



Original Article

The Clinical Significance of the Spectrum of Interactions of the Rare IVS-II-5 G>C (*HBB: c.315+5 G>C*) Variation with Other β -Thalassemia Mutations in Southern China

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Competing interests: The authors declare no conflict of Interest.

Abstract. Background: IVS-II-5 G>C (*HBB: c.315+5 G>C*) is a rare β -thalassemia mutation. However, there is no clear evidence regarding the effect of this defect or co-inheritance of other β -thalassemia mutations on phenotypes.

Methods: The clinical phenotypes associated with compound heterozygosity for the IVS-II-5 G>C mutation and other β -thalassemia mutations, together with the genetic modifiers' potential effect of the genetic modifiers α -thalassemia, were studied in 13 patients. In addition, analyses of red cell indices, hemoglobin component, iron status, and α -globin genes were carried out in 19 heterozygotes.

Results: Next-generation sequencing of 24 undiagnosed patients with transfusion-dependent thalassemia (TDT) or non-transfusion-dependent thalassemia (NTDT) identified 13 carriers of the IVS-II-5 G>C mutation. There was a wide spectrum of phenotypic severity in compound heterozygotes and 6 (46.2%) of 13 were transfusion dependent. Analysis of 19 heterozygotes indicated that most were hematologically normal without appreciable microcytosis or hypochromia, and approximately half had normal hemoglobin A₂ levels at the same time.

Conclusion: Compound heterozygotes for IVS-II-5 G>C and other severe β -thalassemia mutations are phenotypically severe enough to necessitate appropriate therapy and counselling. Co-inheritance of this nucleotide substitution with other β -thalassemia mutations may account for a considerable portion of the incidence of undiagnosed patients with NTDT and TDT in Guangxi. Therefore, the IVS-II-5 G>C mutation can pose serious difficulties in screening and counselling.

Keywords: β -thalassemia; IVS-II-5 G>C; Genotype; Phenotype.

Citation: Liao G., Zhou Y., Yin X., He S., Wu Y., Xiao J., Gen Z. g, Huang Q., Luo G., Yan K g. The clinical significance of the spectrum of interactions of the rare IVS-II-5 G>C (*HBB: c.315+5 G>C*) variation with other β -thalassemia mutations in southern China. *Mediterr J Hematol Infect Dis* 2022, 14(1): e2022034, DOI: <http://dx.doi.org/10.4084/MJHID.2022.034>

Published: May 1, 2022

Received: January 16, 2022

Accepted: April 4, 2022

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Introduction. β -Thalassemia is a genetic hemolytic disease caused by a reduction (β^+ allele) or deletion (β^0 allele) of the beta globin gene. So far, more than 350 pathogenic genetic mutations with a high degree of genetic heterogeneity based on geographical location and race have been associated with β -thalassemia.¹ Further elucidation of the genotype/phenotype relationship could benefit phenotype prediction for genetic counseling of at-risk couples and appropriate clinical treatment for homozygous or compound heterozygous patients. Usually, in the heterozygous state, β -thalassemia is characterized by mild microcytic hypochromic anemia and increased hemoglobin A₂ (HbA₂) levels. However, some β -globin mutations are very mild or silent. Thus, heterozygote carriers have normal hematological indices and electrophoretic fractions. These specific defects to the β -globin gene can be identified by genetic and molecular analyses.^{2,3}

The rare IVS-II-5 G>C (*HBB*: c.315+5 G>C) mutation of the β -globin gene was first observed in a family in Guangxi, China. The proband and her two brothers were compound heterozygous for two mutations: the IVS-II-5 G>C substitution and the deletion -TCTT from codons 41–42 (*HBB*: c.126_129delCTTT). These mutations resulted in severe anemia, requiring regular blood transfusions.⁴ In addition, Zhao et al.⁵ reported a patient with normal red blood cell indices and borderline HbA₂ levels, which was found to be compound heterozygous for the IVS-II-5 G>C and IVS-II-672 A>C (*HBB*: c.316-179 A>C) mutations. There is no clear evidence regarding the effect of this defect or co-inheritance of other β -globin gene mutations on phenotypes. This study describes 32 individuals with the IVS-II-5 G>C mutation, including 19 heterozygous and 13 with co-inheritance of the β^0 -thalassemia mutation. In addition, the clinical and hematological phenotypes of heterozygotes with the very rare IVS-II-5 G>C variant or compound heterozygotes with β^0 -thalassemia mutations and the importance of genetic counseling are discussed.

