

Combined use of gap-PCR and next-generation sequencing improves thalassaemia carrier screening among premarital adults in China

Jianghong Zhao 💿 ,¹ Jia Li,² Qiaohong Lai,¹ Yanping Yu¹

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¹Department of Obstetrics and Gynecology, Xiaolan Hospital Affiliated to Southern Medical University, Zhongshan, Guangdong province, China ²Department of Thyroid and Breast, Shanghai Tenth People's Hospital, Shanghai, China

Correspondence to

Dr Jianghong Zhao, Department of Obstetrics and Gynecology, Southern Medical University, Zhongshan, Guangdong province, China; huangbuoying@163.com and Dr Yanping Yu, Department of Obstetrics and Gynecology, Xiaolan Hospital Affiliated to Southern Medical University, Zhongshan, Guangdong province, China; hbjmsr@163. com

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ABSTRACT

Aims Thalassaemia is one of the most common genetics disorders in the world, especially in southern China. The aim of the present study was to investigate the feasibility of combining the gap-PCR and next-generation sequencing (NGS) for thalassaemia carrier screening in the Chinese population.

Methods Blood samples were obtained from 944 prepregnancy couples; thalassaemia carrier screening was performed by using a routine haematological method and a combination of gap-PCR and NGS method.

Results We found that the α thalassaemia carrier rate was 11% (207/1888); the β thalassaemia carrier rate was 3.7% (70/1888); the composite α thalassaemia and β thalassaemia carrier rate was 0.4% (8/1888). We also identified seven novel mutations, including *HBA1*: c.412A>G, -50 (G>A), *HBB*: c.*+129T>A, *HBB*: c.-64G>C, *HBB*: c.-180G>C, *HBB*: c.*+5G>A and *HBB*: c.-113A>G. By comparing the combined gap-PCR and NGS method, the MCV+MCH and HbA2 detection strategy showed a lower sensitivity of 61.05% (105/172) and a higher missed diagnosis ratio of 38.95% (67/172) for α thalassaemia mutations. The sensitivity was improved with the MCV+MCH and HbA2 detection screen when compared with MCV+MCH detection for β thalassaemia (98.51% vs 85.90%).

Conclusions Our study suggests the combined gap-PCR and NGS method is a cost-effective method for the thalassaemia carrier screening, particularly for the α thalassaemia mutation carriers.

INTRODUCTION

Thalassaemia is one of the most common genetic disorders in the world and is caused by α -globin and β -globin gene mutations. Thalassaemia is distributed mainly in coastal areas of the Mediterranean sea, Africa, the Middle East, India and southeastern Asia.¹ In China, thalassaemia is mainly distributed in southern China, particularly in Guangxi, Guangdong and Hainan provinces.^{2–4} Most patients with severe thalassaemia may die in utero or during early childhood if untreated. Thus, effective preconception screening is essential for reducing the incidence of thalassaemia in high-prevalence regions of thalassaemia.

Over 1530 mutations ranging from singlenucleotide variations to large rearrangements are associated with thalassaemia or abnormal haemoglobin variants (HbVar database for human HbVar and thalassaemia mutations). Moreover, several variants at various loci have been identified to modify β thalassaemia phenotypes.⁵⁻⁷ The traditional method uses haematological and biochemical tests and subsequent molecular genetic tests to screen for thalassaemia carriers with phenotypic traits associated with thalassaemia.^{1 8} Whereas a phenotypic screening approach has a certain weakness in carrier testing, many carriers who have normal or borderline red cell indices and/or HbA2 levels may be missed.^{8 9}

Next-generation sequencing (NGS) allows for multiplex and high-throughput detection of genetic variants¹⁰ and is commonly used for diagnosis of genetic disorders.^{11 12} Two recent studies have shown that NGS largely improved thalassaemia carrier screening and had a higher sensitivity and specificity than traditional methods; however, these results might be limited to certain ethnic population or rely only on the NGS technique.^{13 14} In the present study, we applied the gap-PCR and NGS techniques to test more than 300 mutations associated with thalassaemia in a cohort of 944 couples pretyped by routine screening methods. Our results suggested that the combined method outperformed traditional methods in the detection of pathogenic or likely pathogenic variants, thus providing an effective platform for application in clinical management of monogenic diseases in a large-scale population.

MATERIALS AND METHODS Samples and demographic data

A total of 944 prepregnancy couples between August 2017 and August 2018 were screened for thalassaemia mutations in Xiaolan People's Hospital of Zhongshan. Their age ranged from 23 to 40 years. All individuals provided informed written consent.

Haematological phenotype analysis

We collected 8 mL blood samples from each individual for routine blood examinations. An automated haematology analyser was used to detect mean corpuscular volume (MCV) and mean corpuscular haemoglobin (MCH). Participants with an MCV of <82 fL or an MCH of <27 pg underwent Hemoglobin A2 (HbA2) test by the alkaline agarose gel electrophoresis (Helena Spife 3000). An HbA2 value between 2.5% and 3.5% was considered normal. When HbA2 was $\leq 2.5\%$, α thalassaemia was suspected, whereas when HbA2 was $\geq 3.5\%$, β thalassaemia was considered.¹³

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Reverse dot blot hybridisation (RDB)

Suspected α thalassaemia and β thalassaemia carriers with positive HbA2 results underwent routine genotyping tests. RDB was performed to detect common α thalassaemia or β thalassaemia mutations, including non-deletion-type thalassemia hemoglobin Constant Spring (CS) (HBA2: c.427T>C), Quong Sze (QS) (HBA2: c.377T>C), Westmead (WS) (HBA2: c.369C>G) and 17 β thalassaemia mutations, Int (*HBB*: c.2T>G), CD41-42 (HBB: c.124 127delTTCT), CD31(HBB: c.94delC), CD14-15 (HBB: c.45 46insG), CD17 (HBB: c.52A>T), CD71-72 (HBB: c.216 217insA), IVS-I-1 (HBB: c.92+1G>T), CD43 (HBB: c.130G>T), CD27/28 (HBB: c.84 85insC), IVS-II-654 (HBB: c.316-197C>T), -28 (HBB: c.-78A>G), -29 (HBB: c.-79A>G), IVS-1-5 (HBB: c.92+5G>C), BE (HBB: c.79G>A), -32 (HBB: c.-82C>A), -30 (HBB: c.-80T>C) and HBB: c.-50A>C. In the RDB assay, the amplified target DNA is first fixed to a nylon membrane to form a filter-fixed DNA dot; the dot is then hybridised to allele-specific oligonucleotide (ASO) probes whose 5' terminal was conjugated with either P-labelled deoxynucleoside triphosphates, biotin or horseradish peroxidase. All procedures of RDB were conducted following the kit's instructions (YanengBio, Shenzhen, China).

