjgb/rkpcgb/). In order to achieve statistical significance, the sample sizes were calculated to be at least 1,953 and 695 on the basis of the estimated carrier rates of 1/48 in Guangdong and 1/940 in Shaanxi by using the online sample size calculator EPITOOLS (https://epitools.ausvet.com.au/twoproportions), with a confidence level of 95% and the power of 0.99. In the case of sample loss and

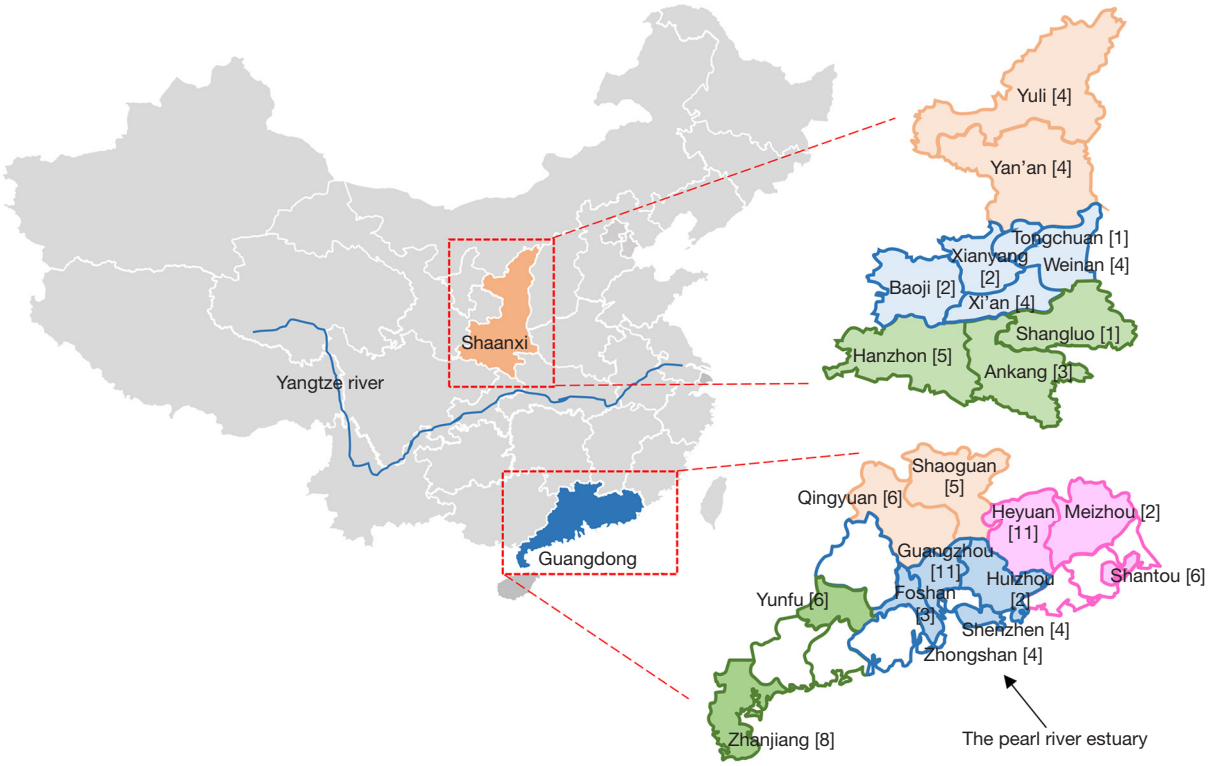

Figure 1 Geographic distribution of the mutated *SLC25A13* alleles in different cities of the Guangdong and Shaanxi provinces of China. Guangdong Province comprises of 4 regions: north (orange), west (green), east (pink), and the Pearl River Delta area (blue). Shaanxi Province comprises of 3 areas: north (light orange), center (light blue) and south (light green). The mutated *SLC25A13* alleles from different cities are marked in parentheses. This figure was generated by means of the software WPS Office PowerPoint 2019. The base map was created by incrementally assembling the outlines of the Chinese administrative regions, which could be downloaded via the URL link http://www.900ppt.com/.

a higher carrier rates of CD in Shaanxi, more participants were added to enlarge the sample size. A computer-based randomization was used to select samples from each of the regions. The number of samples from each region was in accordance with the population distribution.