Subjects and Methods.

Subjects. The subjects were selected from undiagnosed patients presenting with transfusion dependent thalassemia (TDT) or non-transfusion dependent thalassemia (NTDT) and relatives. The gap-polymerase chain reaction (Gap-PCR) and reverse dot-blot (RDB) technique were previously performed to investigate the incidences of 17 common types of Chinese β -thalassemia mutations [-28 A>G (*HBB*: c.-78 A>G), -29 A>G (*HBB*: c.-79 A>G), -30 T>C (*HBB*: c.-80 T>C), -32 C>A (*HBB*: c.-82 C>A), codons 14/15 +G (*HBB*: c.45_46insG), codon 17 A>T (*HBB*: c.52 A>T), codon 26 G>A (HbE or *HBB*: c.79 G>A), codons 27–28 +C (*HBB*: c.84_85G),

codon 31 -C (*HBB*: c.94delC), codons 41–42 -TCTT (*HBB*: c.126_129delCTTT), codon 43 G>T (*HBB*: c.130 G>T), codons 71-72 +A (*HBB*: c.216_217insA), IVS-I-1 G>T (*HBB*: c.(0).92+1 G>T), IVS-I-5 G>C (*HBB*: c.92+5 G>C), IVS-II-654 C>T (*HBB*: c.316-197 C>T), Cap+40-43 -AAAC (*HBB*: c.11_-8delAAAC), and codon ATG>AGG (*HBB*: c.2 T>G)] and six common Chinese α -thalassemia mutations [-^{SEA} (South East Asian) deletion, - $\alpha^{3.7}$ (rightward) and - $\alpha^{4.2}$ (leftward) deletions and Hb Constant Spring (Hb CS; *HBA2*: c.427 T>C), Hb Quong Sze (Hb QS; *HBA2*: c.377 T>C) and Hb Westmead (*HBA2*: c.369 C>G) point mutations] in the undiagnosed patients.⁶

Genotypic analysis using traditional methods. The family members were analyzed with Gap-PCR and RDB methods on the common types of Chinese thalassemia mutations. In addition, the Multiplex ligation-dependent probe amplification was conducted to identify variations in α and β gene copy numbers for the undiagnosed patients and their family members.

Genetic sequencing. Genomic DNA was extracted from peripheral blood samples using the MagCore® Genomic DNA Whole Blood Kit (ATRI DA B.V., Amersfoort, Netherlands). A library was constructed using the Twist Library Preparation Kit Twist Comprehensive Exome 96 Reactions Kit (Twist Bioscience, South San Francisco, CA, USA). Next-generation sequencing (NGS) was performed to screen potential variants using the Illumina NovaSeq sequencing platform (Illumina, Inc., San Diego, CA, USA) with a sequencing read length of PE150. This procedure included whole-exome sequencing for nearly 700 genes related to hematological hereditary and immunodeficiency disorders based on NGS. In addition, Sanger sequencing was performed to confirm the presence or absence of these mutations.

Hematological analysis. Complete blood counts were analyzed using an XE 5000 automatic blood cell analyzer (Sysmex Corporation, Kobe, Japan). Fetal hemoglobin (HbF) and HbA₂ levels were quantified by high-pressure liquid chromatography (HPLC) (VARIANT II Hemoglobin Testing System; Bio-Rad Laboratories, Hercules, CA, USA) and capillary electrophoresis (Sebia, Lisses, France). Serum iron and ferritin levels were measured using a Cobas 6000 chemistry analyzer (Roche Diagnostics, Mannheim, Germany). Total iron-binding capacity was determined using a BN ProSpec® System (Siemens Healthineers, Erlangen, Germany).

Results. Of the 24 undiagnosed patients with TDT or NTDT, 13 (54.2%) were compound heterozygous for the IVS-II-5 G>C mutation and a second β^0 gene mutation (-

Table 1. Hematological indices, molecular, and clinical findings in the patients with IVS-II-5 G>C and other β -thalassemia mutations.