Gap-PCR tests and NGS screening

As for suspected α thalassaemia and β thalassaemia carriers, multiplex gap-PCR was used to detect deletion-type α thalas-saemia $\alpha^{3.7}$, $-\alpha^{4.2}$, $-{}^{\text{SEA}}$, $-{}^{\text{FIL}}$ and $-{}^{\text{THAI}}$ and β thalassaemia deletions (Chinese Ggamma (Agammadeltabeta) 0, South-East Asia type hereditary persistence of fetal hemoglobin (SEA-HPFH) and Taiwanese). NGS was applied to detect all mutations in HBA1, HBA2 and HBB genes.

Genomic DNA was extracted from 200 µL blood samples using the Kingfisher Flex (Thermo Scientific, Rockford, Illinois, USA) and isolated using the GenMag Nucleic Acid Isolation kit (Magnetic bead method) (GenMagBio, Beijing, China). DNA extracts were arrayed in 96-well plates, and the concentration was quantified by Nanodrop-8000 (Thermo Scientific, Waltham, MA, USA). We restricted our analysis to samples with a DNA concentration $>20 \,\mu$ g/mL and an A260:A280 ratio between 1.8 and 2.0. We designed forward primers and specific reverse primers to amplify HBA1, HBA2 and HBB genes. PCR indexes were used to distinguish the amplified products. The primers used in this study came from a published article¹⁵ and patent (patent ID CN108796054A). The HBA1 amplicon size was 919 bp; the HBA2 amplicon size was 914 bp; and the HBB amplicon size was 599 and 771 bp. PCR reactions were performed in 96-well plates, with each sample corresponding to one library. Ninety-six kinds of index sequences were designed, corresponding to each well of the plate. Ninety-six samples were pooled into one tube for further library construction. The detailed experimental protocol was described in a previous study.^{13 15} ABI 9700 Thermal Cycler (Life Technologies, Foster City, California, USA) was used in PCR reactions. We adopted NGS library preparation protocol for library construction, including purified genomic DNA (Qiagen DNA Purification kit, Hilden, Germany), DNA quantification (NanoDrop 8000 UV-Vis Spectrophotometer, Thermo Fisher Scientific, Waltham, MA, USA), DNA fragmentation (excluding copy number variation amplicons), blunt-ended fragmentation (Enzymatics kits, Qiagen, Hilden, Germany), 3'-dA overhang, paired-end adapters ligation, DNA fragment separation (copy number variations not included) and size selection using magnetic beads. Sequencing was performed using the paired-end tag (PE100)

protocol with an BGISEQ-500 machine (Beijing Genomics Institute, shenzhen, China).

Bioinformatics analyses

We identified α thalassaemia and β thalassaemia deletion genotypes according to the expected amplicon sizes for the deletion junction fragments by agarose gel electrophoresis. For transcripts used to call genetic variants, we required that the sequence depth should be greater than $50 \times$. We developed a bioinformatics pipeline to detect Hb gene point mutations. First, we Protected filtered low-quality reads and classified remaining reads based on adapter information (index primer). Then, we aligned reads to target region reference using BWA software¹⁶ and obtained consensus sequence by SAMtools.¹⁷ Coverage, depth and length were recorded for each consensus using ReSeqTools.¹⁸ Finally, Z were recorded for each consensus using ReseqTools.³⁵ Finally, single-nucleotide variation and indel results were filtered based on sequencing quality and depth. Mutation types were annotated according to the HbVar database of human HbVar and thalassaemia mutations. **Validation of NGS methods** We applied two different strategies to validate the gap-PCR and NGS approach for detection of thalassaemia carriers. First, we

ð compared four α thalassaemia mutations and eight β thalassaemia mutations detected by NGS with the Sanger sequencing uses related results. The mutations analysed included α thalassaemia mutations Hemoglobin (Hb) WS, Hb CS, Hb QS and alpha2 codon 30 del GAG and β thalassaemia mutations Hb E, codons 41/42 (-TTCT), IVS-II-654 (C>T), codon 17 (A>T), -28 (A>G), -50 (G>A), codons 71/72 (+A) and codon 43 (G>T). Second, to text we compared thalassaemia mutations detected by gap-PCR and NGS with routine genotyping tests (the gap-PCR and RDB method) in 184 individuals with positive HbA2 results.

RESULTS

NGS methodological validation

and data mining, A We initially performed the validation experiment by comparing results generated by NGS and Sanger sequencing independently. Sanger sequencing confirmed 4 α thalassaemia mutations and 8 β thalassaemia mutations detected as positive by NGS results from l training, 12 samples were found to completely match the NGS results (online supplementary figures 1 and 2). Then, among the 184 individuals who underwent routine genotyping and gap-PCR , and similar technologies and NGS tests, 152 showed positive α thalassaemia mutations and β thalassaemia mutations. Thalassaemia mutations detected by the gap-PCR and NGS were all successfully confirmed by gap-PCR and RDB (online supplementary table S1).

Combined gap-PCR and NGS screening reveals thalassaemia mutation carriers

We applied the combined gap-PCR and NGS method to 944 couples and found that 15.1% (285/1888) of couples were thalassaemia mutation carriers. Of these, the α thalassaemia carrier rate was 11% (207/1888); the β thalassaemia carrier rate was 3.7% (70/1888); and the composite α thalassaemia and β thalassaemia carrier rate was 0.4% (8/1888). Moreover, 2.9% (27/944) of couples were both estimated as thalassaemia mutation carriers. We identified 20 distinct genotypes of α thalassaemia mutations (online supplementary table S2 and table 1). Among these genotypes, $-\frac{SEA}{\alpha\alpha}$ was the most common genotype and accounted for 44.4% of all cases (92/207). $-\alpha^{3.7}/\alpha\alpha$ was the second most common genotype, with an incidence rate of 25.6% (53/207). In addition, we identified 12 carriers with rare