Besides the 2,428 samples in our previous study (9), we collected an additional 981 blood samples for health examination from 4 peripheral cities (Heyuan, Shaoguan, Shantou, and Yunfu) in Guangdong and 2,746 of these samples from 10 cities (Yan'an, Yulin, Weinan, Xianyang, Baoji, Xi'an, Tongchuan, Hanzhong, Shangluo, and Ankang) in Shaanxi between June 2016 and December 2018 (*Figure 1*).

This study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and was approved by the Medical Ethics Committee of the First Affiliated Hospital, Jinan University, Guangdong, China (NO.: KY-2019-052, 2015-038, 2014-004). Participants were genotyped retrospectively using blood samples previously collected for the purpose of health examination. The data related to individual identification were anonymized during the entire study process. Therefore, informed consent was waived.

Genetic analysis

Genomic DNA was purified from the peripheral blood samples. The mutations c.1638_1660dup and c.615+5G>A were detected using established polymerase chain reaction (PCR) and PCR-restriction fragment length polymorphism methods, as in previous publications (12,14). The mutations c.852_855del and c.1751-5_1751-4ins(2684) were screened using newly-developed multiplex PCR approaches as described below.

Four primers, MuI-3NF (5'-TGGCAATTTGAATA ACATCAGATGAC-3', forward), MuI-NR1-AP2 (5'-AC TATGGGGCCGTTCAATGTCTGCTAAGGTCA TA-3', reverse), MuI-AF4-AP2 (5'-ACGCGTGTGTTG TTTTTTCCCCTACAGACGAC-3', forward) and MuI-9AR (5'-CAGTGACACCAACACGGGATTCT-3', reverse), were designed for the multiplex PCR amplification of mutation c.852_855del (*Figure 2A*); the optimal concentrations of the 4 primers were set at a ratio of 1:1:0.5:0.5, respectively. The PCR cycling condition included an initial denaturation step of 94 ℃ for 5 min, 35 cycles of denaturation at 94 ℃ for 30 sec, annealing at 57 ℃ for 30 sec and extension at 72 ℃ for 1 min, as well as a final extension at 72 ℃ for 7 min.

For the multiplex PCR amplification of mutation c.1751-5_1751-4ins(2684), 1 forward primer MuXIX-2UF (5'-GCCAAACCACTTACAGCGGAGT-3') along with 2 reverse primers MuXIX-2UR (5'-TTATGACAGAGAGCA GCACTGGTTC-3') and MuXIX-1R (5'-TCCCTACGAC AACAGAGCATTAGC-3') were designed (*Figure 2B*), and their optimal concentrations were set at a ratio of 5–6:4–5:1, respectively. The temperature condition was as follows: 94 ℃ for 5 min, followed by 35 cycles of 94 ℃ for 30 sec, 60 ℃ for 30 sec and 72 ℃ for 40 sec, as well as a final extension step of 72 ℃ for 7 min.

Calculation of the allele frequencies, carrier frequencies and CD morbidity rates

Geographically, Guangdong Province includes the Pearl River Delta (Guangzhou, Shenzhen, Foshan, Huizhou, and Zhongshan), along with northern (Qingyuan and Shaoguan), eastern (Meizhou, Heyuan, and Shantou), and western (Zhanjiang and Yunfu) regions (*Figure 1*); meanwhile, Shaanxi consists of northern (Yulin and Yan'an), central (Weinan, Xi'an, Xianyang, Baoji, and Tongchuan), and southern (Hanzhong, Ankang, and Shangluo) areas (*Figure 1*).

The mutated *SLC25A13* allele frequencies, carrier frequencies and CD morbidity rates in different areas were calculated based on the Hardy-Weinberg equilibrium. The genotypes *AA* (healthy individual), *AB* (carrier) and *BB* (patient) of a biallelic genetic marker were expected to have the relative frequencies of p^2, $2pq$ and q^2, with p and q being the *A* (wild type) and *B* (mutant) allele frequency, respectively; thus, $p+q=1$. The values of $2pq$ and q^2 represented as the carrier frequency of SLC25A13 variants and CD morbidity rate, respectively (15,16).