Case no.	Phenotype	Sex	¹ Age (yr)	² Hb (g/dL)	³ HbF (%)	³ HbA ₂ (%)	Splenectomy	BT	α -genotype	β -genotype
1	NTDT	M	4.5	6.3	24.5	6.1	Yes	6 monthly	$\alpha\alpha/\alpha\alpha$	$\beta^{CD41-42}/\beta^{IVS-2-5}$
2	NTDT	M	3.0	7.3	8.6	5.9	No	yearly	$\alpha\alpha/\alpha\alpha$	$\beta^{CD41-42}/\beta^{IVS-2-5}$
3	NTDT	F	4.0	6.9	21.6	6.5	Yes	3–4 months	$\alpha\alpha/\alpha\alpha$	$\beta^{CD41-42}/\beta^{IVS-2-5}$
4	NTDT	F	6.5	7.2	20.1	6.4	Yes	Never	$\alpha\alpha/\alpha\alpha$	$\beta^{CD41-42}/\beta^{IVS-2-5}$
5	NTDT	F	3.5	7.9	13.2	5.6	Yes	Infrequent	$\alpha\alpha/\alpha\alpha$	$\beta^{CD41-42}/\beta^{IVS-2-5}$
6	NTDT	F	5.0	3.3	11.8	3.5	No	Infrequent	$\alpha\alpha/\alpha\alpha$	$\beta^{CD41-42}/\beta^{IVS-2-5}$
7	TDT	F	0.5	6.2	19.3	5.2	No	Regular	$\alpha\alpha/\alpha\alpha$	$\beta^{CD41-42}/\beta^{IVS-2-5}$
8	NTDT	F	10.0	5.5	3.8	4.1	Yes	Infrequent	$\alpha\alpha/\alpha\alpha$	$\beta^{CD17}/\beta^{IVS-2-5}$
9	TDT	M	2.0	4.7	17.5	4.9	No	Regular	$\alpha\alpha/\alpha\alpha$	$\beta^{CD17}/\beta^{IVS-2-5}$
10	TDT	F	0.8	6.2	11.3	5.1	No	Regular	$\alpha\alpha/\alpha\alpha$	$\beta^{CD17}/\beta^{IVS-2-5}$
11	TDT	M	0.5	4.0	12.5	4.2	Yes	Regular	$\alpha\alpha/\alpha\alpha$	$\beta^{CD17}/\beta^{IVS-2-5}$
12	TDT	M	0.5	6.4	9.7	4.6	No	Regular	$\alpha\alpha/\alpha\alpha$	$\beta^{CD17}/\beta^{IVS-2-5}$
13	TDT	M	0.3	5.3	14.6	3.9	No	Regular	$\alpha\alpha/\alpha\alpha$	$\beta^{CD71-72}/\beta^{IVS-2-5}$

Abbreviations: ¹Age at onset; ²Pretransfusion hemoglobin; ³Hemoglobin analysis before first blood transfusions; NTDT: non-transfusion dependent thalassemia; TDT: transfusion dependent thalassemia; HbF: fetal hemoglobin; HbA₂: hemoglobin A₂; BT: blood transfusions.

TCTT at codons 41–42 in seven, A>T at codon 17 in five, and +A at codons 71–72 in one). The clinical phenotype was TDT in six patients and NTDT in seven. The details of the genotypes and phenotypes are described in **Table 1**.

The age of onset, pretransfusion hemoglobin (Hb) levels, and number and frequency of transfusions were obtained from the patient's records. All patients were compound heterozygotes and at diagnosis before the age of 5 years, except two at ages of 6.5 and 10. Seven patients had received variable numbers of blood transfusions before being referred. Hemoglobin analysis before the first transfusion found that these patients had increased levels of HbF (range, 3.8%–24.5%) and HbA₂ (3.5%–6.5%) to various degrees. The mean pretransfusion Hb concentration of the six patients with the TDT phenotype was 5.5 (range, 4.0–6.4) g/dL. All patients had mild to moderate splenomegaly at the time of presentation. During the follow-up period, six patients underwent splenectomy (**Table 1**).

