BMJ Open

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| Journal: | *BMJ Open* |
| Manuscript ID | bmjopen-2015-010047 |
| Article Type: | Research |
| Date Submitted by the Author: | 20-Sep-2015 |
| Complete List of Authors: | Wang, Shiyun; Shanghai Diabetes Institute, Shanghai Key Laboratory of Diabetes Mellitus, Shanghai Clinical Center for Diabetes, Shanghai Jiao Tong University Affiliated Sixth,<br>Zhang, Rong; Shanghai Diabetes Institute, Shanghai Key Laboratory of Diabetes Mellitus, Shanghai Clinical Center for Diabetes, Shanghai Jiao Tong University Affiliated Sixth,<br>Xiang, Guangxin; National Engineering Research Center for Beijing Biochip Technology,<br>Li, Yang; National Engineering Research Center for Beijing Biochip Technology,<br>Hou, Xuhong; Shanghai Diabetes Institute, Shanghai Key Laboratory of Diabetes Mellitus, Shanghai Clinical Center for Diabetes, Shanghai Jiao Tong University Affiliated Sixth,<br>Jiang, Fusong; Shanghai Diabetes Institute, Shanghai Key Laboratory of Diabetes Mellitus, Shanghai Clinical Center for Diabetes, Shanghai Jiao Tong University Affiliated Sixth,<br>Jiang, Feng; Shanghai Diabetes Institute, Shanghai Key Laboratory of Diabetes Mellitus, Shanghai Clinical Center for Diabetes, Shanghai Jiao Tong University Affiliated Sixth,<br>Hu, Cheng; Shanghai Diabetes Institute, Shanghai Key Laboratory of Diabetes Mellitus, Shanghai Clinical Center for Diabetes, Shanghai Jiao Tong University Affiliated Sixth,<br>Jia, Weiping; Shanghai Jiaotong University Affiliated Sixth People's Hospital, |
| Primary Subject Heading: | Haematology (incl blood transfusion) |
| Secondary Subject Heading: | Epidemiology, Genetics and genomics, Haematology (incl blood transfusion) |
| Keywords: | EPIDEMIOLOGY, Antenatal < GENETICS, Anaemia < HAEMATOLOGY |
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Research article

# Mutation Screening for Thalassaemia in the Jino Ethnic Minority Population of Yunnan Province, Southwest China

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**Keywords:**

thalassaemia, gene mutation, haemoglobin, anaemia, Jino, Yunnan Province

**Word count: 1,800**

# ABSTRACT

## Objectives

This study aimed to detect α- and β-thalassaemia mutations in the Jino ethnic minority population of Yunnan Province, southwest China.

## Design

A total of 1,613 Jino adults were continuously recruited from February 2012 to April 2012. Fasting venous blood samples were obtained to determine haematological parameters. Haemoglobin analysis was conducted via high-performance liquid chromatography. Participants with hypochromic microcytic anaemia or positive haemoglobin analysis profiles were confirmed via α- and β-globin genetic testing, including DNA microarray analysis, direct sequencing methods and multiplex gap-polymerase chain reaction assays.

## Setting

Shanghai Diabetes Institute, Shanghai Key Laboratory of Diabetes Mellitus, Shanghai Jiao Tong University Affiliated Sixth People's Hospital

## Results

We found 363 suspected cases via primary screening of haematological parameters and haemoglobin analysis. After further genetic testing, six types of α- and β-thalassaemia mutations were detected in 203 out of 363 individuals. Both of $\alpha^0$- and $\alpha^+$-thalassaemia mutations, - -$^{SEA}$ and - $\alpha^{3.7}$ were identified. Additionally, 13 Hb E carriers had coexisting $\alpha^0$ or $\alpha^+$-thalassaemia deletions. Clinical haematological parameters indicated that in this study, carriers of all thalassaemic genotypes had

more severe hypochromic microcytic anaemia compared with non-thalassaemic individuals.

## Conclusion

Our results provide information on the Jino ethnic minority that may be useful for further genetic counselling, prenatal screening and clinical diagnosis of thalassaemia in this region.

## Strengths and limitations of this study:

1. As Jino, the last ethnic minority confirmed in China, was reported to have a high prevalence of thalassaemia according to a previous research of children under 10 years old, this study aimed to detect the mutations of α- and β-thalassaemia in Jino adults.
2. α- and β-thalassaemia mutation spectrum shown in this research may help to explain further genotype – phenotype correlations and to establish a thalassaemia prevention program in this area.
3. The sample size of controls we used in the genetic testing was relatively small and may not have the validity to detect the other genotypes of silent α-thalassaemia from this group.

# INTRODUCTION

As a group of monogenic disorders, thalassaemia is a serious health problem worldwide, especially in Mediterranean areas, Southeast Asia and southern China [1-3]. Yunnan Province, which is located along the border areas of China-Myanmar-Laos, is notable for its ethnic diversity. According to a survey of children under 10 years old, several ethnic minorities in this region have a high prevalence of thalassaemia, with the prevalence of α-thalassaemia (α-thal) being highest (22.1%) in Dai from Xishuangbanna and the prevalence of β-thalassaemia (β-thal) being highest in Achang (40.6%) [4].

Jino is the last ethnic minority confirmed in China, and the prevalences of α-thal and β-thal among Jino children are 3.1% and 29.3%, respectively. Thalassaemic children can exhibit various clinical symptoms; some are asymptomatic carriers, whereas others have severe haemolytic anaemia [5]. Blood transfusion therapy, which is needed for severe carriers, imposes a heavy burden on families and public health management [6]. Although genetic screening is essential to prevent and control this inherited disease, systematic investigations of thalassaemia mutations in Jino adults are rare.

The Jino population comprises nearly 20,000 individuals, and most (approximately 90%) live around Jino Mountain, which is located in east-central Yunnan Province [7]. A large number of thalassaemic mutations have been found in the general population worldwide [8-10]; however, little is known regarding this isolated population. Indeed, the molecular mechanism and genetic variations of thalassaemia in Jino individuals

may be different from those in other ethnicities. Our study aimed to detect α-thal and β-thal gene mutations in Jino adults to provide basic information for further prenatal consulting and thalassaemia diagnosis.

# MATERIALS AND METHODS

## Participants and clinical screening

This cross-sectional study was conducted in eight villages of Jino Mountain Township in southern Yunnan Province, China (Fig. 1).

A total of 1,613 Jino adults, including 762 males and 851 females, were randomly selected from February 2012 to April 2012 (haematological and demographic characteristics of the total population included in the study are described in Table 1). Haematological parameters were measured, including haemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular haemoglobin (MCH), red blood cell (RBC), and red cell distribution width (RDW). Haemoglobin was analysed by HPLC (high-performance liquid chromatography) using the VARIANT II$^{TM}$ haemoglobin analysing system (Bio-Rad Laboratories, Hercules, CA, USA). α- and β-globin genetic testing was performed in participants (n=363) with hypochromic microcytic anaemia (MCV<80 fl and/or MCH<27 pg) and/or positive HPLC profiles. The remaining participants (n=1250) were considered normal, and controls (n=50) were randomly selected from this group for further genetic testing.

**Table 1. Haematological and demographic characteristics of 1,613 Jino ethnic minority adults included in the study.**

| Parameter | Total | Males | Females |
|---|---|---|---|
| Samples(n) | 1613 | 762 | 851 |
| Age(years) | 40.43 ± 14.78 | 40.11 ± 15.21 | 40.71 ± 14.38 |
| BMI(kg/m$^2$) | 21.79 ± 3.20 | 22.12 ± 3.17 | 21.50 ± 3.21 |
| RBC($10^{12}$/L) | 4.85 ± 0.56 | 5.08 ± 0.57 | 4.65 ± 0.47 |
| RDW(%) | 12.76 ± 1.29 | 12.60 ± 1.10 | 12.89 ± 1.42 |
| MCV(fL) | 83.42 ± 8.08 | 84.97 ± 7.54 | 82.03 ± 8.30 |
| MCH(pg) | 29.21 ± 3.26 | 29.91 ± 3.07 | 28.59 ± 3.30 |
| HCT(%) | 40.25 ± 4.36 | 42.88 ± 3.74 | 37.90 ± 3.44 |
| Hb(g/dL) | 14.09 ± 1.70 | 15.09 ± 1.49 | 13.20 ± 1.34 |

The data are shown as the means ± SD; BMI: body mass index; RBC: red blood cell; RDW: red cell distribution width; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; HCT: haematocrit; Hb: haemoglobin

## Genetic testing

Genomic DNA was extracted from venous blood leukocytes. Three methods were utilized to detect thalassaemic mutations.

A CapitalBio Thalassaemia Gene Mutation Detection Kit (CapitalBio, Beijing, China) was used to determine 25 common mutations in globin genes in the Chinese population via DNA microarray. Six α-thal gene mutations and nineteen β-thal gene mutations were included. A BioMixer™ II Microarray Hybridization Station (CapitalBio, Beijing, China) was used for hybridization after multiplex polymerase chain reaction amplification. Then, chips were scanned using a LuxScan™ 10K-B

Microarray Scanner (CapitalBio, Beijing, China).

