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Characterization of glucose-6-phosphate dehydrogenase deficiency and identification of a novel haplotype 487G>A/IVS5-612(G>C) in the Achang population of southwestern China

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The prevalence of glucose-6-phosphate dehydrogenase (G6PD) deficiency and its gene mutations were studied in the Achang population from Lianghe County in Southwestern China. We found that 7.31% (19 of 260) males and 4.35% (10 of 230) females had G6PD deficiency. The molecular analysis of *G6PD* gene exons 2—13 was performed by a PCR-DHPLC-Sequencing or PCR-Sequencing. Sixteen independent subjects with *G6PD* Mahidol (487G>A) and the new polymorphism IVS5-612 (G>C), which combined into a novel haplotype, were identified accounting for 84.2% (16/19). And 100% Achang *G6PD* Mahidol were linked to the IVS5-612 C. The percentage of *G6PD* Mahidol in the Achang group is close to that in the Myanmar population (91.3% 73/80), which implies that there are some gene flows between Achang and Myanmar populations. Interestingly, *G6PD* Canton (1376G>T) and *G6PD* Kaiping (1388G>A), which were the most common *G6PD* variants from other ethnic groups in China, were not found in this Achang group, suggesting that there are different *G6PD* mutation profiles in the Achang group and other ethnic groups in China. Our findings appear to be the first documented report on the G6PD genetics of the AChang people, which will provide important clues to the Achang ethnic group origin and will help prevention and treatment of malaria in this area.

glucose-6-phosphate dehydrogenase deficiency, Achang population, *G6PD* Mahidol, gene mutation, Myanmar population

Glucose-6-phosphate dehydrogenase (G6PD, EC 1.1.1.49; MIM# 305900) is a key enzyme in the pentose phosphate pathway. One of the major functions of this pathway is to generate NADPH, an important hydrogen-donor for many kinds of biosynthesis reactions in cells[1]. G6PD deficiency is one of the most common enzymopathies affecting over 400 million people in the world, with a high prevalence in tropical and subtropical regions where malaria is endemic. Today, it is generally accepted that G6PD deficiency is a result of malarial selection in many parts of the world. The core haplotypes carrying the proposed protective mutation stand out and show significant evidence of selection[2]. Malaria caused by Plasmodium infection has threatened humans since the establishment of slash-and-burn agriculture and killed over two million people annually[3]. Over 3 billion people living in more than 100 countries or territories are at risk[4]. In China, malaria remains a serious public health problem. Yunnan Province, located in the southwest of China, is one of the areas where malaria has been endemic with high transmission of *Plasmodium falciparum*, which is the most deadly malaria parasite[5]. For example, Dehong Dai-Jingpo Autonomous Prefecture is a high malaria endemic area with an average prevalence of 113.16/10000 from 1951 to

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2001[6]. The malaria incidence in this area was 37.03/10000 in 2003, which increased 37.72% compared with that of 2002[7].

The clinical presentations of G6PD deficiency are rather diverse including neonatal jaundice and acute hemolytic anemia induced by infection and certain drugs such as asprimaquine and certain foods such as fava beans[8]. In addition, chronic non-spherocytic hemolytic anemia is usually associated with a more severe form of G6PD deficiency[9]. To date, over 150 different variants of *G6PD* have been identified at the DNA level[10], including 21 from Chinese populations of different ethnicities, such as Han, Tai, Bai, etc.[11]. Molecular analysis has revealed that each ethnic Chinese population has a characteristic profile of deficient variants. However, there is still no information about the prevalence and molecular variants of G6PD deficiency in the Achang ethnic Chinese group, located mainly in Dehong Dai-Jingpo Autonomous Prefecture in Yunnan. Genetic characterization of G6PD deficiency in this Achang group will help prevention and treatment of malaria and understanding of the origin of the Achang ethnic group.

In this work, we investigated the prevalence of G6PD deficiency and identified *G6PD* gene mutations and polymorphism in the Achang ethnic group. Genetic analysis was made by PCR combined with denaturing high-performance liquid chromatography and sequencing or direct PCR sequencing. A novel haplotype, *G6PD* Mahidol (487G>A) /IVS5-612(G>C) was identified.

1 Materials and methods

1.1 Sample collection

This study was approved by the Ministry of Health of China and followed the Guidelines for Human Research setup by the university.