Compound heterozygotes for the IVS-II-5 G>C and -TCTT at codons 41–42. Of the seven patients with the IVS-II-5 G>C and -TCTT at codons 41–42 genotype, 6 (85.7%) had the NTDT phenotype. One had never received a transfusion, three required occasional blood transfusions, and one received 3–4 blood transfusions each year after disease onset, but transfusions were no longer necessary after splenectomy. Another patient with TDT received 1–2 transfusions per month.

Compound heterozygotes for the IVS-II-5 G>C and A>T at codon 17 mutations. Of the five patients with the IVS-II-5 G>C and A>T at codon 17 genotype, 4 (80.0%) had the TDT phenotype, including three who required

monthly blood transfusions, while the fourth died from anemic heart disease. The remaining patient had the NTDT phenotype and rarely required transfusion at the disease onset.

Compound heterozygotes for the IVS-II-5 G>C and +A at codons 71–72. One case with the IVS-II-5 G>C and +A at codons 71–72 genotype required one blood transfusion per month at disease onset. This patient received hematopoietic stem cell transplantation, and Hb levels remained at >7 g/dL afterwards.

IVS-II-5 G>C in a heterozygous state. Nineteen individuals (six men and 13 women; age range, 6–68 years) heterozygous for the IVS-II-5 G>C mutation were screened from 42 relatives. None had co-inherited α -thalassemia. Iron deficiency anemia was ruled out in 15 carriers, as Hb levels were 11.0–15.0 g/dL. The mean corpuscular volume (MCV) was > 80 (reference range, 80–100) fL in 12 (63.2%) individuals and the mean corpuscular hemoglobin (MCH) was > 27 (reference range, 27–34) pg in 11 (57.9%). HbA₂ levels measured by HPLC ranged from 2.7% to 3.9%. When defining HbA₂ \geq 4.0% as abnormal, 3.5%–4.0% as critical, and <3.5% as normal, 13 (68.4%) of the 19 patients had normal HbA₂ levels, while six were considered critical. Eight patients with MCV and HbA₂ were completely normal (cases 1, 4, 5, 8, 12, 13, 16, and 17), which included five who received capillary electrophoresis, suggested that HbA₂ levels were in the normal range (<3.5%) (**Table 2**).

Discussion. Molecular defects causing β -thalassemia are mainly due to point mutations, few deletions, dysregulation of the globin gene, or an insert in the

Table 2. Hematological indices and molecular findings in the patients with heterozygous IVS-II-5 G>C mutation.