To validate β-thal mutations, three fragments of the β-globin gene were amplified. The first fragment was amplified with 5'-CCT AAG CCA GTG CCA GAA GAG C-3' as the forward primer and 5'-TGC CCA GTT TCT ATT GGT CTC C-3' as the reverse primer, the second fragment was amplified with 5'-TAG AAA CTG GGC ATG TGG AG-3' as the forward primer and 5'-TGT ACC CTG TTA CTT ATC CC-3' as the reverse primer, and the third fragment was amplified with 5'-TCA GGG CAA TAA TGA TAC AA-3' as the forward primer and 5'-TTA GTA GTT GGA CTT AGG GA-3' as the reverse primer. The fragments were sequenced using a 3500 Genetic Analyser (Applied Biosystems, Foster City, CA, USA).

Moreover, we also confirmed three α-thal deletions [i.e., the Southeast Asian deletion (- $-^{SEA}$), rightward deletion (- $\alpha^{3.7}$), and leftward deletion (- $\alpha^{4.2}$)] via multiplex gap-polymerase chain reaction assays [11, 12].

## Statistical analysis

A statistical analysis was carried out using SAS for Windows (version 9.2; SAS Institute, Cary, NC, USA). All quantitative traits were tested for normality, and skewed quantitative traits were logarithmically transformed to approximate univariate normality. The data are shown as the means ± standard deviation (SD). The quantitative traits (RBC, Hb, MCV, MCH, RDW) were compared between two groups by using Wilcoxon test, and ANOVA tests were performed for comparing the differences in three subgroups of thalassaemia carriers (α-Thal, β-Thal and αβ-Thal). Two-tailed statistical significance was considered at $p<0.05$.

# RESULTS

## Mutations identified in Jino

Due to mutations in different globin genes, we observed three groups of thalassaemic carriers, including individuals with only α-thal gene mutations or β-thal gene mutations and individuals with combined αβ-thalassaemia (αβ-thal) gene mutations. Six different thalassaemia mutations were detected in 203 individuals among 363 suspected cases. No mutations were observed in the 50 controls. Table 2 shows the allele frequency of α- and β-thalassaemia mutations found in our study.

**Table 2. Allele frequency of α- and β-Thalassaemia mutations found in our study.**

| Mutation | Phenotype | n | Number of Alleles | Allele Frequency (ratio) |
|---|---|---|---|---|
| α-thalassaemia | | | | |
| $--^{SEA}$ | $\alpha^0/\alpha$ | 42 | 42 | 70.00% (42/60) |
| $-\alpha^{3.7}$ | $\alpha^+/\alpha$ | 16 | 16 | 30.00% (18/60) |
| $-\alpha^{3.7}/-\alpha^{3.7}$ | $\alpha^+/\alpha^+$ | 1 | 2 | — |
| β-thalassaemia | | | | |
| codon 17 | $\beta^0/\beta^A$ | 20 | 20 | 12.35% (20/162) |
| Hb E | $\beta^E/\beta^A$ | 132 | 132 | 87.65% (142/162) |
| Hb E/ Hb E | $\beta^E/\beta^E$ | 5 | 10 | — |

α: the normal α-globin chain; $\alpha^0$: the α-globin chain is totally deletion; $\alpha^+$: the α-globin chain is partly deletion; $\beta^A$: the normal β-globin chain; $\beta^E$: the abnormal β-globin chain of Hb E mutation; $\beta^0$: the β-globin chain is totally deletion.

## Mutations in the α-thal gene

None of the three common non-deletion $\alpha^+$-thal genes were found in the 203 participants with thalassaemia mutations. Forty-six of the 203 participants carried α-thal gene deletions only; - -$^{SEA}$ and - $\alpha^{3.7}$ were observed, accounting for 16.7% (34/203) and 5.9% (12/203) of the mutations, respectively. Among these individuals, we identified both $\alpha^+/\alpha$ and $\alpha^+/\alpha^+$ for - $\alpha^{3.7}$ and $\alpha^0/\alpha$ for - -$^{SEA}$ (gel electrophoresis results are shown in Fig. 2). However, no - $\alpha^{4.2}$ mutations were observed.

## Mutations in the β-thal gene

We observed mutations in codon 17 (A>T) (Fig. 3) and Hb E [codon 26 (G>A)] (Fig. 4). Codon 17 (A>T), which accounted for 9.9% (20/203) of mutations, was found to be $\beta^0/\beta^A$ in this population. Participants with only the Hb E [codon 26 (G>A)] variant, either $\beta^E/\beta^A$ or $\beta^E/\beta^E$, accounted for 61.1% (124/203) of mutations. Furthermore, 13 Hb E carriers harboured - -$^{SEA}$ (n=8) or - $\alpha^{3.7}$ (n=5) at a combined frequency of 6.4% (13/203).

## Haematological features of different thalassaemia genotypes

The haematological data of different thalassaemia genotypes are summarized in Table 3. Compared with normal individuals, thalassaemic carriers had significantly lower Hb, MCV and MCH levels ($p$<0.001, respectively) and higher RBC and RDW levels ($p$<0.001, respectively). Furthermore, we compared the differences in those five indexes among three groups of carriers (α-thal, β-thal and αβ-thal). Significant

**Table 3. Haematological data of 1,613 Jino ethnic minority individuals with different thalassaemia subtypes.**

| Thalassaemia types | n | RBC($10^{12}$/L) | Hb(g/dL) | MCV(fL) | MCH(pg) | RDW(%) |
|---|---|---|---|---|---|---|
| α-Thalassaemia | 46 | 5.63 ± 0.78 | 13.03 ± 1.61 | 68.99 ± 6.50 | 23.33 ± 2.45 | 13.64 ± 1.38 |
| $\alpha^0/\alpha$ | 34 | 5.88 ± 0.68 | 12.98 ± 1.62 | 65.69 ± 2.65 | 22.08 ± 0.81 | 14.01 ± 1.37 |
| $\alpha^+/\alpha$ | 11 | 4.86 ± 0.58 | 13.19 ± 1.70 | 78.96 ± 4.43 | 27.14 ± 1.87 | 12.52 ± 0.74 |
| $\alpha^+/\alpha^+$ | 1 | 5.34 | 12.7 | 71.5 | 23.8 | 13.1 |
| β-Thalassaemia | 144 | 5.27 ± 0.48 | 13.31 ± 1.70 | 72.46 ± 7.09 | 25.29 ± 2.75 | 13.21 ± 0.96 |
| $\beta^0/\beta^A$ | 20 | 5.41 ± 0.38 | 10.68 ± 0.53 | 58.70 ± 2.31 | 19.77 ± 0.83 | 14.23 ± 0.40 |
| $\beta^E/\beta^A$ | 120 | 5.23 ± 0.48 | 13.77 ± 1.41 | 75.17 ± 3.88 | 26.34 ± 1.49 | 12.98 ± 0.85 |
| $\beta^E/\beta^E$ | 4 | 5.91 ± 0.51 | 12.55 ± 1.22 | 60.23 ± 3.61 | 21.23 ± 1.15 | 15.00 ± 0.57 |
| αβ-Thalassaemia | 13 | 5.67 ± 0.70 | 13.63 ± 1.67 | 69.40 ± 6.85 | 24.17 ± 2.59 | 13.12 ± 0.92 |
| $\beta^E/\beta^A$ with $\alpha^0/\alpha$ | 7 | 6.02 ± 0.69 | 13.57 ± 1.58 | 65.94 ± 2.82 | 22.57 ± 1.07 | 13.66 ± 0.71 |
| $\beta^E/\beta^A$ with $\alpha^+/\alpha$ | 5 | 5.21 ± 0.53 | 14.08 ± 1.88 | 76.46 ± 3.86 | 27.00 ± 1.33 | 12.22 ± 0.36 |
| $\beta^E/\beta^E$ with $\beta^0/\beta^A$ | 1 | 5.57 | 13.8 | 58.3 | 21.2 | 11.8 |
| Total thalassaemia | 203 | 5.38 ± 0.60 | 13.27 ± 1.68 | 71.48 ± 7.08 | 24.77 ± 2.79 | 13.30 ± 1.08 |
| Non-thalassaemia | 1410 | 4.78 ± 0.51 | 14.21 ± 1.67 | 85.14 ± 6.64 | 29.85 ± 2.79 | 12.68 ± 1.30 |
| $P^a$ value | | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| $P^b$ value | | 0.0012 | >0.05 | 0.0111 | 0.0002 | 0.0573 |

The data are shown as the n, means ± SD, medians (interquartile range) or raw data when necessary; RBC: red blood cell; Hb: haemoglobin; MCV: mean

corpuscular volume; MCH: mean corpuscular haemoglobin; RDW: red cell distribution width.

[a] Non-thalassaemic individuals compared with thalassaemia group.

[b] Compare among three subgroups of thalassaemia (α-Thal, β-Thal and αβ-Thal).

differences in MCV ($p$=0.0111), MCH ($p$=0.0002) and RBC ($p$=0.0012) were observed between the groups. MCV and MCH levels in the α-thal group were significantly lower than those in the β-thal group ($p$<0.05), whereas RBC levels were higher ($p$<0.05). In contrast, no difference was observed between the αβ-thal and α-thal groups/β-thal groups. Moreover, there was a tendency towards increased RDW levels in the α-thal group compared with the β-thal group ($p$=0.0573).