A total of 490 ethnic Chinese Achang individuals (260 males and 230 females) from three Achang villages in Lianghe County of Dehong Dai-Jingpo Autonomous Prefecture in Yunnan Province of China (Figure 1) were the subjects of this study. These people in villages might be considered a genetic isolate, because they have their own characteristic physical, cultural and genetic traits and they practice strict endogamy. Local staffs assisted the gathering of residents to perform rapid on-site examinations for G6PD deficiency. Among the examined residents, there were two individuals who had a history of hemolysis needed blood transfusions. Subject 1 was a one and half year old boy who had acute hemolysis at eight-months-old without notable remote causes. Subject 2 was a fourteen-years-old girl who had acute hemolysis after eating fava beans at four-years-old. Both of them had been treated with two or three blood transfusions.

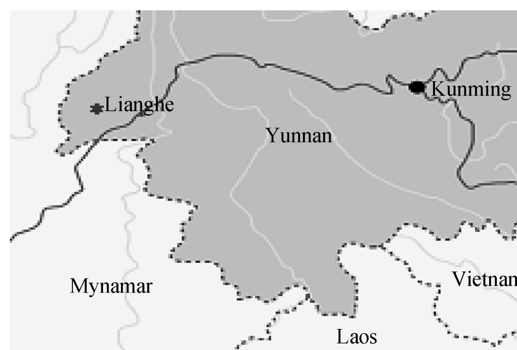

Figure 1 The geographic location of Lianghe County where Achang ethnic group is gathering.

1.2 G6PD activity assay

The Nitroblue tetrazolium paper strip and G6PD/6PGD ratio method, which was an accepted method for the screening of G6PD deficiency in the Chinese population, was used to screen for G6PD deficiency in this study[12]. When a low G6PD activity was identified for an individual or normal individuals used as polymorphism controls, an informed consent was obtained before 2 mL of venous blood was taken to perform *G6PD* genetic analysis. The purpose of the DNA analysis was also explained to each individual before blood collection. The collected blood samples were stored at 4℃ and brought back to the research laboratory in Kunming City by airplane. G6PD activity was confirmed in the laboratory by the WHO recommended standard method[13] within 24 hours of samples collected. In our laboratory, the reference range of G6PD activity in normal males was 6.47 ± 2.24 IU/g Hb (mean ± SD) and in normal females was 5.83 ± 1.94 IU/g Hb. G6PD deficiency was defined as G6PD activity less than 1.5 IU/g Hb[13].

1.3 DNA extraction and PCR amplification

Genomic DNA was isolated from the peripheral blood leucocytes of 19 unrelated G6PD deficient subjects and 10 normal Achang subjects with PureGene DNA Purification Kit (Gentra, USA). *G6PD* gene exons 2－13 were amplified with PCR. Each PCR reaction contained 25 μL of 1×buffer, 100 μmol/L dNTP, 200 nmol/L primers[14－16], 25－50 ng genomic DNA, and 1.5 U Taq

DNA polymerase. The reaction was performed under the following cycle conditions: 95℃ for 4 min followed by 30 cycles of 94℃ for 30 s and 58℃—63℃ for 30 s followed by 72℃ for 1 min. Subsequently, 72℃ for 10 min was used for extension. 5 μL of the PCR product was used to check the product integrity by agarose gel electrophoresis, and the remaining PCR product was subjected to DHPLC analysis.

1.4 Screening of *G6PD* mutations by DHPLC

G6PD variants were detected by denaturing high-performance liquid chromatography (DHPLC) heteroduplex formation analysis on an automated HPLC instrument (Transgenomic 3500 A) equipped with a DNA-Sep Column and the automated WAVE DNA Fragment Analysis System (Transgenomic, Omaha, NE, USA). A mixture of 5 μL amplicon from each G6PD deficient subject and a normal Achang subject control in 1:1 ratio was heated at 95℃ for 5 min followed by a stepwise cooling program (−1℃/1 min) until the temperature reached 24℃. The DNA samples were then injected into the Transgenomic 3500 A DHPLC analytical instrument pre-equilibrated with buffer A (0.1 mol/L TEAA, pH 7.0) in the presence of different percentages (48.8%—56.3%) of buffer B (0.1 mol/L TEAA, 25% acetonitrile, pH 7.0) for different primers. The temperature required for successful resolution of heteroduplex molecules was determined by use of the DHPLC melting algorithm followed by empirical optimization. Samples were eluted isocratically with different percentages (48.8%—56.3%) of buffer B for different primers over a 4—5 min period at a constant flow rate of 0.9 mL/min. Heterozygous profiles were identified by a shorter retention time compared with normal controls. Table 1 shows the appropriate experimental conditions used in PCR-DHPLC analysis.