Table 1 α -Globin gene mutation spectrum					
Mutation type	HGVS name	Number	Ratio (%)	Class	
SEA	NG_000006.1:g.26264_ 45564del19301	96	42.1	Common	
-α ^{3.7}	NG_000006.1:g.34164_ 37967del3804	61	26.8	Common	
Hb Westmead	<i>HBA2</i> : c.369C>G	24	10.5	Common	
-α ^{4.2}	NA	20	8.8	Common	
Hb Constant Spring	<i>HBA2</i> : c.427T>C	9	3.9	Common	
Hb Quong Sze	<i>HBA2</i> : c.377T>C	4	1.8	Common	
Hb G-Honolulu	<i>HBA2</i> : c.91G>C	4	1.8	Rare	
Hb Hekinan II	<i>HBA1</i> : c.84G>T	3	1.3	Rare	
HBA2: c.46G>A	<i>HBA2</i> : c.46G>A	2	0.9	Rare	
Hb Owari	<i>HBA1</i> : c.364G>A	2	0.9	Rare	
HBA2: c.190G>A	<i>HBA2</i> : c.190G>A	1	0.4	Rare	
HBA1: c.412A>G	<i>HBA1</i> : c.412A>G	1	0.4	Novel	
Alpha2 codon 30 del GAG	<i>HBA2</i> : c.91_93delGAG	1	0.4	Rare	
Total		228	100		

genotypes, such as Hb Hekinan II and Hb Owari. We identified 14 distinct genotypes of B thalassaemia mutations (online supplementary table S3 and table 1). The most common genotypes were codons 41/42 (-TTCT) (34.3%, 24/70) and IVS-II-654 (C>T) (15.7%, 11/70). We also identified 14 β thalassaemia carriers with rare genotypes which were not detected in previous studies. 13 19 20 For example, we found that five β thalassaemia carriers had a -50 (G>A) mutation. In addition, we identified eight composite α thalassaemia and β thalassaemia carriers (online supplementary table S4). Of these, two cases showed a composite $-\alpha^{3.7}/\alpha\alpha$ and codons 41/42 (-TTCT) genotype, and remaining cases showed a unique genotype (online supplementary table S4 and table 2).

Mutation spectrum of α -globin and β -globin genes

We identified 13 distinct mutations in α -globin genes (table 1). Most recurrent mutations were consistent with the report of Guangdong province.²⁰ The most recurrent two mutations were -^{SEA} and $-\alpha^{3.7}$, accounting for approximately 68.9% of all mutations (157/228). Unexpectedly, we noted that the mutation rate of Hb Westmead was higher than $-\alpha^{4.2}$ in Zhongshan city, which

Table 2 β-Globin gene mutation spectrum					
Mutation type	HGVS name	Number	Ratio (%)	Class	
Codons 41/42 (-TTCT)	HBB: c.124_127delTTCT	29	35.8	Common	
IVS-II-654 (C>T)	HBB: c.316–197C>T	14	17.3	Common	
Codon 17 (A>T)	<i>HBB</i> : c.52A>T	8	9.9	Common	
-28 (A>G)	<i>HBB</i> : c78A>G	7	8.6	Common	
-50 (G>A)	<i>HBB</i> : c100G>A	5	6.2	Novel	
Hb E	<i>HBB</i> : c.79G>A	4	4.9	Common	
<i>HBB</i> : c.*+129T>A	<i>HBB</i> : c.*+129T>A	3	3.7	Novel	
Codons 71/72 (+A)	HBB: c.216_217insA	3	3.7	Common	
Codon 43 (G>T)	<i>HBB</i> : c.130G>T	2	2.5	Common	
<i>HBB</i> : c64G>C	<i>HBB</i> : c64G>C	2	2.5	Novel	
<i>HBB</i> : c180G>C	<i>HBB</i> : c180G>C	1	1.2	Novel	
<i>HBB</i> : c.*+5G>A	<i>HBB</i> : c.*+5G>A	1	1.2	Novel	
Hb New York	<i>HBB</i> : c.341T>A	1	1.2	Rare	
HBB: c113A>G	<i>HBB</i> : c113A>G	1	1.2	Novel	
Total		81	100		



Figure 1 Diagram of work flow and screening results. MCH, mean corpuscular haemoglobin: MCV, mean corpuscular volume: NGS, nextgeneration sequencing; RDB, reverse dot blot hybridisation.

was discordant with that of Guangdong province.²⁰ We additionally identified seven rare mutations, such as Hb Hekinan II, Hb G-Honolulu and Hb Owari, accounting for 6.1% (14/228) of all mutations. Of these, the HBA1: c.412A>G variant was first identified as a novel α -globin gene mutation.

, AI training, and We identified 14 distinct mutations in β -globin genes (table 2). Among these mutations, codons 41/42 (-TTCT) and IVS-II-654 (C>T) were most prevalent, accounting for 53.1% (43/81) of all mutations. This was consistent with previous reports.¹⁹ We additionally identified seven rare mutations, such as -50 (G>A) and Hb New York, accounting for 17.3% (14/81) of all mutations. Of these, -50 (G>A), HBB: c.*+129T>A, HBB: c.-64G>C, HBB: c.-180G>C, HBB: c.*+5G>A and HBB: c.-113A>G were identified as β -globin gene mutations.

Comparison analysis of traditional methods and the combined method

Based on MCV and MCH (MM) metrics, 18% (340/1888) of the people showed an abnormal haematological phenotype. Of these, 339 individuals further had a haemoglobin electrophoresis test based on data availability. As a result, 120 and 64 individuals were suspected of being the α thalassaemia and β thalassaemia carriers, respectively (figure 1). We estimated the detection rates of α thalassaemia mutations to be 73.49% by MM (158/215) and

65.12% (112/172) by haemoglobin electrophoresis. The detection rates of B thalassaemia mutations by MM were 85.90% (67/78) and 95.65% (66/69) by haemoglobin electrophoresis (online supplementary table S5). Compared with the combined