Case no.	Sex /Age	Hb (g/dL)	RBC (10 ⁹ /L)	MCV (fL)	MCH (pg)	MCHC (g/dL)	HPLC		CE		SI (μmol/L)	SF (ng/ml)	TIBC (μmol/L)	α-globin Genotype	β-globin Genotype
							HbF	HbA ₂	HbF	HbA ₂					
1	F-27	12.4	4.58	82.0	27.0	32.8	0.3	3.1	0	3.1	21.0	65.3	15.3	αα/αα	β ^{IVS-2-5} /β ^N
2	F-41	11.4	4.46	76.0	25.5	33.6	0.4	3.6	0	3.6	19.0	59.4	12.0	αα/αα	β ^{IVS-2-5} /β ^N
3	F-32	12.9	4.99	78.0	26.4	33.4	0.6	3.0	0	3.2	10.0	58.3	20.7	αα/αα	β ^{IVS-2-5} /β ^N
4	F-49	12.4	4.56	83.0	27.3	33.0	0.2	3.4	0	3.3	23.0	59.3	49.8	αα/αα	β ^{IVS-2-5} /β ^N
5	F-62	13.2	4.64	85.0	28.5	33.6	1.9	3.2	1.5	3.1	19.0	81.0	425.3	αα/αα	β ^{IVS-2-5} /β ^N
6	M-68	15.6	6.98	68.0	22.4	32.8	0.3	2.9	0	2.8	23.0	67.5	354.9	αα/αα	β ^{IVS-2-5} /β ^N
7	M-6	11.9	6.27	61.0	19.2	31.3	0.3	3.3	0	3.2	22.0	62.1	65.1	αα/αα	β ^{IVS-2-5} /β ^N
8	F-39	12.0	4.24	86.0	28.3	32.9	0.3	3.4	0	2.8	17.0	59.7	13.9	αα/αα	β ^{IVS-2-5} /β ^N
9	M-7	12.5	4.96	79.0	25.8	33.5	0.9	3.5	0.3	3.6	19.0	70.2	29.6	αα/αα	β ^{IVS-2-5} /β ^N
10	M-67	12.1	4.31	84.0	28.2	33.4	0.1	3.7	0	3.0	23.0	54.1	235.0	αα/αα	β ^{IVS-2-5} /β ^N
11	F-37	13.9	5.30	78.0	26.3	33.7	0.2	3.4	0	3.7	15.0	59.6	45.9	αα/αα	β ^{IVS-2-5} /β ^N
12	M-28	15.6	5.68	81.0	27.5	34.0	0.3	3.4	0	3.2	-	-	-	αα/αα	β ^{IVS-2-5} /β ^N
13	F-51	13.5	4.87	88.0	27.7	31.6	0.4	3.4	-	-	-	-	-	αα/αα	β ^{IVS-2-5} /β ^N
14	M-66	15.8	5.30	84.0	29.8	35.3	1.3	3.7	-	-	-	-	-	αα/αα	β ^{IVS-2-5} /β ^N
15	F-58	14.1	5.24	83.0	27.0	32.7	0.5	3.5	-	-	-	-	-	αα/αα	β ^{IVS-2-5} /β ^N
16	F-34	12.3	4.48	81.0	27.5	33.4	0.0	2.7	-	-	-	82.0	-	αα/αα	β ^{IVS-2-5} /β ^N
17	F-36	12.7	4.70	82.0	27.2	32.9	0.0	3.4	-	-	-	74.5	-	αα/αα	β ^{IVS-2-5} /β ^N
18	F-24	12.0	4.80	80.0	26.0	33.6	0.0	3.4	-	-	-	55.0	-	αα/αα	β ^{IVS-2-5} /β ^N
19	F-20	11.0	4.80	78.0	25.3	32.8	0.0	3.9	-	-	-	80.0	-	αα/αα	β ^{IVS-2-5} /β ^N

Abbreviations: Hb: hemoglobin (11.5-15.0 g/dL); RBC: red blood cell count (3.8-5.1 10⁹/L); MCV: mean corpuscular volume (80-100 fL); MCH: mean corpuscular hemoglobin (27-34 pg); MCHC: mean corpuscular hemoglobin concentration (31.6-35.4 d/dL); HPLC: high-pressure liquid chromatography; CE: capillary electrophoresis; HbF: fetal hemoglobin (<2 %); HbA₂: hemoglobin A₂ (2-4 %); SI: serum iron (12-30 umol/L); SF: serum ferritin (11-306.8 ng/ml); TIBC: total iron binding capacity (45-75 umol/L). Reference range values are shown in parenthesis.

coding region. A previous study conducted in Guangxi province, China, revealed the presence of few frequent mutations and a large number of rare defects associated with molecular heterogeneity. A large-scale epidemiological survey conducted in Guangxi reported that the carrier rate of the IVS-II-5 G>C mutation was 0.02%.⁷ No IVS-II-5 G>C mutation was detected in the screening of 47,500 people in Baise city⁸ or 130,318 in Yulin city in Guangxi.⁹ Only one case with the IVS-II-5 G>C mutation was identified among 189,414 individuals screened in Fujian, China.¹⁰ These studies confirmed that the IVS-II-5 G>C mutation is rare in the Chinese population. However, in the present study, 13 (54.2%) of the 24 undiagnosed patients with TDT or NTDT had co-inherited the IVS-II-5G>C mutation, which included 7 (53.8%) from Hechi City. In addition, 13 (68.4%) of the 19 heterozygous were from Hechi City, which was not included in the large-scale epidemiological survey previously conducted in Guangxi. Hence, it seemed that the mutation was concentrated in Hechi city. However, a large-scale epidemiological investigation is needed to confirm this suspicion.