1.5 DNA sequencing

The DNA sequences of PCR products purified with Agarose Gel DNA Purification Kit (TaKaRa) were read on the ABI PRISM^{TM} 3730XL DNA Sequencer (PE Biosystems, CT, USA). Both strands of each exon were sequenced. The results were compared with human *G6PD* gene DNA sequences (EMBL X55448).

2 Results

2.1 Prevalence of G6PD deficiency

From the 490 Achang subjects, we found that 19 out of 260 males had deficient G6PD with a prevalence of G6PD deficiency of 7.31% (19 of 260) and that 10 out of 230 females had deficient G6PD with a prevalence of 4.35% (10 of 230). G6PD enzymatic activity assay showed that 24.14% in G6PD-deficient individuals had severe enzyme deficiency including subjects 1 and 2 with less than 10% of normal enzymatic activities. Others had moderate enzyme deficiency with 10%—60% of normal enzymatic activity.

2.2 *G6PD* mutations and polymorphism

We found that mutation, *G6PD* Mahidol G487A

Table 1 Primer and DHPLC conditions for screening *G6PD* variants

Exon	Primer sequence	Size (bp)	T_m (℃)	Buffer B (%)	Partially denaturing temperature (℃)
2	5′-TGTCACCCTGGTGTGAGA-3′ 5′-GCCCTGCAACAATTAGTTGGAA-3′	313	60	55.1	61.4
3,4	5′-AGGATGATGTATGTAGGTCG-3′ 5′-CCGAAGCTGGCCATGCTGGG-3′	378	60	52.3	57.4
5	5′-ACACACGGACTCAAAGAGAG-3′ 5′-CCCGGACACGCTCATAGAGT-3′	362	60	56.3	61.9
6	5′-TGGGAGGGCGTCTGAATGAT-3′ 5′-CGGGGAGGCAGTGGGCCAGG-3′	307	60	53.9	63.1
7	5′-GGGTGACCCCTCACATGTGGCCCCT-3′ 5′-GGCTCTGCCACCCTGTGCCAGCCT-3′	249	58	54.1	59.6
8	5′-TTGGGGTCCCCATGCCCTTG -3′ 5′-TGCCTCGTCACAGATGGGCC-3′	231	53	48.8	53.3
9	5′-TCCCTGCACCCCAACTCAAC-3′ 5′-AGTGCGTGAGTGTCTCAGT-3′	333	60	54.6	61.7
10	5′-ACTGAGACACTCACG-3′ 5′-CACCATGTGGAGTCCCCCGG-3′	333	63	49.6	54.6
11,12	5′-TGGCATCAGCAAGACACTCTCTC-3′ 5′-CCCTTTCCTCACCTGCCATAAA-3′	318	62	53.2	63.0
13	5′-CTATATTTATGGCAGGTGAGG -3′ 5′-GCAGCTGAGGTCAATGGTC-3′	299	58	55.3	61.8

(163Gly > Ser) in exon 6 of the *G6PD* gene, was the most common variant in the Achang ethnic group accounting for 84.2% (16/19) of the mutations. The amplification fragment of exon 6 of *G6PD* gene was 307 bp (Figure 2(a)). Three different types of DHPLC chromatograms of PCR products of exon 6 were shown in Figure 2(b). DNA direct sequencing revealed that each chromatogram represented WT, G487A homozygote, and G487A heterozygote in exon 6, respectively (Figure 2(c)). 100% *G6PD* Mahidol was linked to the polymorphism IVS5-612 C in these subjects, but the normal Achang people were always linked to IVS5-612 G (Figure 2(d)). The specific polymorphism in intron 5 of *G6PD* gene, IVS5-612 G>C, was found here firstly in China. So, G487A and IVS5-612 (G>C) combined into a novel haplotype of G6PD deficiency in the Achang population, which was submitted to GenBank successfully (Accession number: EF190463).