Statistical analysis

By using the statistical software SPSS version 23.0 (IBM Corp., Chicago, IL, USA), the distributions of the 4 *SLC25A13* variants were compared via Chi-square and Fisher's exact tests among different geographic regions, with a P value <0.05 indicating statistical significance.

Results

Mutation screening

To evaluate the reliability of the new PCR methods in this study, the 4 prevalent *SLC25A13* mutations were detected in the 200 samples of Qingyuan city, and the results were completely consistent with those in the previous study by using HybProbe assay and HRMA approaches (9).

This study detected 13 individuals with the c.852_855del, 1 with the c.1638_1660dup, and 4 with the c.615+5G>A mutation in the 981 additional samples from Guangdong (*Table 1*). Therefore, together with the screening findings in our previous study of 2,428 samples (9), a total of 68 mutant *SLC25A13* alleles were detected in 6,818 independent alleles (3,409 samples). Additionally, a total of 29 mutated *SLC25A13* alleles in 28 individuals were detected among the 5,492 alleles from Shaanxi. Among them, there were 13, 9, 5, and 2 alleles bearing c.852_855del, c.1751-5_1751-4ins(2684), c.1638_1660dup, and c.615+5G>A, respectively (*Table 2*). Of note, 1 individual from Xi'an was identified as a homozygote of the c.852_855del mutation, who was apparently healthy but with a specific fondness for high-protein food.

Carrier frequencies and theoretical morbidity rates

The mutated *SLC25A13* allele frequencies in the Guangdong and Shaanxi were 0.997% (68/6,818) and 0.528% (29/5,492), the carrier frequencies were 1/51 and 1/95, and the theoretical morbidity rates were 1/10,053 and 1/35,865, respectively. According to the latest population data and the above theoretical morbidity rates in the Guangdong and Shaanxi provinces, it was estimated that at least 10,345 and 1,032 CD patients were distributed in the different cities of these 2 provinces, respectively (*Table 2*).