Table 3 Sensiti	Table 3 Sensitivity and specificity of MCV+MCH and HbA2 levels for thalassaemia carriers screened by NGS							
MM and HbA2	Sensitivity (95% CI)	Specificity (95% Cl)	PPV (95% CI)	NPV (95% CI)	True positive	False positive	True negative	False negative
α thalassaemia	61.05% (53.33% to 68.38%)	87.65 % (84.05% to 90.70%)	67.74% (61.21% to 73.64%)	84.12 % (81.41% to 86.51%)	105	50	355	67
αα/SEA	86.81% (78.10% to 93.00%)	84.36% (80.82% to 87.48%)	50.97% (45.44% to 56.47%)	97.16 % (95.27% to 98.30%)	79	76	410	12
αα/-α3.7	34.21% (19.63% to 51.35%)	73.65% (69.72% to 77.33%)	8.39% (5.45% to 12.70%)	94.08 % (92.62% to 95.26%)	13	142	397	25
αα/-α4.2	37.50% (15.20% to 64.57%)	73.44% (69.58% to 77.05%)	3.87% (2.06% to 7.14%)	97.63 % (96.56% to 98.37%)	6	149	412	10
$\boldsymbol{\beta}$ thalassaemia	98.51% (91.96% to 99.96%)	99.61 % (98.59% to 99.95%)	97.06% (89.22% to 99.25%)	99.8 % (98.64% to 99.97%)	66	2	508	1
Codons 41/42 (- TTCT)	96.55% (82.24% to 99.91%)	92.7 % (90.19% to 94.73%)	41.18% (34.01% to 48.74%)	99.8% (98.67% to 99.97%)	28	40	508	1
IVS-II-654 (C>T)	100.00% (76.84% to 100.00%)	90.41 % (87.67% to 92.71%)	20.59% (16.75% to 25.04%)	100.00 %	14	54	509	0
–28 (A>G)	100.00% (59.04% to 100.00%)	89.3 % (86.47% to 91.71%)	10.29% (8.30% to 12.70%)	100.00 %	7	61	509	0
Codon 17 (A>T)	100.00% (63.06% to 100.00%)	89.46 % (86.64% to 91.86%)	11.76% (9.50% to 14.48%)	100.00%	8	60	509	0

CI, Confidence interval; MCH, mean corpuscular haemoglobin; MCV, mean corpuscular volume; MM, MCV and MCH; NGS, next-generation sequencing; NPV, negative predictive value: PPV. positive predictive value.

method results, the MCV+MCH and HbA2 detection strategy resulted in a lower sensitivity of 61.05% (105/172) and a high missed diagnosis ratio of 38.95% (67/172) for α thalassaemia. The sensitivity improved with the MM and HbA2 detection screen when compared with MM detection for $\boldsymbol{\beta}$ thalassaemia (98.51% vs 85.90%; table 3 and online supplementary tables S5 and S6). Notably, we identified six high-risk couples who have same and abnormal α thalassaemia genotype via NGS. Of these, only three couples were discovered through traditional methods. This demonstrated that the combined approach largely increased the sensitivity and outperformed conventional haematological methods in screening for thalassaemia mutation carriers.

DISCUSSION

In the present study, we revealed that the α thalassaemia carrier rate was 11% and the ß thalassaemia carrier rate was 3.7%, and the composite α thalassaemia and β thalassaemia carrier rate was 0.4% in the Chinese population. The most common α thalassaemia genotype is --SEA/ $\alpha\alpha$, followed by other less common α thalassaemia genotypes, including $-\alpha 3.7/\alpha \alpha$, Hb Westmead and $-\alpha 4.2/\alpha \alpha$. The prevalent genotypes of β thalassaemia are codons 41/42 (-TTCT), IVS-II-654 (C>T) and codon 17 (A>T). Among β -globin mutants, codons 41/42 show a higher carrier frequency than codon 17. All these findings are in line with previous studies.¹³ ¹⁴ Additionally, we detected some rare α -globin and β -globin gene mutations unpublished by previous studies,⁵ ⁶ representing 6.1% and 17.3% of all mutations, respectively. For example, we identified four cases with an Hb G-Honolulu mutation. This mutation was first identified in a Chinese woman in Singapore and was subsequently observed in southern China.²¹ We also identified seven novel mutations, including HBA1: c.412A>G, -50 (G>A), HBB: c.*+129T>A, HBB: c.-64G>C, HBB: c.-180G>C, HBB: c.*+5G>A and HBB: c.-113A>G. Patients with these novel variants showed normal values in haematological indexes. Therefore, it remains to be further elucidated in future studies if these variants are pathogenic

Through comparison analysis, we demonstrated that conventional haematological methods have lower sensitivity, especially

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Protected by copyright, including for uses related for α thalassaemia detection, and a large fraction of thalassaemia variants with clinical significance like --SEA, $-\alpha 3.7$ and $-\alpha 4.2$ were not detected because the patients usually have normal values in MCV, MCH and HbA2 indexes. The sensitivity of MM to text combined with HbA2 improved when compared with MM in the detection for β thalassaemia mutations (92.75% vs 85.90%). These findings are consistent with a previous study.¹³ Although and there are some similar studies on the application of NGS to the detection of thalassaemia mutations in China, He et al's¹³ and detection of thalassaemia mutations in China, He *et al*'s¹³ and a Yao *et al*'s³ studies focused on the spectrum of thalassaemia amutations and evaluated the performance of NGS in screening for thalassaemia mutations in Chinese ethnic minorities. Zhang *et al*'s study²² lacked quantitative measures of the performance \mathbf{Q} of NGS in the general Chinese population. For the first time, ۷ our study provided an in-depth comparison between traditional haematological methods and combined gap-PCR and NGS, which further demonstrated that the combined method outperformed the traditional haematological method in screening for thalassaemia mutations, particularly for α thalassaemia mutation carriers, in the general Chinese population.

The gap-PCR and NGS combined method has various advantages over the traditional haematological screening method. The major factors affecting the application of NGS are the high cost of sequencer, reagent and additional bioinformatics technicians. Whereas the NGS strategy shows a substantial advantage in time and cost in a large-scale population screening, in this study, the NGS had a throughput of 3000 samples per run, and the combined gap-PCR and NGS costs approximately US\$25 for each sample, which is similar to that of the traditional method combined with gap-PCR or PCR-RBD (\$25). Therefore, NGS is cost-efficient to screen for thalassaemia mutations in a largescale population. Third, the combined method detects a wider spectrum of mutations, including common, rare, annotated and novel variants; therefore, it remarkably increases the detection rates of carrier status and therefore improves the detection rate of at-risk couples at a relatively low cost. Given the strengths of the combined gap-PCR and NGS method, we believe it can completely replace the haemoglobin electrophoresis screening

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step. In the prepregnancy or pregnancy screening, if the routine blood tests suggest small cell low-pigment anaemia, the gap-PCR and NGS combined detection should be carried out for the patients. Based on the results of gap-PCR and NGS combined detection, prenatal counselling can be more accurate so as to avoid abortion of fetuses with severe thalassaemia at late pregnancy or before pregnancy.