# DISCUSSION

Thalassaemia is a common monogenic disease with a relatively high prevalence in Southeast Asia. In China, this disease is mainly prevalent in areas near the southern bank of the Yangtze River, such as Guangdong, Guangxi, Fujian and Yunnan Provinces [13-15]. Prenatal screening and related molecular diagnoses are crucial for preventing and treating thalassaemia. Many thalassaemia studies have been conducted in Yunnan Province [16, 17]. However, data on the Jino population are limited because this population is the last ethnic minority confirmed in China.

We randomly selected 1,613 Jino adults from eight villages of Jino Mountain Township in Jinghong, Southern Yunnan. Among the gene mutations identified, the most prevalent α-thal and β-thal genotypes in this region were - -$^{SEA}$ and Hb E, in agreement with previous data from Yunnan Province [18,19]. According to our results, the overall prevalence of thalassaemia in Jino was nearly 12.6%, which is similar to the prevalence observed in Kunming [20]. The prevalence of αβ-thal (8%) in our

population was equal to that in the Li population in Hainan Province (7.99%), where thalassaemia prevalence is high [21]. Although Yunnan Province has a high prevalence of thalassaemia with diverse genotypes, globin gene mutations among the Jino population are relatively limited.

Hb E, a type of haemoglobinopathy, can be observed in most regions of southeast China [22]. Due to a point mutation in the β-globin gene, the balance of various globin products is disrupted, leading to a structural haemoglobin variant. Although Hb E carriers may only have slight anaemia, their offspring will exhibit severe clinical symptoms in the presence of other β-thal types [23]. Therefore, potential Hb E carriers should undergo genetic testing and prenatal counselling. HPLC is often used as an efficient primary screening method to detect abnormal Hb, as was done in this study and previous studies [24,25]. In our study, 95.6% (131/137) of Hb E carriers were identified by HPLC, and 13 of these individuals had concomitant α-thal deletions.

Different genotypes lead to different clinical phenotypes [26]. We found that thalassaemic carriers had significantly lower MCV and MCH levels. Regarding those with β-thal mutations, MCV and MCH levels were significantly decreased in codon 17 carriers compared with Hb E carriers, suggesting that a nonsense mutation in the β-globin gene causes greater erythrocyte impairment. Hypochromic microcytic anaemia was moderate in individuals with $\beta^E/\beta^A$ and $\alpha^+/\alpha$ compared with $\beta^E/\beta^A$ carriers. This paradox may be explained by the fact that changes in the α- and β-globin chains could balance each other out when both mutations coexist in an individual. Accordingly, rapidly estimating the genetic state of an illness based on haematological parameters is difficult. Therefore, genetic screening of both α- and β-globin gene mutations in potential parents is of utmost importance to prevent births with severe defects [27].

In conclusion, this study revealed α- and β-thalassaemia mutations in the Jino ethnic minority population in Yunnan Province. Of these mutations, - -$^{SEA}$ and Hb E were the most prevalent α-thal and β-thal gene mutation types. In addition, data based on clinical haematological parameter analysis indicated that the severity of hypochromic microcytic anaemia is associated with the genotype of thalassaemia. Our results provide evidence that may be useful for further genetic counselling, prenatal screening and clinical diagnosis of thalassaemia in this region.

## Contributors

WJ and CH conceived and designed the experiments. SW and RZ performed the experiments and analyzed the data. GX and YL contributed materials and analysis tools. SW prepared the article. CH and WJ revised the manuscript. All the authors have read and approved the final version of this manuscript.

## Acknowledgements

We thank all of the participants for their dedication. We acknowledge the skillful technical support of all nursing and medical staff at the Shanghai Clinical Center for Diabetes.

## Funding

This work was financially supported by a grant from the National Natural Science Foundation of China (91331110).

## Competing interests

None declared.

## Ethics approval

According to the Helsinki Declaration II, ethical approval for the study was granted

by the Institutional Review Board of Shanghai Jiao Tong University affiliated with the Sixth People's Hospital, Shanghai, China. Written informed consent was obtained from all participants.

## Data sharing statement

No additional data are available.

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# Figure legends

**Fig. 1: Geographical location of the Jino ethnic minority populations in Yunnan Province, Southwest China.** The solid black triangle represents Jino Mountain. The solid black circles represent the eight villages of Jino Mountain Township where the 1,613 subjects were randomly selected.

**Fig. 2: Three α-thal deletions.** Lane 1 represents homozygous - $\alpha^{3.7}$; lanes 2 and 3 represent heterozygous - $\alpha^{3.7}$; and lanes 4 and 5 represent heterozygous - $-^{SEA}$. The 2.0 kb, 1.7 kb, 1.4 kb, and 1.2 kb marked bands represent the - $\alpha^{3.7}$, α α, - $\alpha^{4.2}$,and - $-^{SEA}$ genotypes, respectively.

**Fig. 3: Heterozygous codon 17 (A>T) mutation (a) and the corresponding normal sequence of β-globin.** Red arrows indicate the position of this point mutation.

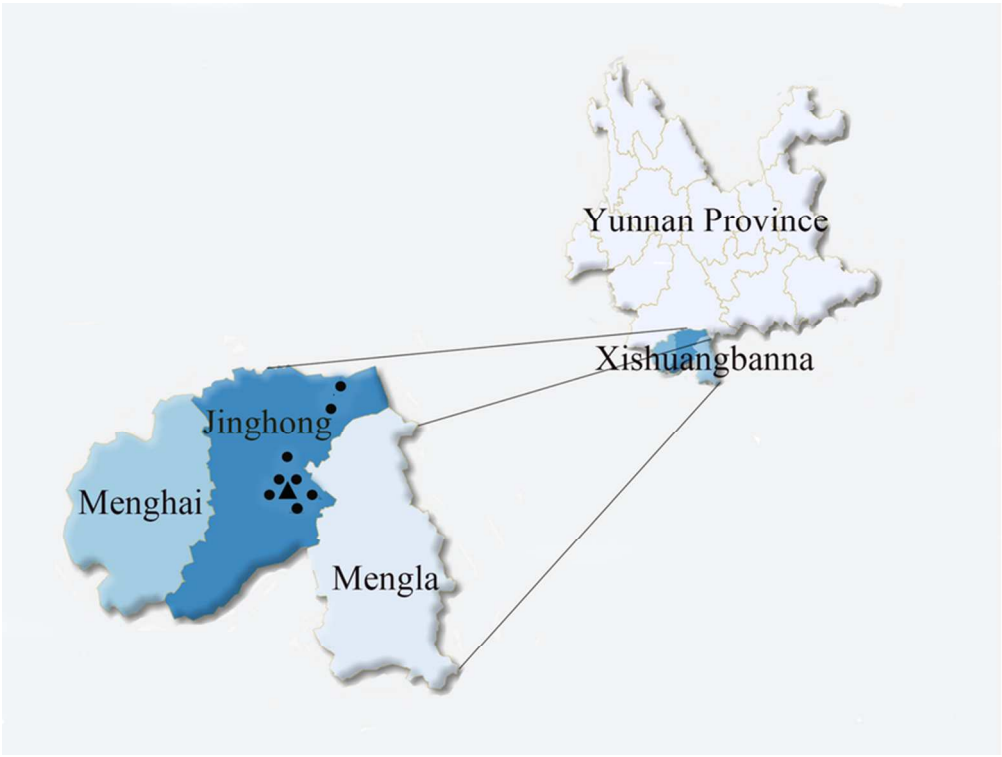
**Fig. 4: Heterozygous codon 26 (G>A) mutation (a) and the corresponding normal sequence of β-globin.** Red arrows indicate the position of this point mutation.