There have been relatively few reported cases of co-inheritance of the IVS-II-5 G>C mutation compounded with other β-thalassemia mutations. Thus, the impact on phenotype remains unclear.^{4,5,11} Three cases previously reported with the IVS-II-5 G>C mutation compounded with -TCTT at codons 41-42 had transfusion-dependent thalassemia. Only one (14.3%) of seven patients in the present study had the TDT phenotype, while 6 (85.7%) had the NTDT phenotype. Interestingly, one patient required 3-4 blood transfusions per year but no longer

after splenectomy. In addition, blood transfusions were no longer needed after splenectomy in three patients, as Hb levels were maintained at >6.8 g/dL.¹¹ The efficacy of splenectomy for the treatment of thalassemia is dependent on the destruction site of red blood cells. However, the efficacy of splenectomy is poor in some patients with ineffective hematopoiesis,¹² suggesting a greater extent of erythrocyte destruction in the spleen of patients with the IVS-II-5 G>C mutation as compared to those with other types of β-thalassemia. Hence, splenectomy is relatively effective in these patients. Clinical manifestations were more severe with the IVS-II-5 G>C/codon 17 A>T genotype, as most patients had the TDT phenotype. In patients with the IVS-II-5 G>C and +A at codons 71-72, our data were insufficient to predict the phenotype as only one was diagnosed with TDT. Among the patients with severe disease in the present study, none carried both IVS-II-5 and β⁺-thalassemia mutations, which indirectly confirmed that the clinical symptoms might not be severe for patients with IVS II-5 G>C and β⁺-thalassemia mutations. Zhao et al.⁵ reported a case of compounded heterozygosity for IVS II-5 G>C and IVS-II-672 A>C with normal Hb and MCV levels, although there is no evidence that the IVS-II-672 A>C sequence variant is pathogenic. In addition, although the influence of co-inheritance of α-thalassemia on the phenotype was ruled out, the influence of other globin gene modifications on the severity of clinical presentation remains unclear.

Analysis of heterozygotes with the IVS-II-5 G>C mutation showed that in 12 (63.2%) of 19 individuals, MCV was > 80 (reference range, 80-100) fL and in 11

(57.9%) of 19, MCH was >27 (reference range, 27–34) pg. When screening β -thalassemia carriers, it is not uncommon to identify individuals with normal or mildly reduced red cell indices. Subjects with this phenotype may be carriers of very mild or silent β -gene mutations associated with high residual β -globin chain output. Unlike the IVS-II-1 G>T mutation, which causes β^0 -thalassemia, the IVS-II-5 G>C mutation did not completely abolish normal splicing.¹¹ Jiang et al.¹¹ found that normally spliced RNA was the dominant form of the IVS-II-5 G>C mutation. Therefore, co-inheritance of α -thalassemia has a significant effect on red cell indices, particularly the MCV and MCH, which may be normalized.¹³ Furthermore, mutations to the KLF1 gene have been associated with normal MCV and MCH.¹⁴ Although co-inheritance of α -thalassemia was excluded from our cohort, and other factors may contribute to differences in RBC parameters between individuals. The HbA₂ levels of all heterozygotes were <4.0%, of which 13 (68.4%) were below the diagnostic cutoff of 3.5%. Of the 19 heterozygotes for the IVS-II-5 G>C mutation, 8 (42.1%) were silent with completely normal HbA₂ and MCV levels. Because whole blood cell and Hb composition analysis are required for a diagnosis of anemia and screening of thalassemia, the key RBC parameters and the constitution of Hb were compared among patients with other β^+ -thalassemia mutations or IVS-II-5 G>C carriers (**Figure 1**). Significant

differences in HbA₂, HbF, MCV, MCH, and MCHC values were evident between patients with other β^+ -thalassemia mutations (IVS-II-654 C>T or -28 A>G) and IVS-II-5 G>C carriers ($P<0.001$). IVS-II-5 G>C carriers have significantly higher Hb levels than IVS-II-654 C>T carriers ($P=0.002$). Thus, the IVS-II-5 G>C mutation is responsible for milder anemia than other mutations described in China, although the variant was described as the β^+ hematological phenotype in a prior publication.¹¹ Based solely on the screening results, there was no indication of β -thalassemia, which is prone to a missed diagnosis. In addition, the IVS-II-5 G>C defect is undetectable by traditional methods. DNA sequencing is usually required, which prevents the detection of more patients and missed diagnoses, and may eventually have adverse consequences in genetic counseling. Thus, it is recommended that if a partner is diagnosed as a carrier of thalassemia and indices and HbA₂ are borderline, then molecular screening for the IVS-II-5 G>C mutation by NGS should be performed.