Though our study demonstrated the combined gap-PCR and NGS method outperformed routine haematological method in the detection of the thalassaemia mutations, it might have certain limitations. First of all, the study population was relatively small and confined to the Zhongshan city from Guangdong province. The results might have regional bias, therefore, further studies are required to validate the findings in a larger sample population from multiple regions of South China. Secondly, the pathogenicity of novel thalassaemia mutations remained unknown; their phenotypic effect should be validated in future studies. In summary, we systematically characterised thalassaemia carrier rate and mutation spectrum of α -globin and β -globin genes using gap-PCR and NGS among Chinese people. Our study has great value for preventing major thalassaemia in China while laying the groundwork for clinical application of the gap-PCR and NGS combined method to thalassaemia carrier screening.

Take home messages

- ► The first comprehensive survey of thalassaemia gene mutations was performed in the Zhongshan region.
- The α thalassaemia carrier rate was 11% (207/1888); the β thalassaemia carrier rate was 3.7% (70/1888); and the composite α thalassaemia and β thalassaemia carrier rate was 0.4% (8/1888). Seven novel mutations were identified, including *HBA1*: c.412A>G, -50 (G>A), *HBB*: c.*+129T>A, *HBB*: c.-64G>C, *HBB*: c.-180G>C, *HBB*: c.*+5G>A and *HBB*: c.-113A>G.
- The combined gap-PCR and NGS method outperformed the MCV+MCH and HbA2 method and MCV+MCH method in the detection of α thalassaemia and β thalassaemia mutations.

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Contributors YY designed the study and wrote the article; JZ and QL performed routine thalassemia analysis; QL performed the specific gap-PCR amplification and the DNA sequencing; JL wrote the manuscript and provided technical support. All authors approved the final manuscript.

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Competing interests None declared.

Patient consent for publication Not required.

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Provenance and peer review Not commissioned; externally peer reviewed.

Data availability statement Data are available upon reasonable request. All data relevant to the study are included in the article or uploaded as supplementary information.

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ORCID iD

Jianghong Zhao http://orcid.org/0000-0002-4074-9476

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2 Supplementary Figure 1. Validation of NGS method for the α-thalassemia mutations Hb Westmead (A),

- 1 Hb Constant Spring (B), Hb Quong Sze (C) and alpha2 Codon 30 del GAG (D). The wildtype (above)
- 2 and mutant (bottom) of each mutation were shown in the red rectangle.





Supplementary Figure2. Validation of NGS method for the β-thalassemia mutations Hb E(A), Codons
41/42 (-TTCT) (B), IVS-II-654 (C>T) (C), Codon 17 (A>T) (D), -28 (A>G) (E), -50 (G>A) (F), Codons
71/72 (+A) (G) and Codon 43 (G>T) (H). The wildtype (above) and mutant (bottom) of each mutation
were shown in the red rectangle.

Case ID	α_Gap-PCR	β_SNP(NGS)	New variants (NGS)	Routine genotyping test	HbA2
Case1	αα/αα	IVS-II-654 (C>T)	Ν	IVS-II-654 (C>T)	5.3
Case2	αα/SEA	Ν	Ν	αα/SEA	2.4
Case3	αα/SEA	Ν	Ν	αα/SEA	2.3
Case4	αα/αα	Codons 41/42 (-TTCT)	Ν	Codons 41/42 (-TTCT)	5.6
Case5	αα/αα	-28 (A>G)	HBA1:c.412A>G(Thr>Ala)	-28 (A>G)	5.2
Case6	αα/-α3.7	Ν	Ν	αα/-α3.7	2.4
Case7	αα/-α4.2	Ν	Ν	αα/-α4.2	2.3
Case8	αα/-α3.7	Codons 41/42 (-TTCT)	Ν	$\alpha\alpha$ /- α 3.7 and Codons 41/42 (-TTCT)	5.8
Case9	αα/SEA	Ν	Ν	αα/SEA	2.1
Case10	αα/αα	-28 (A>G)	Ν	-28 (A>G)	5.2
Case11	αα/SEA	Ν	Ν	αα/SEA	2.2
Case12	αα/αα	Hb E	Ν	Hb E	3.5
Case13	αα/αα	Codons 41/42 (-TTCT)	Ν	Codons 41/42 (-TTCT)	5.4
Case14	αα/SEA	Ν	Ν	αα/SEA	2.3
Case15	αα/αα	IVS-II-654 (C>T)	Ν	IVS-II-654 (C>T)	4.9
Case16	αα/αα	Codon 17 (A>T)	Ν	Codon 17 (A>T)	6.1
Case17	αα/SEA	Ν	Ν	αα/SEA	2.4
Case18	αα/SEA	Ν	Ν	αα/SEA	2.3
Case19	αα/αα	-28 (A>G)	Ν	-28 (A>G)	5.4
Case20	αα/αα	Codons 41/42 (-TTCT)	Ν	Codons 41/42 (-TTCT)	5.4
Case21	αα/SEA	Ν	Ν	αα/SEA	2.1
Case22	αα/SEA	Ν	Ν	αα/SEA	1.8

Table S1. Comparison of thalassemia mutations detected by gap-PCR & NGS with routine genotyping tests (the gap-PCR & RDB method) in 152 individuals with thalassemia mutations.

Case23	αα/αα	Codons 41/42 (-TTCT)	Ν	
Case24	αα/SEA	Ν	Ν	
Case25	αα/-α3.7	Ν	Ν	
Case26	αα/SEA	Ν	Ν	
Case27	αα/αα	Codon 17 (A>T)	Ν	
Case28	αα/SEA	Ν	Ν	
Case29	αα/SEA	Ν	Ν	
Case30	αα/SEA	Codons 41/42 (-TTCT)	Ν	
Case31	αα/SEA	Ν	Ν	
Case32	αα/SEA	Ν	Ν	
Case33	-α4.2/SEA	Ν	Ν	
Case34	αα/αα	IVS-II-654 (C>T)	Ν	
Case35	αα/-α4.2	Ν	Ν	
Case36	αα/SEA	Ν	Ν	
Case37	αα/SEA	Ν	Ν	
Case38	αα/SEA	Ν	Ν	
Case39	αα/SEA	Ν	Ν	
Case40	αα/SEA	Ν	Ν	
Case41	αα/SEA	Ν	Ν	
Case42	αα/αα	Codon 43 (G>T)	Ν	
Case43	αα/SEA	Ν	Ν	
Case44	αα/-α3.7	Ν	Ν	
Case45	αα/αα	Codons 41/42 (-TTCT)	Ν	
Case46	αα/αα	-28 (A>G)	Ν	
Case47	αα/SEA	Ν	Ν	
Case48	αα/αα	Codon 17 (A>T)	Ν	