We did not find *G6PD* Canton G1376T (459 Arg > Leu) in exon 11 nor the *G6PD* Kaiping G1388A (463 Arg>His) in exon 12, which account for over 50%—60% *G6PD* deficient variants in the other Chinese population. Moreover, no mutations were found in exons 2—13 of the *G6PD* gene of 3 (15.8%) Achang individuals who showed deficient G6PD enzymatic activities.

3 Discussion

We found an overall prevalence of G6PD deficiency of 7.31% in males and 4.35% in females from screening 490 Achang individuals from villages in Lianghe County of Dehong Dai-Jingpo Autonomous Prefecture of Yunnan Province. The prevalences of G6PD deficiency among the 5 ethnic groups in Yunnan of China, Myanmar and Thailand are listed in Table 2. The prevalences of G6PD deficiency in Achang male and female were lower than that found in Dai (male: 17.4%; female: 16.9%) and Jinuo (male: 14.3%) ethnic groups, but higher than that in Bai group (male: 1.13%; female:

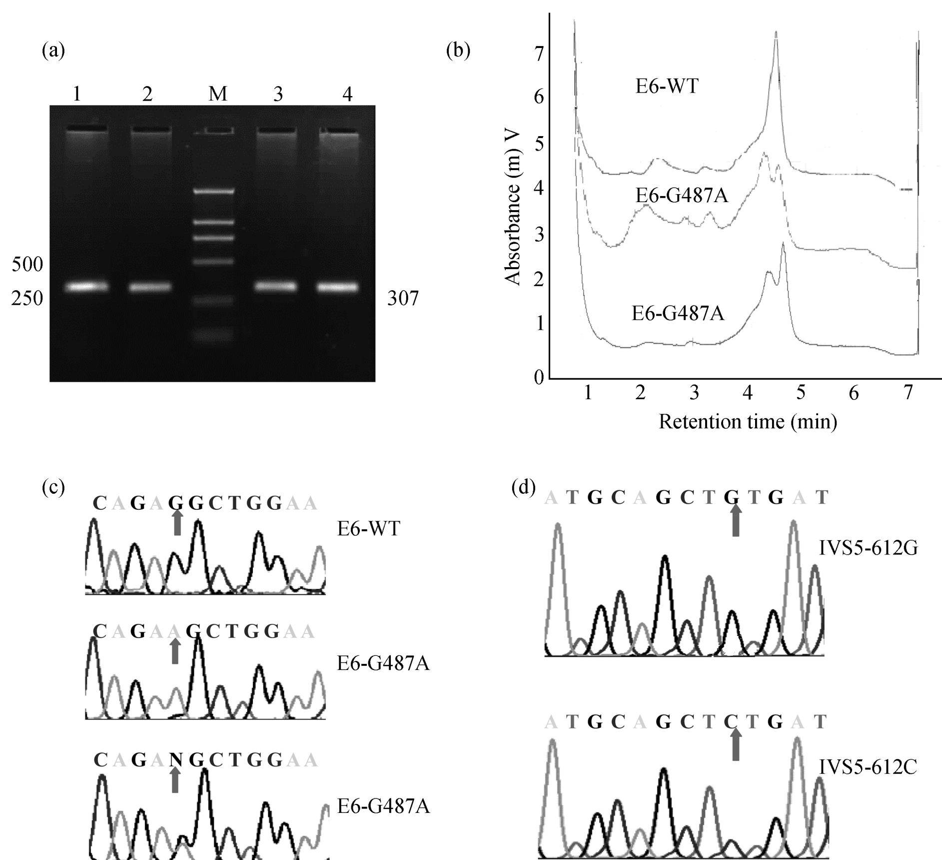

Figure 2 Identification of the *G6PD* haplotype 487 G>A/IVS5-612(G>C) in Achang ethnic group. (a) PCR products from exon 6 of the *G6PD* gene from Achang people; (b) elution peak patterns for screening mutation exon 6 of the *G6PD* gene; (c) part DNA sequences for the *G6PD* mutation G487A for confirming the distinct DNA elution peak patterns; (d) part DNA sequences in intron 5.

Table 2 Prevalence of G6PD deficiency among the different ethnic groups

Different groups	Screening		G6PD deficiency		Total	% of G6PD deficiency		Reference
	male	female	male	female		male	female	
Dai	362	142	63	24	504	17.4	16.9	11
Bai	3792	1500	43	23	5292	1.13	1.53	11
Yi	293	149	18	5	442	6.14	3.36	11
Jinuo	322	UD	46	UD[a)]	322	14.3	UD[a)]	11
Achang	260	230	19	10	490	7.31	4.35	present study
Thailand	350	172	39	10	522	11.1	5.80	17
Myanmar	1000		121		1000	11.00		18
Total	8572		421		8572	4.8		

a) Undetection.