# Table legends

**Table 1.** Haematological and demographic characteristics of 1,613 Jino ethnic minority adults included in the study.

**Table 2.** Allele frequency of α- and β-Thalassaemia mutations found in our study.

**Table 3.** Haematological data of 1,613 Jino ethnic minority individuals with different thalassaemia subtypes.

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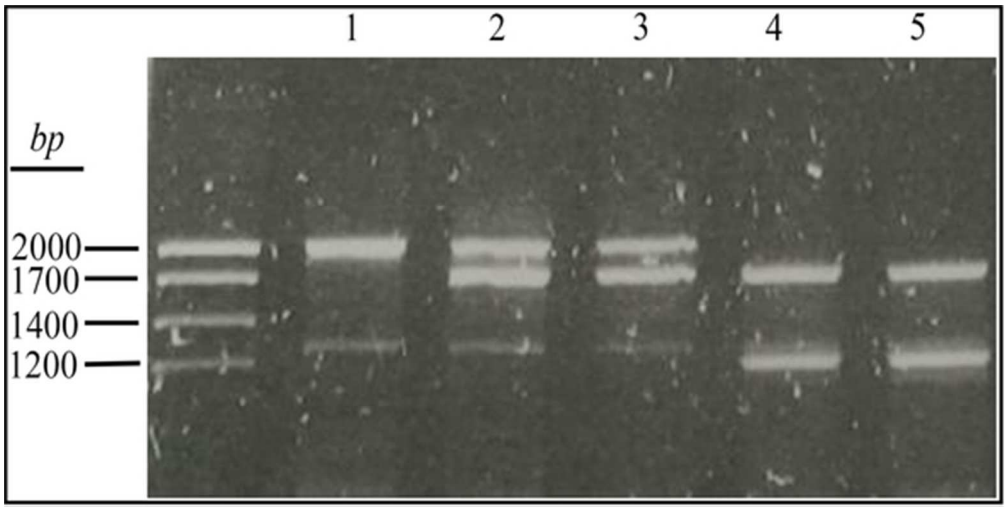


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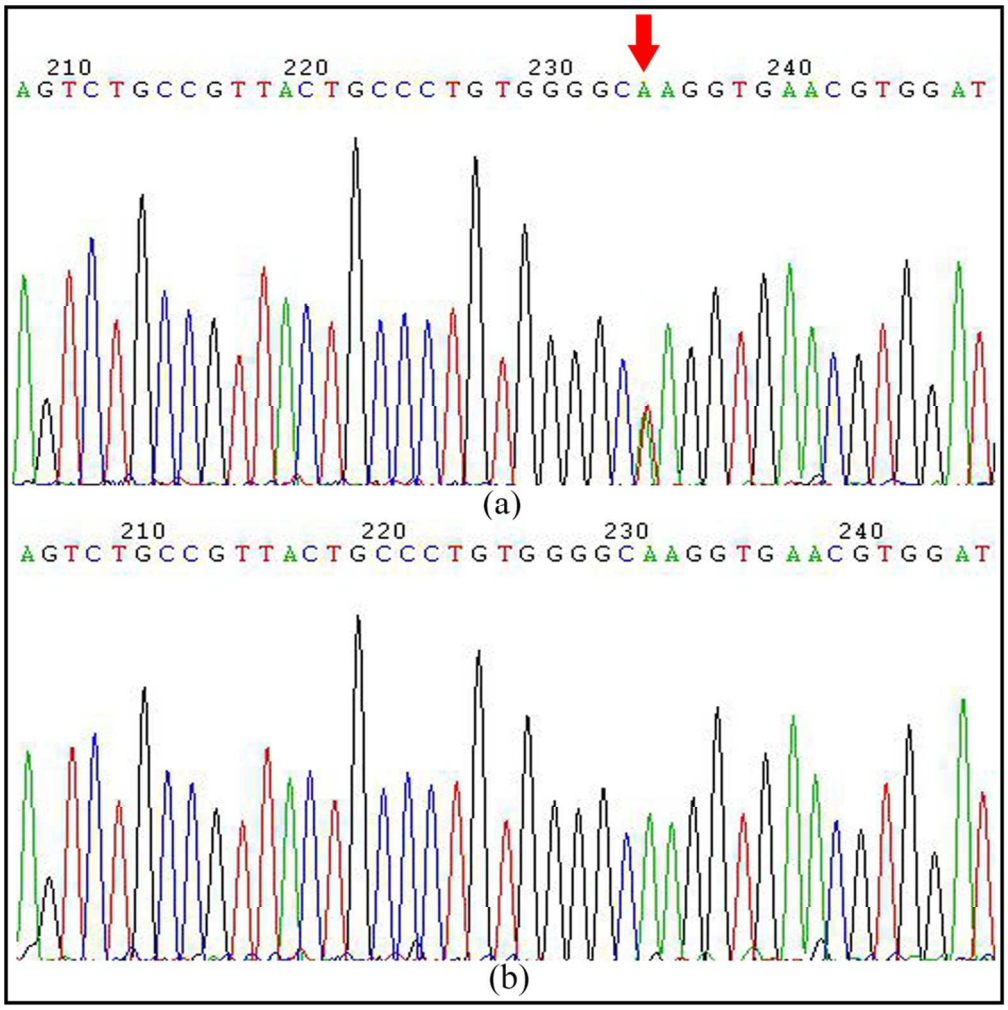


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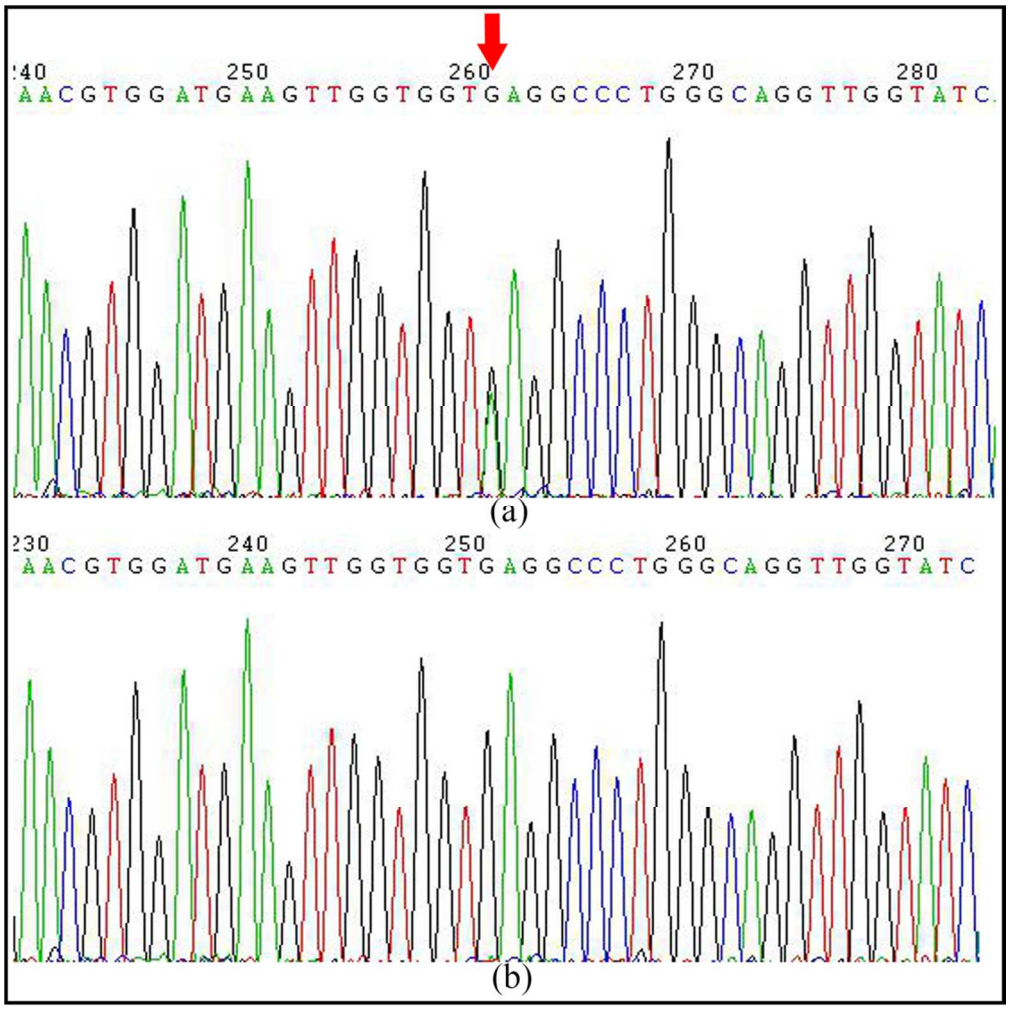


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BMJ Open

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|---|---|
| Journal: | *BMJ Open* |
| Manuscript ID | bmjopen-2015-010047.R1 |
| Article Type: | Research |
| Date Submitted by the Author: | 04-Nov-2015 |
| Complete List of Authors: | Wang, Shiyun; Shanghai Diabetes Institute, Shanghai Key Laboratory of Diabetes Mellitus, Shanghai Clinical Center for Diabetes, Shanghai Jiao Tong University Affiliated Sixth,<br>Zhang, Rong; Shanghai Diabetes Institute, Shanghai Key Laboratory of Diabetes Mellitus, Shanghai Clinical Center for Diabetes, Shanghai Jiao Tong University Affiliated Sixth,<br>Xiang, Guangxin; National Engineering Research Center for Beijing Biochip Technology,<br>Li, Yang; National Engineering Research Center for Beijing Biochip Technology,<br>Hou, Xuhong; Shanghai Diabetes Institute, Shanghai Key Laboratory of Diabetes Mellitus, Shanghai Clinical Center for Diabetes, Shanghai Jiao Tong University Affiliated Sixth,<br>Jiang, Fusong; Shanghai Diabetes Institute, Shanghai Key Laboratory of Diabetes Mellitus, Shanghai Clinical Center for Diabetes, Shanghai Jiao Tong University Affiliated Sixth,<br>Jiang, Feng; Shanghai Diabetes Institute, Shanghai Key Laboratory of Diabetes Mellitus, Shanghai Clinical Center for Diabetes, Shanghai Jiao Tong University Affiliated Sixth,<br>Hu, Cheng; Shanghai Diabetes Institute, Shanghai Key Laboratory of Diabetes Mellitus, Shanghai Clinical Center for Diabetes, Shanghai Jiao Tong University Affiliated Sixth,<br>Jia, Weiping; Shanghai Jiaotong University Affiliated Sixth People's Hospital, |
| Primary Subject Heading: | Haematology (incl blood transfusion) |
| Secondary Subject Heading: | Epidemiology, Genetics and genomics, Haematology (incl blood transfusion) |
| Keywords: | EPIDEMIOLOGY, Antenatal < GENETICS, Anaemia < HAEMATOLOGY |
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Research article

# Mutation Screening for Thalassaemia in the Jino Ethnic Minority Population of Yunnan Province, Southwest China

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**Keywords:**

thalassaemia, gene mutation, haemoglobin, anaemia, Jino, Yunnan Province

**Word count: 2,129**

# ABSTRACT

## Objectives

This study aimed to detect α- and β-thalassaemia mutations in the Jino ethnic minority population of Yunnan Province, southwest China.