Codons 41/42 (-TTCT)	5.7
αα/SEA	2.1
Hb Constant Spring (Hb CS)	1.7
αα/SEA	2.2
Codon 17 (A>T)	5.5
αα/SEA	2.1
αα/SEA	2.3
αα/SEA and Codons 41/42 (-TTCT)	
αα/SEA	2.4
αα/SEA	2
-α4.2/SEA	1.3
IVS-II-654 (C>T)	5.1
αα/-α4.2	2.4
αα/SEA	2.4
αα/SEA	2.1
αα/SEA	2.3
αα/SEA	2.4
αα/SEA	2.2
αα/SEA	2.2
Codon 43 (G>T)	4.9
αα/SEA	2.1
αα/-α3.7	2.3
41-42/N	5.7
-28 (A>G)	5.4
αα/SEA	2.1
Codon 17 (A>T)	5.5

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Case49	αα/SEA	Ν	Ν	αα/SEA	2.4
Case50	αα/-α3.7	Ν	Ν	αα/-α3.7	2.3
Case51	αα/αα	Codons 41/42 (-TTCT)	Ν	Codons 41/42 (-TTCT)	5.5
Case52	αα/αα	Codons 71/72 (+A)	Ν	Hb Westmead and Codons 71/72 (+A)	5.6
Case53	αα/αα	Codons 41/42 (-TTCT)	Ν	Codons 41/42 (-TTCT)	5.5
Case54	αα/SEA	Ν	Ν	αα/SEA	2.2
Case55	αα/SEA	Ν	Ν	αα/SEA	2.2
Case56	-α3.7/SEA	Ν	Ν	-α3.7/SEA	1.1
Case57	αα/SEA	Ν	Ν	αα/SEA	2.1
Case58	αα/-α3.7	Ν	Ν	αα/-α3.7	2.4
Case59	αα/αα	Codon 17 (A>T)	Ν	Codon 17 (A>T)	5.8
Case60	αα/αα	Hb E	Ν	Hb E	3.6
Case61	αα/αα	-28 (A>G)	Ν	-28 (A>G)	6
Case62	αα/SEA	Ν	Ν	αα/SEA	2.1
Case63	αα/αα	Hb E	Ν	Hb E	3.8
Case64	αα/SEA	Ν	Ν	αα/SEA	2.3
Case65	αα/αα	Codons 71/72 (+A)	Ν	Codons 71/72 (+A)	5.9
Case66	αα/SEA	Ν	Ν	αα/SEA	2.2
Case67	αα/αα	Codons 41/42 (-TTCT)	Ν	Codons 41/42 (-TTCT)	5.7
Case68	αα/SEA	Ν	Ν	αα/SEA	2.4
Case69	αα/αα	Ν	Hb Hekinan II	Hb Westmead	1.8
Case70	αα/SEA	Ν	Ν	αα/SEA	2.2
Case71	αα/SEA	Ν	Ν	αα/SEA	2.2
Case72	αα/SEA	Ν	Ν	αα/SEA	2.4
Case73	αα/SEA	Ν	Ν	αα/SEA	2.4
Case74	αα/SEA	Ν	Ν	αα/SEA	2.4

Case75	αα/SEA	Ν	Ν	αα/SEA	2.4
Case76	αα/αα	IVS-II-654 (C>T)	Ν	IVS-II-654 (C>T)	5
Case77	αα/αα	Codons 41/42 (-TTCT)	Ν	Codons 41/42 (-TTCT)	6.4
Case78	αα/SEA	Ν	Ν	αα/SEA	2.3
Case79	αα/αα	Codons 41/42 (-TTCT)	Ν	Codons 41/42 (-TTCT)	5.4
Case80	αα/SEA	Ν	Ν	αα/SEA	1.7
Case81	αα/αα	IVS-II-654 (C>T)	Ν	IVS-II-654 (C>T)	5.2
Case82	αα/αα	Codon 43 (G>T)	Ν	Codon 43 (G>T)	5.5
Case83	αα/αα	Codon 17 (A>T)	Ν	Codon 17 (A>T)	5.7
Case84	αα/SEA	Ν	Ν	αα/SEA	2.2
Case85	αα/αα	IVS-II-654 (C>T)	Hb New York	IVS-II-654 (C>T)	4.6
Case86	αα/SEA	Ν	Ν	αα/SEA	1.1
Case87	αα/SEA	Ν	Ν	αα/SEA	2
Case88	αα/SEA	Ν	Ν	αα/SEA	2.2
Case89	αα/SEA	Ν	Ν	αα/SEA	2.4
Case90	αα/SEA	Ν	Ν	αα/SEA	2.2
Case91	αα/SEA	Ν	Ν	αα/SEA	2.4
Case92	αα/αα	Codons 41/42 (-TTCT)	Ν	Codons 41/42 (-TTCT)	4.8
Case93	αα/αα	Codons 41/42 (-TTCT)	Ν	Codons 41/42 (-TTCT)	5.5
Case94	αα/αα	Codons 41/42 (-TTCT)	HBB:c.*+5G>A	Codons 41/42 (-TTCT)	5.6
Case95	αα/-α3.7	Ν	Ν	αα/-α3.7	2.4
Case96	αα/αα	Codons 41/42 (-TTCT)	Ν	Codons 41/42 (-TTCT)	5.7
Case97	αα/αα	IVS-II-654 (C>T)	Ν	IVS-II-654 (C>T)	4.8
Case98	αα/SEA	Ν	Ν	αα/SEA	2.2
Case99	αα/SEA	Ν	Ν	αα/SEA	2.1
Case100	αα/αα	IVS-II-654 (C>T)	HBA2:c.46G>A(Gly>Ser)	IVS-II-654 (C>T)	5.5