The mutated alleles, carrier frequencies, and morbidity

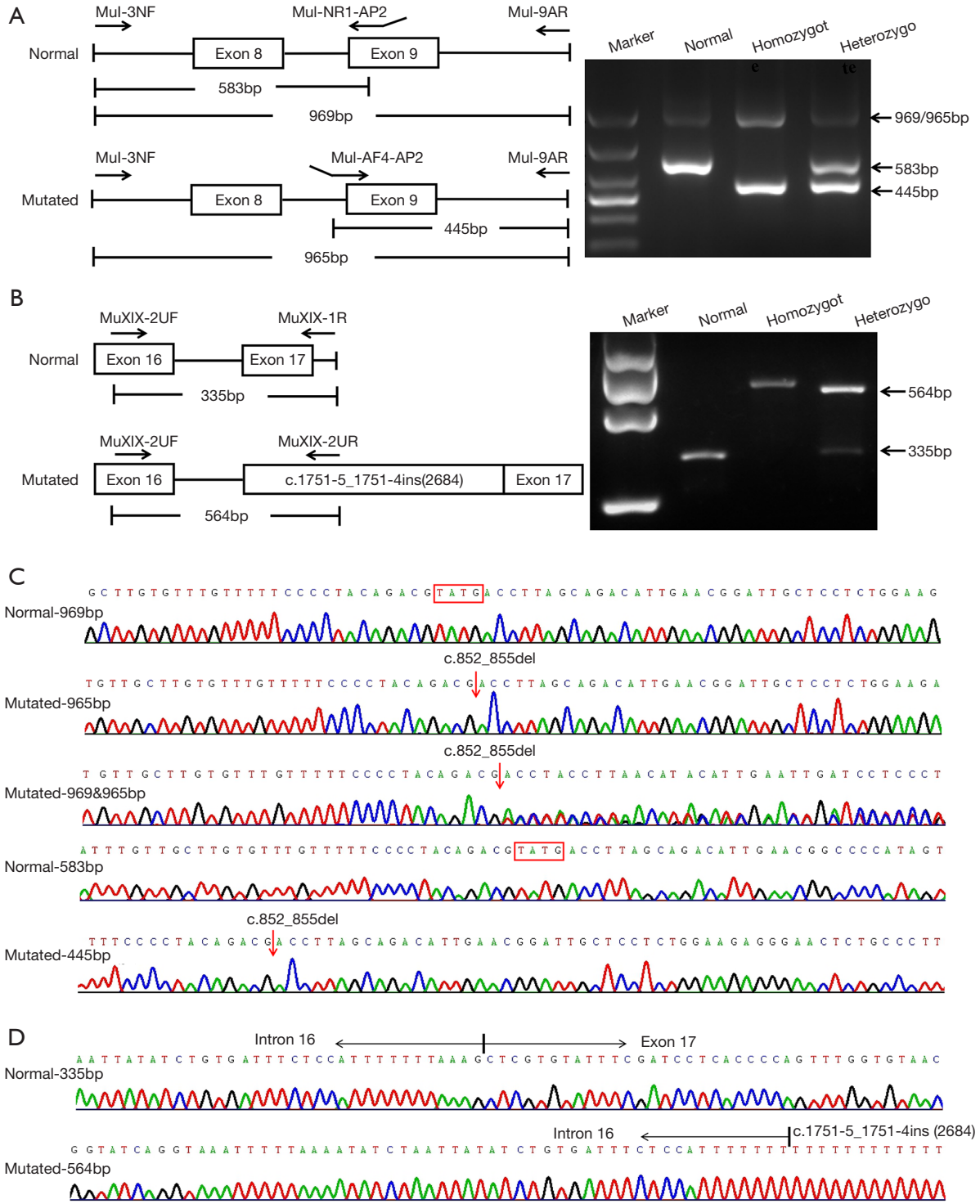

Figure 2 Novel approaches developed for the screening of the mutations c.852_855del and c.1751-5_1751-4ins(2684). (A) On c.852_855del screening, the wild-type *SLC25A13* genotype exhibited 2 bands of 583 bp and 969 bp in size, the homozygote showed 2 bands of 445 bp and 965 bp, while the heterozygote showed all 4 bands. In this figure, the 2 bands of 965 bp and 969 bp in sizes represent the PCR products with and without the mutation c.852_855del, respectively. (B) PCR products of the wild-type and homozygote of c.1751-5_1751-4ins(2684) only exhibited a band of 335 bp and 564 bp, respectively, while that of the heterozygote presented with both bands. (C) Sanger sequencing of PCR products of c.852_855del. (D) Sanger sequencing of PCR products of c.1751-5_1751-4ins(2684).

Table 1 Distribution of the 4 prevalent *SLC25A13* mutations in different areas of Guangdong Province

Regions	Cities	New report					Published report (9)					Total					Mutated alleles	Carrier frequencies	Morbidity rates
		I	III	X	XIX	Sample sizes	I	III	X	XIX	Sample sizes	I	III	X	XIX	Sample sizes			
Pearl River Delta	Guangzhou						10		1		599	10		1		599	11	1/55	1/11,861
	Shenzhen						3			1	306	3			1	306	4	1/77	1/23,409
	Foshan						3				223	3				223	3	1/75	1/22,102
	Huizhou						2				190	2				190	2	1/96	1/36,100
	Zhongshan						3	1			240	3	1			240	4	1/61	1/14,400
	Total						21	1	1	1	1,558	21	1	1	1	1,558	24	1/65	1/16,857
North	Qingyuan						6				200	6				200	6	1/34	1/4,444
	Shaoguan	5				230						5				230	5	1/47	1/8,464
	Total	5				230	6				200	11				430	11	1/40	1/6,112
East	Meizhou						1		1		213	1		1		213	2	1/107	1/45,369
	Heyuan	1				200	9			1	197	10			1	397	11	1/37	1/5,210
	Shantou	2	1	3		200						2	1	3		200	6	1/34	1/4,444
	Total	3	1	3		400	10		1	1	410	13	1	4	1	810	19	1/43	1/7,270
West	Zhanjiang						4	1	3		260	4	1	3		260	8	1/33	1/4,225
	Yunfu	5		1		351						5		1		351	6	1/59	1/13,689
	Total	5		1		351	4	1	3		260	9	1	4		611	14	1/44	1/7,619
	In total	13	1	4		981	41	2	5	2	2,428	54	3	9	2	3,409	68	1/51	1/10,053

I: c.852_855del; III: c.1638_1660dup; X: c.615+5G>A; XIX: c.1751-5_1751-4ins(2684).

rates among different administrative cities and geographic regions were shown in *Tables 2* and *3*.