## Design

A total of 1,613 Jino adults were continuously recruited from February 2012 to April 2012. Fasting venous blood samples were obtained to determine haematological parameters. Haemoglobin analysis was conducted via high-performance liquid chromatography. Participants with hypochromic microcytic anaemia or positive haemoglobin analysis profiles were confirmed via α- and β-globin genetic testing, including DNA microarray analysis, direct sequencing methods and multiplex gap-polymerase chain reaction assays.

## Setting

Shanghai Diabetes Institute, Shanghai Key Laboratory of Diabetes Mellitus, Shanghai Jiao Tong University Affiliated Sixth People's Hospital

## Results

We found 363 suspected cases via primary screening of haematological parameters and haemoglobin analysis. After further genetic testing, four types of α- and β-thalassaemia mutations were detected in 203 out of 363 individuals. Both of $\alpha^0$- and $\alpha^+$-thalassaemia mutations, $--^{SEA}$ and $-\alpha^{3.7}$ were identified. β-thalassaemia mutations included CD17 (HBB:c.52A > T) and CD26 (HbE or HBB:c.79G>A). Additionally, 13 HbE carriers had coexisting $\alpha^0$ or $\alpha^+$-thalassaemia deletions. Clinical

haematological parameters indicated that in this study, carriers of all thalassaemic genotypes had more severe hypochromic microcytic anaemia compared with non-thalassaemic individuals.

## Conclusion

Our results provide information on the Jino ethnic minority that may be useful for further genetic counselling, prenatal screening and clinical diagnosis of thalassaemia in this region.

## Strengths and limitations of this study:

1. As Jino, the last ethnic minority confirmed in China, was reported to have a high prevalence of thalassaemia according to the previous research of children under 10 years old, this study aimed to detect the mutations of α- and β-thalassaemia in Jino adults.
2. α- and β-thalassaemia mutation spectrum shown in this research may help to explain further genotype – phenotype correlations and to establish a thalassaemia prevention program in this area.
3. The sample size we used in the genetic testing was relatively small and may not have the validity to identify the rare thalassaemia from this ethnic group.

# INTRODUCTION

As a group of monogenic disorders, thalassaemia is a serious health problem worldwide, especially in Mediterranean areas, Southeast Asia and southern China [1-3]. Yunnan Province, which is located along the border areas of China-Myanmar-Laos, is notable for its ethnic diversity. According to a previous study of children under 10 years old, several ethnic minorities in this region have a high prevalence of thalassaemia, with the prevalence of α-thalassaemia (α-thal) being highest (22.1%) in Dai from Xishuangbanna and the prevalence of β-thalassaemia (β-thal) being highest in Achang (40.6%) [4].

Jino is the last ethnic minority confirmed in China, and the prevalence of α-thal and β-thal among Jino children are 3.1% and 29.3%, respectively. Thalassaemic children may exhibit various clinical symptoms; some are asymptomatic carriers, whereas others have severe haemolytic anaemia [5]. Blood transfusion therapy, which is needed for severe carriers, imposes a heavy burden on families and public health management [6]. Although genetic screening is essential to prevent and control this inherited disease, systematic investigations of thalassaemia mutations in Jino adults are rare.

The Jino population comprises nearly 20,000 individuals, and most (approximately 90%) live around Jino Mountain, which is located in east-central Yunnan Province [7]. A large number of thalassaemic mutations have been found in the general population worldwide [8-10]; however, little is known regarding this isolated population. Indeed, the molecular mechanism and genetic variations of thalassaemia in Jino individuals

may be different from those in other ethnicities. Our study aimed to detect α-thal and β-thal gene mutations in Jino adults to provide basic information for further prenatal consulting and thalassaemia diagnosis.

# MATERIALS AND METHODS

## Participants and clinical screening

According to the Helsinki Declaration II, ethical approval for the study was granted by the Institutional Review Board of Shanghai Jiao Tong University affiliated with the Sixth People's Hospital, Shanghai, China. This cross-sectional study was conducted between February 2012 to April 2012 in eight villages (Luote, Jiama, Balai, Situ, New Situ, Baka, Baya, Dapingzhang) around Jino Mountain in Jinghong, southern Yunnan Province, China (Fig. 1). List of Jino adults from those eight villages was obtained from local Villager Committee Offices. Participants were sampled by a simple computer program of randomization from those villages. Staffs of local health center who understand both Chinese and Jino language contacted the subjects and introduced the purpose of this study. Oral and written informed consent was obtained from all the individuals. Basic demographic information and fasting venous blood samples were collected by researchers.

A total of 1,613 Jino adults, including 762 males and 851 females, were attending this survey (haematological and demographic characteristics of the total population included in the study are described in Table 1). Haematological parameters were measured, including haemoglobin (Hb), mean corpuscular volume (MCV), mean

corpuscular haemoglobin (MCH), red blood cell (RBC), and red cell distribution width (RDW). Haemoglobin was analysed by HPLC (high-performance liquid chromatography) using the VARIANT II$^{TM}$ haemoglobin analysing system (Bio-Rad Laboratories, Hercules, CA, USA). α- and β-globin genetic testing was performed in participants (n=363) with hypochromic microcytic anaemia (MCV<80 fl and/or MCH<27 pg) and/or positive HPLC profiles. In order to evaluate the validity of primary screening approaches for detection of thalassaemia carriers, part of the individuals with negative screening results (n=50) were randomly selected from remaining participants (n=1250) for further genetic testing.

**Table 1. Haematological and demographic characteristics of 1,613 Jino ethnic minority adults included in the study.**

| Parameter | Total | Males | Females |
|---|---|---|---|
| Samples(n) | 1613 | 762 | 851 |
| Age(years) | 40.43 ± 14.78 | 40.11 ± 15.21 | 40.71 ± 14.38 |
| BMI(kg/m$^2$) | 21.79 ± 3.20 | 22.12 ± 3.17 | 21.50 ± 3.21 |
| RBC($10^{12}$/L) | 4.85 ± 0.56 | 5.08 ± 0.57 | 4.65 ± 0.47 |
| RDW(%) | 12.76 ± 1.29 | 12.60 ± 1.10 | 12.89 ± 1.42 |
| MCV(fL) | 83.42 ± 8.08 | 84.97 ± 7.54 | 82.03 ± 8.30 |
| MCH(pg) | 29.21 ± 3.26 | 29.91 ± 3.07 | 28.59 ± 3.30 |
| HCT(%) | 40.25 ± 4.36 | 42.88 ± 3.74 | 37.90 ± 3.44 |
| Hb(g/dL) | 14.09 ± 1.70 | 15.09 ± 1.49 | 13.20 ± 1.34 |

The data are shown as the means ± SD; BMI: body mass index; RBC: red blood cell; RDW:

red cell distribution width; MCV: mean corpuscular volume; MCH: mean corpuscular haemoglobin; HCT: haematocrit; Hb: haemoglobin

## Genetic testing

Genomic DNA was extracted from venous blood leukocytes. Three methods were utilized to detect thalassaemic mutations.

A CapitalBio Thalassaemia Gene Mutation Detection Kit (CapitalBio, Beijing, China) was used to determine 25 common mutations in globin genes in the Chinese population via DNA microarray. Six α-thal gene mutations and nineteen β-thal gene mutations were included. Among them, there were three α-thal deletions [i.e., the Southeast Asian deletion ($--^{SEA}$), rightward deletion ($-\alpha^{3.7}$), and leftward deletion ($-\alpha^{4.2}$)] and three nondeletional α-thal mutations [i.e., Hb Constant Spring (HBA2:c.427T > C), Hb Quong Sze (HBA2:c.377T > C or HBA1) and Hb Westmead (HBA2:c.369C>G)]. Nineteen β-thal gene mutations were CD14/15 (HBB:c.45_46insG), CD27/28 (HBB:c.84_85insC), CD41/42 (HBB:c.126_129delTCTT), CD71/72 (HBB:c.216_217insA), -32 (HBB:c.-82T>C), -30 (HBB:c.-80T>C), -29 (HBB:c.-79A>G), -28(HBB:c.-78A>G), CD17 (HBB:c.52A>T), CD26 (HBB:c.79G>A), CD30 (HBB:c.91A>G), CD37 (HBB:c.113G>A), CD43 (HBB:c.130G>T), IVS1-1 (HBB:c.92+1G>T), IVS1-5 (HBB:c.92+5G>T), IVS2-5 (HBB:c.315+5G>C), IVS2-654 (HBB:c.316-197C>T), Int (HBB:c.2T>G), CAP (HBB:c.-11_-8delAAAC). A BioMixer™ II Microarray Hybridization Station (CapitalBio, Beijing, China) was used for hybridization after multiplex polymerase chain reaction amplification. Then, chips were scanned using a

LuxScan$^{TM}$ 10K-B Microarray Scanner (CapitalBio, Beijing, China).