1.53%). The prevalences in Achang G6PD deficiency were similar to that found in Yi group (male: 6.14%; female: 3.36%)[11]. Furthermore, the prevalence of G6PD deficiency in Achang group was a little lower compared with populations in Thailand[17] and Myanmar[18], which were about 11%.

Mutation analysis of *G6PD* genes of the 19 Achang G6PD deficient individuals by PCR-DHPLC and sequencing showed that the most common variant was *G6PD* Mahidol 487G>A accounting for 84.2% of the deficiency individuals**.** All of the *G6PD* Mahidol variants in Achang people were associated with IVS5-612 C polymorphism, which resulted in a novel haplotype G487A/IVS5-612 C in G6PD deficiency. *G6PD* Mahidol variant was first characterized biochemically in Thailand[19]. And later the mutation of 487G>A, which causes glycine to serine substitution at amino acid residue 163 of G6PD, was identified[20]. It was reported that *G6PD* Mahidol was the main variant accounting for 91.3% (73/80) in Myanmar[18,21]. In Thailand, *G6PD* Mahidol was found in 17.2% (23/134) of G6PD-deficient subjects in Songkla[22], which was similar to the prevalence of *G6PD* Mahidol (15.1%) in Malaysian Malays[23]. These data implied that heavy gene flows occurred between the Achang group and groups in Myanmar, and in a less degree between Achang group and Thailand or Malaysian Malays populations.

Table 3 Distribution of *G6PD* Mahidol from G6PD-deficient individuals in different ethnic groups

Different groups	G6PD deficiency	*G6PD* Mahidol	% of *G6PD* Mahidol in G6PD deficiency	Reference
Han	20	7	35	24, 25
Dai	55	4	7.2	24, 25
Bai	66	6	9.1	24, 25
Achang	19	16	84.2	present study
Myanmar	50	45	90	18, 21
Thailand	134	23	17.2	22
Total	526	100	19.0	

In one previous study, a total of 18 individuals with *G6PD* Mahidol variant were found in Han, Dai and Bai ethnic groups and *G6PD* Mahidol variant never exceeded 40% in all the ethnic populations studied in Yunnan Province[24,25] (Table 3). But, this variant was found only in Yunnan, not in the neighboring provinces like Guangdong, Guangxi, Hainan and any other Provinces in China. In our study, 16 G6PD deficient Achang individuals were identified as carrying *G6PD* Mahidol variant accounting for 84.2%, which is much higher than the percentage of *G6PD* Mahidol variant in other ethnic populations in Yunnan Province.

The following factors maybe contribute to the higher *G6PD* Mahidol in this Achang group. Firstly, Yunnan Province is located in the southwest of China, close to Southeast Asia, and shares a border with Myanmar, Laos, and Vietnam. Qimu Village in Lianghe County where AChang people live is located in the southwest of Yunnan and it is close to Myanmar (Figure 1). Its location is very convenient for trade with Myanmar, which also contributes to gene flows between the Achang group and groups in Myanmar. In addition, Achang village is situated in the mountain area with inconvenient transportation, which confines the communication and interchange between the Achang people and outside. And practice of strict endogamy in the Achang group also contributed to the preservation of *G6PD* Mahidol variant. Furthermore, the conservation of *G6PD* Mahidol variant in the Achang population is likely related with natural selection. Today, it is generally accepted that the frequencies of low-activity alleles of human G6PD are highly correlated with the prevalence of malaria[2,26]. And Lianghe County is a malaria endemic area. Therefore, high malaria infection in this endemic area in the history likely helped to maintain *G6PD* Mahidol variant at high frequency in the Achang group. However, the further malarial epidemiologic survey in

this population is also required. The identification of G6PD deficient persons will help with the treatment of malaria. For example, malaria patients with G6PD-deficiency should not be given primaquine because it induces hemolysis. Furthermore, practice of strict endogamy also contributed to the preservation of *G6PD* Mahidol variant.