Distribution comparisons of the prevalent SLC25A13 mutations among different geographic regions

The distribution comparison of the 4 prevalent mutations revealed that Guangdong had a higher mutated *SLC25A13* allele frequency than did the Shaanxi population (68/6,818 *vs.* 29/5,492, χ^2=8.570, P=0.003). The mutated allele frequency of c.852_855del was higher in the Guangdong population as compared to that of Shaanxi (54/6,818 *vs.* 13/5,492, χ^2=17.328, P=0.000) while that of c.1751-5_1751-4ins(2684) was lower (2/6818 *vs.* 9/5492, P=0.015). The distribution of c.615+5G>A and c.1638_1660dup between the 2 provinces, as well as all 4 prevalent mutations among different geographic regions within the 2 provinces, did not differed significantly (P>0.05).

Discussion

Melting curve analysis, including the combination of HybProbe assay and High Resolution Melting Assay, has proven to be a convenient and rapid method for the screening of the 4 prevalent *SLC25A13* mutations (9,17-19), but it is technically complex and expensive. Multiplex-PCR consists of multiple sets of primers in a single PCR mixture. By simultaneously amplifying more than 1 fragment in the same reaction, 1-step multiplex PCR was more efficient than simplex PCR (20,21). The c.852_855del and c.1751-5_1751-4ins(2684) mutations are a 4-base deletion in exon 9 and an about 3-kb insertion in intron 16 of the *SLC25A13* gene, respectively, and have been screened by the newly developed multiplex PCR methods. As shown in *Figure 2*,

Table 2 Distribution of the 4 prevalent *SLC25A13* mutations in different areas of Shaanxi Province

Regions	Cities	I	III	X	XIX	Sample sizes	Mutated alleles	Carrier frequencies	Morbidity rates
North	Yulin	2	1			293	3	1/98	1/38,155
	Yan'an	1	1		2	300	4	1/76	1/22,500
	Total	3	2		2	593	7	1/85	1/28,706
Center	Weinan	3			1	500	4	1/126	1/62,500
	Xi'an	2[†]	1		1	174	4	1/44	1/7,569
	Xianyang	1		1		300	2	1/151	1/90,000
	Tongchuan		1			100	1	1/101	1/40,000
	Baoji	1			1	300	2	1/151	1/90,000
	Total	7	2	1	3	1,374	13	1/106	1/44,683
South	Shangluo				1	229	1	1/230	1/209,764
	Hanzhong	1	1	1	2	350	5	1/71	1/19,600
	Ankang	2			1	200	3	1/67	1/17,778
	Total	3	1	1	4	779	9	1/87	1/29,967
In total		13	5	2	9	2,746	29	1/95	1/35,865

I: c.852_855del; III: c.1638_1660dup; X: c.615+5G>A; XIX: c.1751-5_1751-4ins(2684). [†]: Homozygous for the c.852_855del mutation.

Table 3 Comparison of the distribution of the 4 prevalent *SLC25A13* mutations in the Guangdong and Shaanxi provinces

Mutations	Guangdong (n=6,818)	Shaanxi (n=5,492)	χ^2	P value
I	54	13	17.328	0.000
III	3	5	–	0.480
X	9	2	–	0.126
XIX	2	9	–	0.015
Total	68	29	8.570	0.003

In this table, I, III, X and XIX represent the mutations c.852_855del, c.1638_1660dup, c.615+5G>A, and c.1751-5_1751-4ins(2684), respectively.

with 2–3 sets of primers in the same PCR reaction, clear and distinguishable amplified DNA fragments covering the wild-type and mutated *SLC25A13* alleles were obtained. The combination of conventional PCR/PCR-restriction fragment length polymorphism and newly developed multiplex PCR methods for the detection of 4 prevalent mutations proved to be feasible in a large-scale population analysis in the present study.