To validate β-thal mutations, three fragments of the β-globin gene were amplified. The first fragment was amplified with 5'-CCT AAG CCA GTG CCA GAA GAG C-3' as the forward primer and 5'-TGC CCA GTT TCT ATT GGT CTC C-3' as the reverse primer, the second fragment was amplified with 5'-TAG AAA CTG GGC ATG TGG AG-3' as the forward primer and 5'-TGT ACC CTG TTA CTT ATC CC-3' as the reverse primer, and the third fragment was amplified with 5'-TCA GGG CAA TAA TGA TAC AA-3' as the forward primer and 5'-TTA GTA GTT GGA CTT AGG GA-3' as the reverse primer. The fragments were sequenced using a 3500 Genetic Analyser (Applied Biosystems, Foster City, CA, USA).

Moreover, we also confirmed three α-thal deletions [i.e., the Southeast Asian deletion (--$^{SEA}$), rightward deletion (-α$^{3.7}$), and leftward deletion (-α$^{4.2}$)] via multiplex gap-polymerase chain reaction assays. Primers and PCR conditions were designed as described in classical literatures [11,12].

## Statistical analysis

A statistical analysis was carried out using SAS for Windows (version 9.2; SAS Institute, Cary, NC, USA). All quantitative traits were tested for normality, and skewed quantitative traits were logarithmically transformed to approximate univariate normality. The data are shown as the means ± standard deviation (SD). The quantitative traits (RBC, Hb, MCV, MCH, RDW) were compared between two groups by using Wilcoxon test, and ANOVA tests were performed for comparing the differences in three subgroups of thalassaemia carriers (α-Thal, β-Thal and αβ-Thal).

Two-tailed statistical significance was considered at $p<0.05$.

# RESULTS

## Mutations identified in Jino

Due to mutations in different globin genes, we observed three groups of thalassaemic carriers, including individuals with only α-thal gene deletions or β-thal gene mutations and individuals with combined αβ-thalassaemia (αβ-thal) gene mutations. Four different thalassaemia mutations were detected in 203 individuals among 363 suspected cases. No mutations were observed in 50 individuals with negative primary screening results. Table 2 shows the allele frequency of α- and β-thalassaemia mutations found in our study.

**Table 2. Allele frequency of α- and β-Thalassaemia mutations found in our study.**

| Mutation | Phenotype | n | Number of Alleles | Allele Frequency (ratio) |
|---|---|---|---|---|
| α-thalassaemia | | | | |
| $--^{SEA}$ | $\alpha^0/\alpha$ | 42 | 42 | 70.00% (42/60) |
| $-\alpha^{3.7}$ | $\alpha^+/\alpha$ | 16 | 16 | 30.00% (18/60) |
| $-\alpha^{3.7}/-\alpha^{3.7}$ | $\alpha^+/\alpha^+$ | 1 | 2 | — |
| β-thalassaemia | | | | |
| CD17 | $\beta^0/\beta^A$ | 20 | 20 | 12.35% (20/162) |
| HbE | $\beta^E/\beta^A$ | 132 | 132 | 87.65% (142/162) |
| HbE/HbE | $\beta^E/\beta^E$ | 5 | 10 | — |

α: the normal α-globin chain; $\alpha^0$: the α-globin chain is totally deletion; $\alpha^+$: the α-globin chain is partly deletion; $\beta^A$: the normal β-globin chain; $\beta^E$: the abnormal β-globin chain of HbE mutation; $\beta^0$: the β-globin chain is totally deletion.

## Mutations in the α-thal gene

None of the three common nondeletional $\alpha^{+}$-thal mutations, Hb Constant Spring (HBA2:c.427T > C), Hb Quong Sze [HBA2:c.377T > C (or HBA1)] and Hb Westmead (HBA2:c.369C>G), were found in 203 participants with thalassaemia mutations. Forty-six of 203 participants carried α-thal deletions only; $--^{SEA}$ and $-\alpha^{3.7}$ were observed, accounting for 16.7% (34/203) and 5.9% (12/203) of the mutations, respectively. Among these individuals, we identified both $\alpha^{+}/\alpha$ and $\alpha^{+}/\alpha^{+}$ for $-\alpha^{3.7}$ and $\alpha^{0}/\alpha$ for $--^{SEA}$ (gel electrophoresis of PCR amplifying results are shown in Fig. 2). However, no $-\alpha^{4.2}$ deletion was observed.

## Mutations in the β-thal gene

We observed mutations in CD17 (HBB:c.52A > T) (Fig. 3) and CD26 (HbE or HBB:c.79G>A) (Fig. 4). CD17, which accounted for 9.9% (20/203) of mutations, was found to be $\beta^{0}/\beta^{A}$ in this population. Participants with HbE variant only, either $\beta^{E}/\beta^{A}$ or $\beta^{E}/\beta^{E}$, accounted for 61.1% (124/203) of mutations. Furthermore, 13 HbE carriers harboured $--^{SEA}$ (n=8) or $-\alpha^{3.7}$ (n=5) at a combined frequency of 6.4% (13/203).

## Haematological features of different thalassaemia genotypes

The haematological data of different thalassaemia genotypes are summarized in Table 3. Compared with normal individuals, thalassaemic carriers had significantly lower Hb, MCV and MCH levels ($p$<0.001, respectively) and higher RBC and RDW levels ($p$<0.001, respectively). Furthermore, we compared the differences in those five indexes among three groups of carriers (α-thal, β-thal and αβ-thal). Significant

**Table 3. Haematological data of 1,613 Jino ethnic minority individuals with different thalassaemia subtypes.**

| Thalassaemia types | n | RBC($10^{12}$/L) | Hb(g/dL) | MCV(fL) | MCH(pg) | RDW(%) |
|---|---|---|---|---|---|---|
| α-Thalassaemia | 46 | 5.63 ± 0.78 | 13.03 ± 1.61 | 68.99 ± 6.50 | 23.33 ± 2.45 | 13.64 ± 1.38 |
| $\alpha^0/\alpha$ | 34 | 5.88 ± 0.68 | 12.98 ± 1.62 | 65.69 ± 2.65 | 22.08 ± 0.81 | 14.01 ± 1.37 |
| $\alpha^+/\alpha$ | 11 | 4.86 ± 0.58 | 13.19 ± 1.70 | 78.96 ± 4.43 | 27.14 ± 1.87 | 12.52 ± 0.74 |
| $\alpha^+/\alpha^+$ | 1 | 5.34 | 12.7 | 71.5 | 23.8 | 13.1 |
| β-Thalassaemia | 144 | 5.27 ± 0.48 | 13.31 ± 1.70 | 72.46 ± 7.09 | 25.29 ± 2.75 | 13.21 ± 0.96 |
| $\beta^0/\beta^A$ | 20 | 5.41 ± 0.38 | 10.68 ± 0.53 | 58.70 ± 2.31 | 19.77 ± 0.83 | 14.23 ± 0.40 |
| $\beta^E/\beta^A$ | 120 | 5.23 ± 0.48 | 13.77 ± 1.41 | 75.17 ± 3.88 | 26.34 ± 1.49 | 12.98 ± 0.85 |
| $\beta^E/\beta^E$ | 4 | 5.91 ± 0.51 | 12.55 ± 1.22 | 60.23 ± 3.61 | 21.23 ± 1.15 | 15.00 ± 0.57 |
| αβ-Thalassaemia | 13 | 5.67 ± 0.70 | 13.63 ± 1.67 | 69.40 ± 6.85 | 24.17 ± 2.59 | 13.12 ± 0.92 |
| $\beta^E/\beta^A$ with $\alpha^0/\alpha$ | 7 | 6.02 ± 0.69 | 13.57 ± 1.58 | 65.94 ± 2.82 | 22.57 ± 1.07 | 13.66 ± 0.71 |
| $\beta^E/\beta^A$ with $\alpha^+/\alpha$ | 5 | 5.21 ± 0.53 | 14.08 ± 1.88 | 76.46 ± 3.86 | 27.00 ± 1.33 | 12.22 ± 0.36 |
| $\beta^E/\beta^E$ with $\beta^0/\beta^A$ | 1 | 5.57 | 13.8 | 58.3 | 21.2 | 11.8 |
| Total thalassaemia | 203 | 5.38 ± 0.60 | 13.27 ± 1.68 | 71.48 ± 7.08 | 24.77 ± 2.79 | 13.30 ± 1.08 |
| Non-thalassaemia | 1410 | 4.78 ± 0.51 | 14.21 ± 1.67 | 85.14 ± 6.64 | 29.85 ± 2.79 | 12.68 ± 1.30 |
| $P^a$ value | | <0.001 | <0.001 | <0.001 | <0.001 | <0.001 |
| $P^b$ value | | 0.0012 | >0.05 | 0.0111 | 0.0002 | 0.0573 |

The data are shown as the n, means ± SD, medians (interquartile range) or raw data when necessary; RBC: red blood cell; Hb: haemoglobin; MCV: mean

corpuscular volume; MCH: mean corpuscular haemoglobin; RDW: red cell distribution width.