In addition, we demonstrated a new polymorphism of IVS5-612 G>C that was present in 100% of *G6PD* Mahidol in the Achang group, which was the first report in the China population. However, only one Han subject presented the IVS11-93 C/nt 1311 T polymorphism from 18 subjects with *G6PD* Mahidol in Yunnan[25]. Different populations generally show unique *G6PD* polymorphism. For example, of the Khmer group in Cambodia, the haplotype, IVS11-93 C/nt 1311 T, was linked to all *G6PD* Viangchan (871G>A) cases[27], which was the most common variant in the Cambodian population. The association of IVS11-93 T>C with nt 1311 C>T was also studied in the Kurdish population of Western Iran. IVS11-93 C was present in 88.5% of the G6PD deficient individuals and only 7.3% of the Mediterranean alleles were associated with the T form and nt 1311C[28]. Hence the new haplotype G487A/IVS5-612C seems to be a molecular characterization of G6PD deficiency for this Achang group. The different haplotypes of *G6PD* gene in Achang, Han, Cambodian and Kurdish groups suggest that the Achang population has different origin from Han, Cambodian and Kurdish populations.

Interestingly, *G6PD* Canton 1376G>T and *G6PD* Kaiping 1388G>A, which occurred in over 50%—60% G6PD-deficient individuals in Han, Dai, Bai, Yi and Jinuo ethnic groups in China[11], were not found in our study (Table 4). Prevalences of *G6PD* Canton and Kaiping variants in different ethnic groups in China are listed in Table 4. The lack of common *G6PD* Canton and Kaiping variants in the Achang group suggests that there are different *G6PD* mutation profiles in the Achang ethnic group and other ethnic groups in China.

The other 15.8% of G6PD deficient Achang individuals had no identifiable gene mutation in exons 2—13 of *G6PD* gene after PCR sequencing. However, the activities of G6PD enzyme in these subjects were lower than the normal value. DHPLC was a more sensitive and accurate technique used in mutation screening extensively[29,30]. In our study, results of DHPLC were identified by sequencing, so that no identifiable gene mutation was found in some G6PD deficient subjects, which was not because of the method. When there is no mutation in the gene, low G6PD enzyme concentration could lead to low G6PD activity. Correct promoter functioning is needed for the proper expression of *G6PD* that is under the control of some unidentified specific factors and elements[31]. However, no sequence variations were identified in previous studies[32] in the *G6PD* core promoter or in the 5′UTR of G6PD deficient individuals who had no mutations in their exons 2—13. Human *G6PD* gene, as a housekeeping gene, has at least 9 GC boxes in its regulatory 5′-flanking sequence and its promoter function depends on 2 GC boxes that are cell specifically controlled[33]. It is still unclear how human *G6PD* expression is regulated. Understanding of the regulation of *G6PD* gene expression could shed light on the causes of G6PD enzyme deficiency in individuals who have no mutations in the coding regions of *G6PD*.

In summary, we studied the prevalence and molecular genetics of G6PD deficiency in the Achang population at Qimu Village of Lianghe County. We found that the prevalence of G6PD deficiency in the Achang group was 7.31% in males and 4.35% in females. *G6PD* Mahidol was the most common variant accounting for 84.2% in the Achang population, which were linked to the new polymorphism IVS5-612G>C, and so G487A and IVS5-612 C combined into a new haplotype. Higher frequency *G6PD* Mahidol of the Achang group was similar to that of Myanmar population, and the Achang G6PD deficient subjects were lack of *G6PD* Canton or *G6PD* Kaiping. These results demonstrate that some gene flows between Achang and Myanmar populations

Table 4 Distribution of *G6PD* Canton and Kaiping from G6PD-deficient ethnic groups in China

G6PD variant	Nucleotide change	Amino acid change	Han	Dai	Bai	Yi	Jinuo	Achang
Canton	1376, G→T	495Arg→Leu	16/86	21/87	14/66	5/23	12/46	0/19
Kaiping	1388, G→A	463Arg→His	27/86	36/87	28/66	10/23	18/46	0/19
Total			50.0%	65.5%	63.6%	65.2%	65.2%	0
Reference			11	11	11	11	11	present study

occurred during the evolution history of the Achang ethnic group. Also, our results presented different *G6PD* mutation profiles between the Achang group and the other ethnic groups of Chinese population. Up to date, there is little information on the Achang ethnic group origin. Our findings appear to be the first documented report on the G6PD genetics of the Achang people, which will provide some important clues to the Achang ethnic group origin.

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