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Case101	αα/SEA	Ν	Ν	αα/SEA	2.2
Case102	αα/SEA	Ν	Ν	αα/SEA	2.4
Case103	αα/SEA	Ν	Ν	αα/SEA	2.1
Case104	αα/αα	IVS-II-654 (C>T)	Ν	IVS-II-654 (C>T)	4.8
Case105	αα/αα	Codons 41/42 (-TTCT)	HBB:c180G>C	Codons 41/42 (-TTCT)	4.9
Case106	αα/SEA	Ν	Ν	αα/SEA	2.3
Case107	αα/SEA	Ν	Ν	αα/SEA	2.3
Case108	αα/-α4.2	Ν	Hb G-Honolulu	αα/-α4.2	1.7
Case109	αα/αα	Codons 41/42 (-TTCT)	Ν	Codons 41/42 (-TTCT)	5.2
Case110	αα/αα	IVS-II-654 (C>T)	Ν	IVS-II-654 (C>T)	5
Case111	αα/αα	Codons 41/42 (-TTCT)	Ν	Codons 41/42 (-TTCT)	5.6
Case112	αα/αα	Ν	Ν	Hb Quong Sze	1.8
Case113	αα/SEA	Ν	Ν	αα/SEA	2.3
Case114	αα/αα	-28 (A>G)	Ν	-28 (A>G)	5.7
Case115	αα/SEA	Ν	Ν	αα/SEA	2.3
Case116	αα/-α3.7	Ν	Ν	αα/-α3.7	2.4
Case117	αα/αα	Codons 41/42 (-TTCT)	Ν	Codons 41/42 (-TTCT)	5.9
Case118	αα/SEA	Ν	Ν	αα/SEA	2.1
Case119	αα/αα	Codons 41/42 (-TTCT)	Ν	Codons 41/42 (-TTCT)	6.4
Case120	αα/-α3.7	Hb E	Ν	$\alpha\alpha$ /- α 3.7 and Hb E	3.7
Case121	αα/SEA	Ν	Ν	αα/SEA	2.4
Case122	αα/SEA	Ν	Ν	αα/SEA	2.4
Case123	αα/αα	Codon 17 (A>T)	Ν	Codon 17 (A>T)	5.2
Case124	-α3.7/SEA	Ν	Ν	-α3.7/SEA	1.2
Case125	αα/αα	Codons 41/42 (-TTCT)	Ν	Codons 41/42 (-TTCT)	5.4
Case126	αα/SEA	Ν	Ν	αα/SEA	2.2

Case127	αα/-α3.7	Ν	Ν	αα/-α3.7	2.1
Case128	αα/αα	Ν	Ν	αα CS/αα	2.1
Case129	αα/αα	Codons 71/72 (+A)	Ν	Codons 71/72 (+A)	5.3
Case130	αα/-α4.2	Ν	Ν	αα/-α4.2	2.4
Case131	αα/SEA	Ν	Ν	αα/SEA	2.4
Case132	αα/αα	Codons 41/42 (-TTCT)	Ν	Codons 41/42 (-TTCT)	5.9
Case133	αα/αα	Codons 41/42 (-TTCT)	Ν	41-42/N	5.5
Case134	αα/SEA	Ν	Ν	αα/SEA	2.4
Case135	αα/αα	IVS-II-654 (C>T)	Ν	IVS-II-654 (C>T)	5.1
Case136	αα/αα	IVS-II-654 (C>T)	Ν	IVS-II-654 (C>T)	4.7
Case137	αα/SEA	Ν	Ν	αα/SEA	2.2
Case138	αα/SEA	Ν	Ν	αα/SEA	2.3
Case139	αα/SEA	Ν	Ν	αα/SEA	2.4
Case140	αα/SEA	Ν	Ν	αα/SEA	2.3
Case141	αα/-α3.7	Ν	Ν	αα/-α3.7	2.4
Case142	αα/SEA	Ν	Ν	αα/SEA	2.4
Case143	αα/αα	-28 (A>G)	Ν	(-28/N)	5.9
Case144	αα/-α4.2	Ν	Ν	αα/-α4.2	2.3
Case145	αα/-α4.2	IVS-II-654 (C>T)	Ν	$\alpha\alpha/-\alpha4.2$ and IVS-II-654 (C>T)	5.2
Case146	αα/αα	Codons 41/42 (-TTCT)	Ν	Codons 41/42 (-TTCT)	5.3
Case147	αα/αα	IVS-II-654 (C>T)	Ν	IVS-II-654 (C>T)	5.1
Case148	αα/-α3.7	Codons 41/42 (-TTCT)	Ν	$\alpha\alpha$ /- α 3.7 and Codons 41/42 (-TTCT)	5.9
Case149	αα/αα	Codons 41/42 (-TTCT)	Ν	Codons 41/42 (-TTCT)	5.5
Case150	αα/αα	Codons 41/42 (-TTCT)	Ν	Codons 41/42 (-TTCT)	5.5
Case151	αα/SEA	Ν	Ν	αα/SEA	2.3
Case152	αα/αα	Codon 17 (A>T)	Ν	Codon 17 (A>T)	6.3

HBA mutation	cases	constituent ratio (%)
$^{SEA}/\alpha\alpha$	92	44.4
-α ^{3.7/} αα	53	25.6
α ^{WS} α/αα	20	9.7
$-\alpha^{4.2}/\alpha\alpha$	15	7.2
$\alpha^{CS}\alpha/\alpha\alpha$	7	3.4
a ^{QS} a/aa	4	1.9
Hb Hekinan II/αα	2	1.0
$^{SEA}/-\alpha^{3.7}$	2	1.0
αHBA2: c.46G>Aα/αα	1	0.5
Hb Owari/αα	1	0.5
Alpha2 Codon 30 del GAG/αα	1	0.5
$^{SEA}/-\alpha^{4.2}$	1	0.5
$-\alpha^{3.7}/-\alpha^{4.2}$	1	0.5
Hb G-Honolulu/Hb G-Honolulu	1	0.5
HBA2: c.190G>A/αα	1	0.5
α ^{WS} α/Hb Hekinan II	1	0.5
$-\alpha^{4.2}/\alpha^{Hb}$ G-Honolulu α^{Hb} G-Honolulu	1	0.5
$-\alpha^{4.2}$ /Hb Owari	1	0.5
$-\alpha^{3.7}/\alpha^{WS}\alpha^{WS}$	1	0.5
$-\alpha^{3.7}/\alpha^{CS}\alpha^{CS}$	1	0.5
Total	207	100