[a]Non-thalassaemic individuals compared with thalassaemia group.

[b]Compare among three subgroups of thalassaemia (α-Thal, β-Thal and αβ-Thal).

differences in MCV ($p$=0.0111), MCH ($p$=0.0002) and RBC ($p$=0.0012) were observed between those groups. MCV and MCH levels in the α-thal group were significantly lower than those in the β-thal group ($p$<0.05), whereas RBC levels were higher ($p$<0.05). In contrast, no difference was observed between the αβ-thal and α-thal groups/β-thal groups. Moreover, there was a tendency towards increased RDW levels in the α-thal group compared with the β-thal group ($p$=0.0573).

# DISCUSSION

Thalassaemia is a common monogenic disease with a relatively high prevalence in Southeast Asia. In China, this disease is mainly prevalent in areas near the southern bank of the Yangtze River, such as Guangdong, Guangxi, Fujian and Yunnan Provinces [13-15]. Prenatal screening and related molecular diagnoses are crucial for preventing and treating thalassaemia. Many thalassaemia studies have been conducted in Yunnan Province [16, 17]. However, data on the Jino population are limited because this population is the last ethnic minority confirmed in China.

We randomly selected 1,613 Jino adults from eight villages around Jino Mountain in Jinghong, Southern Yunnan. Among the gene mutations identified, the most prevalent α-thal and β-thal genotypes in this region were $--^{SEA}$ and HbE, in agreement with previous data from Yunnan Province [18,19]. According to our results, the overall prevalence of thalassaemia in Jino was nearly 12.6%, which is similar to the prevalence observed in Kunming [20]. Prevalence of αβ-thal (8%) in our population

was equal to that in the Li population in Hainan Province (7.99%), where thalassaemia prevalence is high [21]. Although Yunnan Province has a high prevalence of thalassaemia with diverse genotypes, globin gene mutations spectrum among the Jino population are relatively limited.

HbE, a type of haemoglobinopathy, can be observed in most regions of southeast China [22]. Due to a point mutation in β-globin gene, the balance of various globin products is disrupted, leading to a structural haemoglobin variant. Although HbE carriers may only have slight anaemia, their offspring will exhibit severe clinical symptoms in the presence of other β-thal types [23]. Therefore, potential HbE carriers should undergo genetic testing and prenatal counselling. HPLC is often used as an efficient primary screening method to detect abnormal Hb, as was done in this study and previous studies [24,25]. In our study, 95.6% (131/137) of HbE carriers were identified by HPLC, and 13 of these individuals had concomitant α-thal deletions.

Different genotypes lead to different clinical phenotypes [26]. We found that thalassaemic carriers had significantly lower MCV and MCH levels. Regarding those with β-thal mutations, MCV and MCH levels were significantly decreased in CD17 carriers compared with HbE carriers, suggesting that a nonsense mutation in the β-globin gene causes greater erythrocyte impairment. Hypochromic microcytic anaemia was moderate in individuals with $\beta^E/\beta^A$ and $\alpha^+/\alpha$ compared with $\beta^E/\beta^A$ carriers. This paradox may be explained by the fact that changes in the α- and β-globin chains could balance each other out when both mutations coexist in an individual. Accordingly, rapidly estimating the genetic state of an illness based on haematological parameters is difficult. Therefore, genetic screening of both α- and β-globin gene mutations in potential parents is of utmost importance to prevent births with severe defects [27].

However, there are some limitations in this study. First, the sample size we used in the genetic testing was relatively small and may not have the validity to identify the rare thalassaemia variants from this ethnic group. Second, investigations of population and family structure were not performed in this study, though α-thal and β-thal gene mutations were common among Jino ethnic minority. As a result, further studies about the inbreeding levels and consanguinity structure are warranted to reveal the underlying mechanism of gene flow and then assess the occurrence and persistence of α-thal and β-thal gene mutations, especially co-existing α-thal and β-thal gene mutations within Jino individuals.

In conclusion, this study revealed α- and β-thalassaemia mutations in the Jino ethnic minority population in Yunnan Province. Of these mutations, $--^{SEA}$ and HbE were the most prevalent α-thal and β-thal gene mutation types. In addition, data based on clinical haematological parameter analysis indicated that the severity of hypochromic microcytic anaemia is associated with the genotype of thalassaemia. Our results provide evidence that may be useful for further genetic counselling, prenatal screening and clinical diagnosis of thalassaemia in this region.

# Contributors

WJ and CH conceived and designed the experiments. SW and RZ performed the experiments and analyzed the data. GX, YL, XH, Fusong Jiang and Feng Jiang contributed materials and analysis tools. SW prepared the article. CH and WJ revised the manuscript. All the authors have read and approved the final version of this manuscript.

# Acknowledgements

We thank all of the participants for their dedication. We acknowledge the skillful technical support of all nursing and medical staff at the Shanghai Clinical Center for Diabetes.

## Funding

This work was financially supported by a grant from the National Natural Science Foundation of China (91331110).

## Competing interests

No, there are no competing interests.

## Ethics approval

According to the Helsinki Declaration II, ethical approval for the study was granted by the Institutional Review Board of Shanghai Jiao Tong University affiliated with the Sixth People's Hospital, Shanghai, China. Written and Oral informed consent was obtained from all participants.

## Data sharing statement

No additional data are available.

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# Figure legends

**Fig. 1: Geographical location of the Jino ethnic minority populations in Yunnan Province, Southwest China.** The solid black triangle represents Jino Mountain. The solid black circles represent the eight villages in Jinghong where the 1,613 subjects were randomly selected.

**Fig. 2: Gel electrophoresis of PCR amplify in α-thal deletions.** M: marker, 200 bp DNA Ladder; Lane 1: rightward deletion (genotype of $-\alpha^{3.7}/-\alpha^{3.7}$); Lane 2 and Lane 3: rightward deletion (genotype of $-\alpha^{3.7}/\alpha\alpha$); Lane 4 and Lane 5: Southeast Asia deletion (genotype of $--^{SEA}/\alpha\alpha$).

**Fig. 3: Heterozygous CD17 (A>T) mutation (a) and the corresponding normal sequence of β-globin.** Red arrows indicate the position of this point mutation.

**Fig. 4: Heterozygous CD26 (G>A) mutation (a) and the corresponding normal sequence of β-globin.** Red arrows indicate the position of this point mutation.