In this study, the 4 prevalent *SLC25A13* mutations were screened in the 981 new samples from 4 peripheral cities in Guangdong provinces. The sample size was expanded from 2,428 to 3,409, and the proportions in the 4 geographic regions of Guangdong were thereby made more representative. The 68 carriers identified thus far demonstrated a carrier rate of 1/51 and a theoretical CD morbidity of 1/10,053 in this population, which decreased slightly but might have been more precise than that found in our previous study (9). Data from our previous study showed the mutation carrier rate in the peripheral cities (1/34) to be significantly higher than that in the Pearl River Delta area (1/60). However, when more peripheral cities were enrolled in this study, no significant difference of the carrier rate was found between the 2 areas. The reasonable enlargement of the study population minimized the bias

of the molecular epidemiology study and increased the screening reliability, providing more accurate laboratory evidences for the evaluation of CD effect on the Guangdong population.

For the first time, this study identified 29 mutated *SLC25A13* alleles in the 2746 healthy residents in Shaanxi, a province in the northwest China, with a carrier frequency 1/95 and a CD theoretical morbidity rate of about 1/35,865. Surprisingly, the mutated *SLC25A13* allele frequency in the Shaanxi population was higher (29/5,492 *vs.* 1/1,880, χ^2=7.792, P=0.005) than that previously found in north China (Liaoning, Beijing, Hebei, Shandong, and Henan) (12), suggesting that the CD prevalence might be underestimated at least in northwest China. Among 4 prevalent mutations, c.852_855del (44.82%) and c.1751-5_1751-4ins(2684) (31%) accounted for almost 75%, suggesting that when performing CD genetic testing, analysis of these 2 mutations should be mandatory in this province. Statistical analysis within the present study discovered homogenous distribution of the prevalent *SLC25A13* mutations among different geographic regions in Shaanxi, and hence different molecular targeting might be unnecessary for CD diagnosis in Shaanxi Province.

The distribution comparison revealed that the Guangdong population had a significantly higher mutated *SLC25A13* allele frequency than that of Shaanxi, supporting the notion that different geographic distribution of *SLC25A13* gene mutations exist in China. It is worth noting that the mutated allele frequency of c.852_855del was significantly higher while that of c.1751-5_1751-4ins(2684) was lower in Guangdong compared to Shaanxi. It has been reported that modern humans colonized East Asia via southern and northern routes on both sides of the Himalayas (22,23). In this study, the 2 prevalent mutations with a different geographic distribution might be attributed to the genetic flow which occurred between distinct founding populations, and their relatively higher frequency could be explained as a result of the founder effect. Actually, the c.852_855del and c.1751-5_1751-4ins(2684) mutations are relatively frequent in East Asia (13,18,19,24-26), with the former being reported as having originated around the Guangxi and Yunnan areas in south China (12) and the latter exhibiting high allele frequency (33–45.5%) in Korean (13,26), which is located in the northeast of the Asian continent.

Some limitations in this study should be addressed. Firstly, only focusing on the 4 prevalent mutations might have overlooked other pathogenic *SLC25A13* variants in general human populations. Secondly, there might be sampling bias in the 2 provinces. The proportions of research participants in different regions were similar but not identical with the population distribution. Therefore, further epidemiology study focusing on more *SLC25A13* variants and with larger sample sizes from the 2 provinces is in need.

Conclusions

This study highlighted the feasibility of the combination of the conventional PCR/PCR-restriction fragment length polymorphism and the newly developed multiplex PCR methods for the detection of 4 prevalent *SLC25A13* mutations in a large-scale population. The findings clarify the molecular epidemiological features of CD in Guangdong and Shaanxi populations, providing preliminary but nonetheless significant laboratory evidence for subsequent CD diagnosis and management in these 2 provinces in mainland China.

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Footnote

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Ethical Statement: The authors are accountable for all

aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. The study was conducted in accordance with the Declaration of Helsinki (as revised in 2013) and was approved by the Medical Ethics Committee of the First Affiliated Hospital, Jinan University, Guangdong, China (NO.:KY-2019-052, 2015-038, 2014-004). Participants were genotyped retrospectively using blood samples previously collected for the purpose of health examination. The data related to individual identification were anonymized during the entire study process. Therefore, informed consent was waived.

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