Table S2. Genotypes and constituent ratio of α-thalassemia carriers

HBB mutation	cases	constituent ratio (%)
Codons 41/42 (-TTCT)/β ^A	24	34.3
IVS-II-654 (C>T)/ β^{A}	11	15.7
Codon 17 (A>T)/β ^A	8	11.4
-28 (A>G)/β ^A	6	8.6
-50 (G>A)/β ^A	5	7.1
Hb E/β^A	3	4.3
HBB: c.*+129T>A/ β^{A}	3	4.3
Codons 71/72 (+A)/β ^A	2	2.9
Codon 43 (G>T)/ β^{A}	2	2.9
HBB: c64G>C/ β^{A}	2	2.9
HBB: c180 G>C/Codons 41/42 (-TTCT)	1	1.4
HBB: c.*+5G>A/Codons 41/42 (-TTCT)	1	1.4
Hb New York/IVS-II-654 (C>T)	1	1.4
HBB: c113A>G/ β^{A}	1	1.4
Total	70	100

Table S3. Genotypes and constituent ratio of β-thalassemia carriers

HBA mutation	HBB mutation	cases	constituent ratio (%)
$-\alpha^{3.7}/\alpha\alpha$	Codons 41/42 (-TTCT)/ β^A	2	25.0
$^{SEA}/\alpha\alpha$	Codons 41/42 (-TTCT)/ β^A	1	12.5
$-\alpha^{3.7}/\alpha\alpha$	Hb E/β ^A	1	12.5
$-\alpha^{4.2}/\alpha\alpha$	IVS-II-654 (C>T)/ β^{A}	1	12.5
α ^{WS} α/αα	Codons 71/72 (+A)/β ^A	1	12.5
HBA2: c.46G>A/αα	IVS-II-654 (C>T)/ β^{A}	1	12.5
HBA1: c.412A>G/αα	-28 (A>G)/β ^A	1	12.5
Total	-	8	100

Table S4. Genotypes and constituent ratio of 8 carriers of composite α- and β-thalassemia mutations

ММ	Sensitivity	Specificity	PPV	NPV	True	False	True	False
	(%95 CI)	(%95 CI)	(%95 CI)	(%95 CI)	positive	positive	negative	negative
α -thalassemia	73.49%	89.12%	46.47%	96.32%	158	182	1491	57
	(67.06%- 79.26%)	(87.53%- 90.57%)	(42.55%- 50.44%)	(95.44%- 97.03%)				
α α /SEA	97.85%	86.13 %	26.76%	99.87 %	91	249	1546	2
	(92.45%- 99.74%)	(84.44%- 87.69%)	(24.49%- 29.16%)	(99.49%- 99.97%)				
α α /-α3.7	55.17%	82.20 %	9.41%	98.20 %	32	308	1422	26
	(41.54%- 68.26%)	(80.31%- 83.97%)	(7.46%- 11.80%)	(97.62%- 98.65%)				
α α /-α4.2	83.33%	82.62%	4.41%	99.81 %	15	325	1545	3
	(58.58%- 96.42%)	(80.83%- 84.31%)	(3.54%- 5.48%)	(99.46%- 99.93%)				
β -thalassemia	85.90%	84.92 %	19.71%	99.29 %	67	273	1537	11
	(76.17%- 92.74%)	(83.18%- 86.54%)	(17.56%- 22.04%)	(98.78%- 99.59%)				
Codons 41/42 (-TTCT)	96.55%	83.22%	8.24%	99.94 %	28	312	1547	1
	(82.24%- 99.91%)	(81.44%- 84.89%)	(7.36%- 9.21%)	(99.56%- 99.99%)				

 Table S5. Sensitivity and specificity of MM for thalassemia carriers screened by NGS

IVS-II-654 (C>T)	100.00%	82.60 %	4.12%	100.00 %	14	326	1548	0
	(76.84%- 100.00%)	(80.81%- 84.29%)	(3.75%- 4.53%)					
-28 (A>G)	100.00%	82.30 %	2.06%	100.00 %	7	333	1548	0
	(59.04%- 100.00%)	(80.50%- 84.00%)	(1.87%- 2.26%)					
Codon 17 (A>T)	100.00%	82.34 %	2.35%	100.00 %	8	332	1548	0
	(63.06%- 100.00%)	(80.54%- 84.04%)	(2.14%- 2.59%)					

HbA2	Sensitivity	Specificity	PPV	NPV	True	False	True	False
	(%95 CI)	(%95 CI)	(%95 CI)	(%95 CI)	positive	positive	negative	negative
α -thalassemia	65.12%	77.04 %	54.63%	83.87 %	112	93	312	60
	(57.49%- 72.21%)	(72.63% -81.05%)	(49.42%- 59.75%)	(80.81%-86.53%)				
α α /SEA	86.81%	74.07 %	38.54%	96.77 %	79	126	360	12
	(78.10% -93.00%)	(69.94%- 77.92%)	(34.59%- 42.64%)	(94.64%-98.08%)				
α α /-α3.7	39.47%	64.75 %	7.32%	93.82 %	15	190	349	23
	(24.04%-56.61%)	(60.55%- 68.79%)	(4.98%-10.63%)	(92.10%-95.18%)				
α α /-α4.2	37.50%	64.53 %	2.93%	97.31 %	6	199	362	10
	(15.20%-64.57%)	(60.41%- 68.49%)	(1.56%-5.42%)	(96.10%-98.15%)				
β -thalassemia	95.65%	99.61%	97.06%	99.41 %	66	2	506	3
	(87.82%- 99.09%)	(98.59%-99.95%)	(89.21%-99.25%)	(98.24%-99.80%)				
Codons 41/42 (-TTCT)	96.55%	92.7 %	41.18%	99.80%	28	40	508	1
	(82.24%-99.91%)	(90.19%-94.73%)	(34.01%-48.74%)	(98.67%- 99.97%)				

 Table S6. Sensitivity and specificity of HbA2 for thalassemia carriers screened by NGS

IVS-II-654 (C>T)	100.00%	90.41 %	20.59%	100.00 %	14	54	509	0
	(76.84%- 100.00%)	(87.67%-92.71%)	(16.75%-25.04%)					
-28 (A>G)	100.00%	89.3 %	10.29%	100.00 %	7	61	509	0
	(59.04%- 100.00%)	(86.47%-91.71%)	(8.30%-12.70%)					
Codon 17 (A>T)	100.00%	89.46 %	11.76%	100.00 %	8	60	509	0
	(63.06%- 100.00%)	(86.64%-91.86%)	(9.50%-14.48%)					