# Table legends

**Table 1.** Haematological and demographic characteristics of 1,613 Jino ethnic minority adults included in the study.

**Table 2.** Allele frequency of α- and β-Thalassaemia mutations found in our study.

**Table 3.** Haematological data of 1,613 Jino ethnic minority individuals with different thalassaemia subtypes.

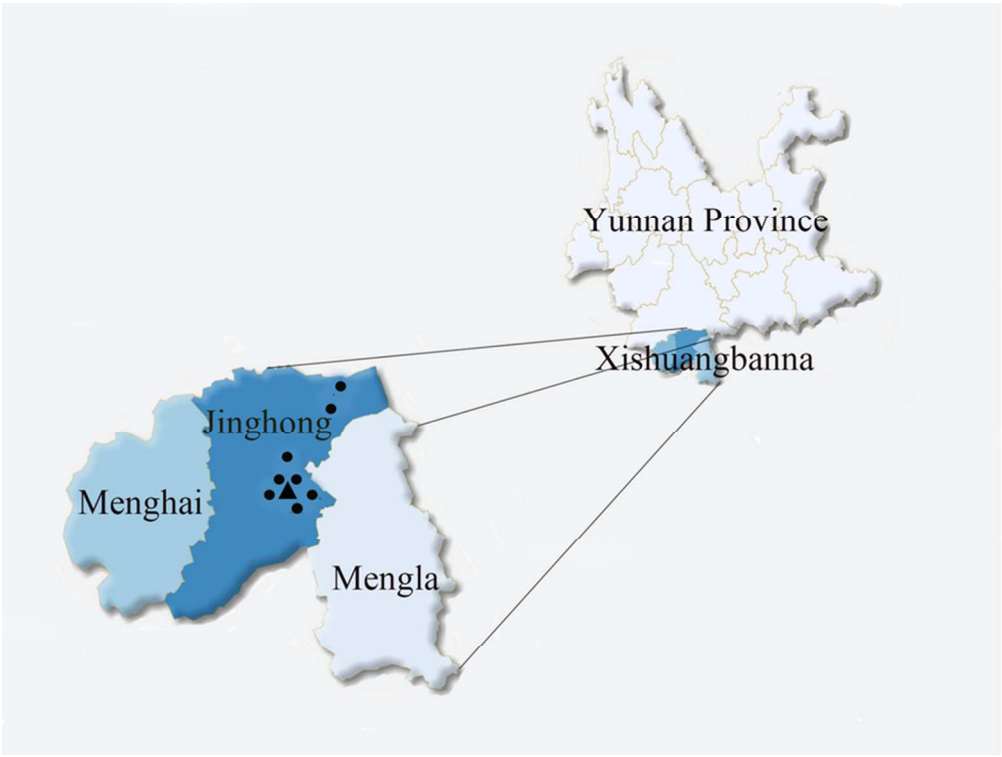


Fig. 1: Geographical location of the Jino ethnic minority populations in Yunnan Province, Southwest China. The solid black triangle represents Jino Mountain. The solid black circles represent the eight villages in Jinghong where the 1,613 subjects were randomly selected.
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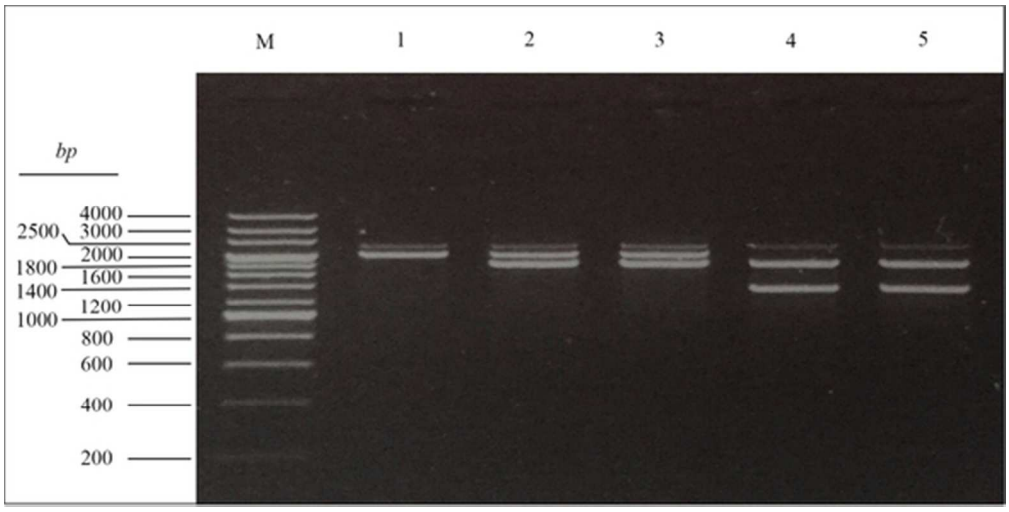


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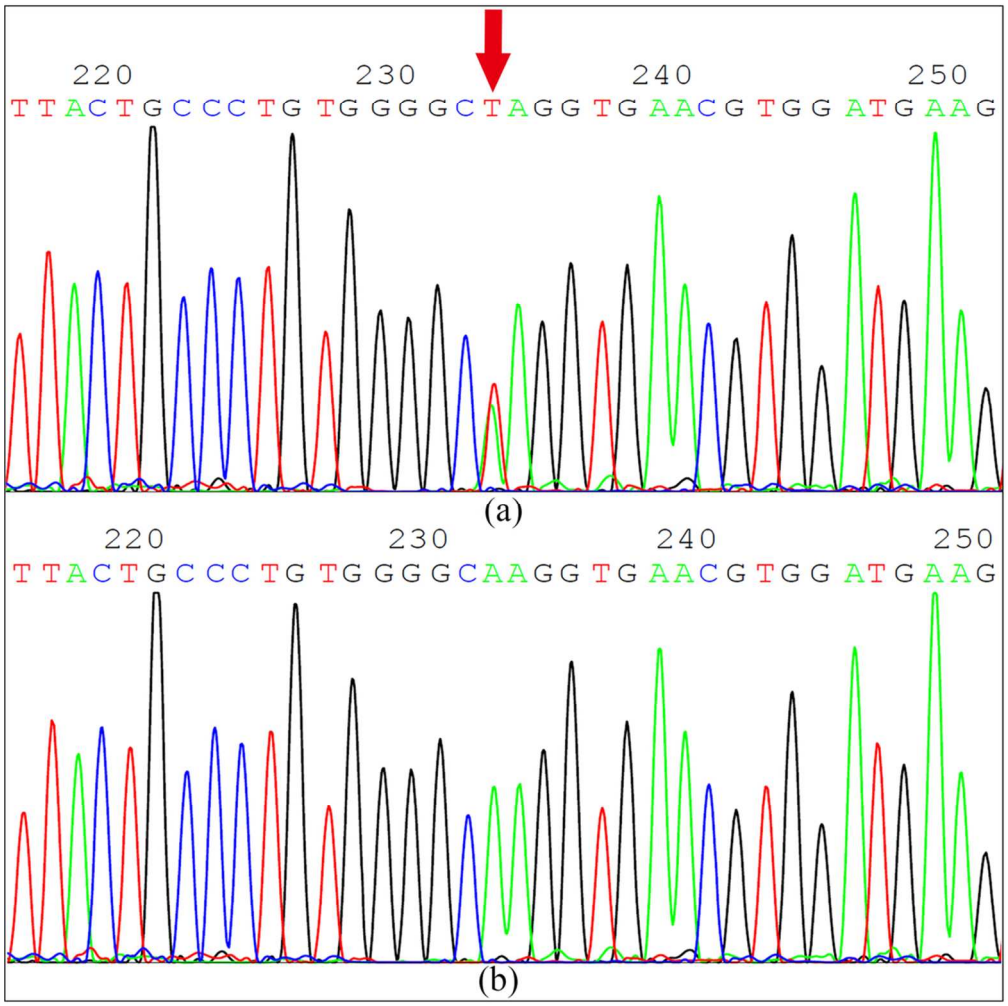


Fig. 3: Heterozygous CD17 (A>T) mutation (a) and the corresponding normal sequence of β-globin. Red arrows indicate the position of this point mutation.
99x99mm (300 x 300 DPI)

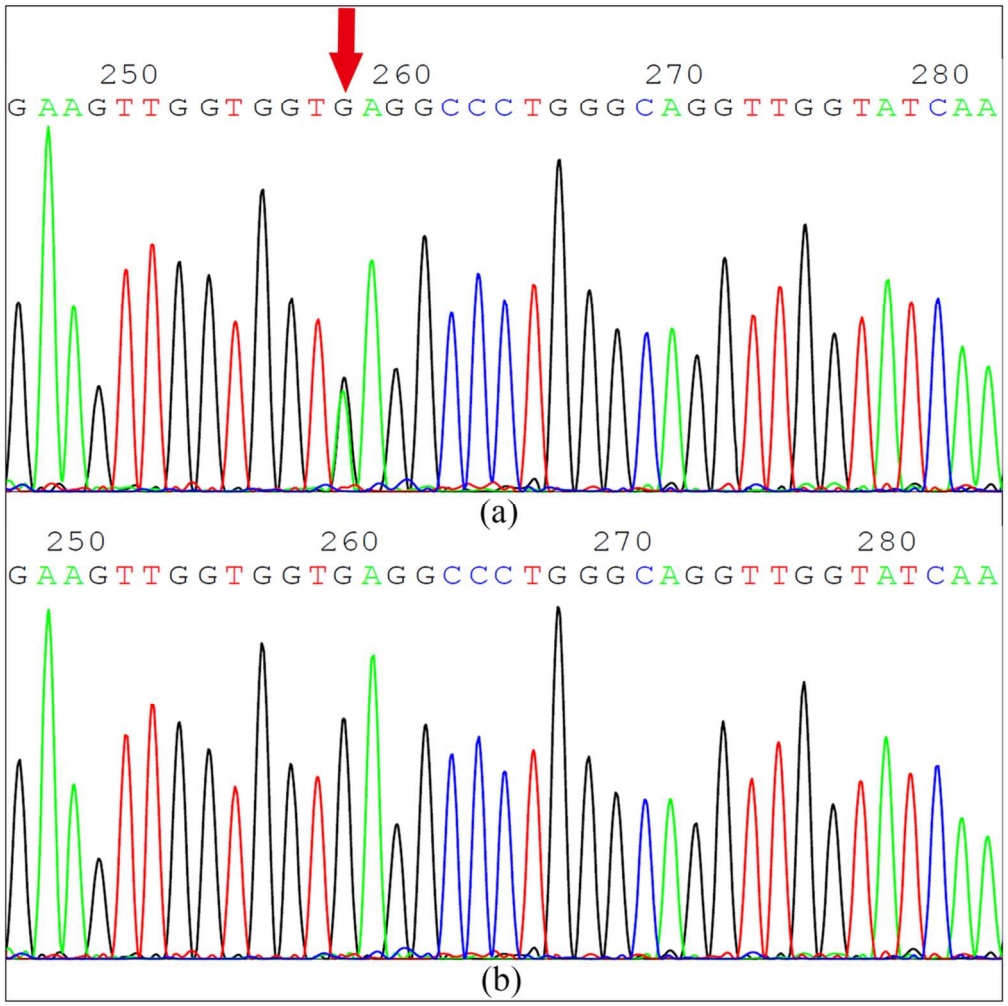


Fig. 4: Heterozygous CD26 (G>A) mutation (a) and the corresponding normal sequence of β-globin. Red arrows indicate the position of this point mutation.
99x99mm (300 x 300 DPI)