Conclusions. In conclusion, the present study results showed that co-inheritance of the IVS-II-5 G>C defect with other mutations to globin genes may account for a considerable portion of the incidence of undiagnosed moderate and severe β -thalassemia in Guangxi. On the other hand, MCV and HbA₂ levels in most patients heterozygous for IVS-II-5 G>C were normal and, thus,

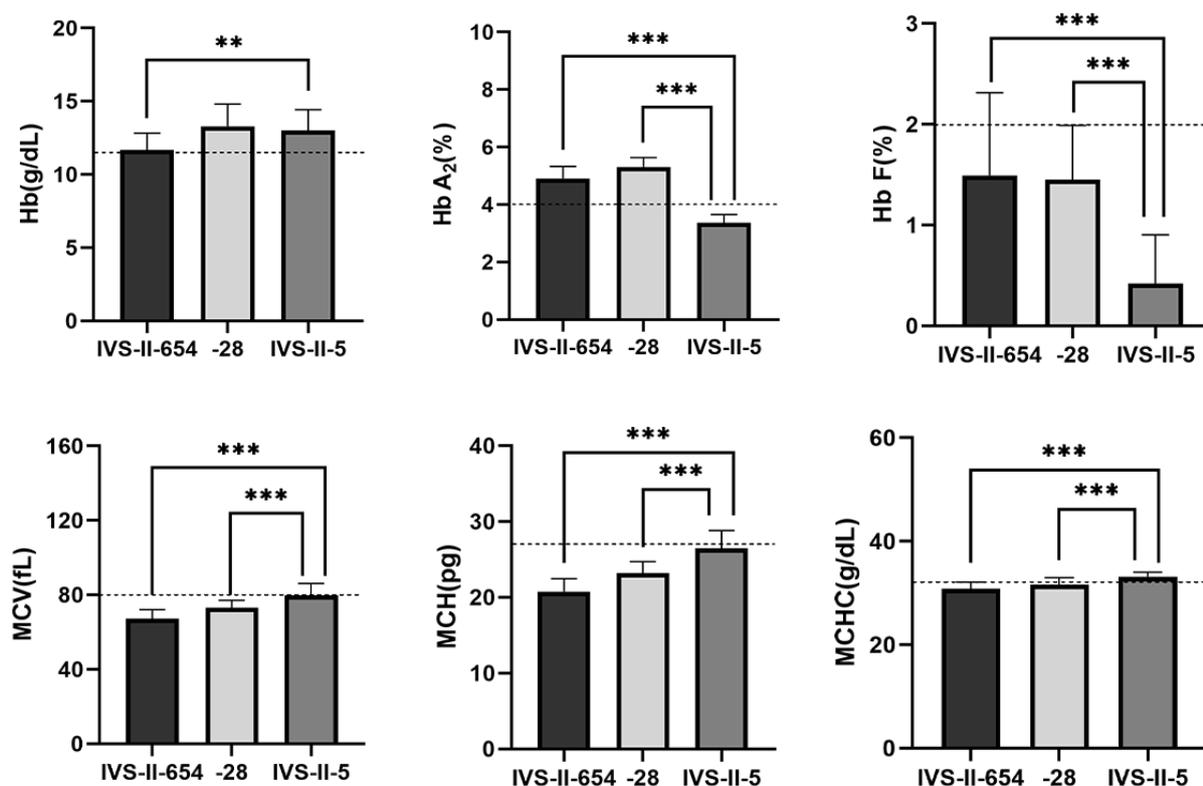


Figure 1. Comparisons of Hb, HbA₂, HbF, MCV, MCH, and MCHC levels between IVS-II-654 C>T (n=20), -28 A>G (n=21), and IVS-II-5 G>C (n=19) carriers. Data are presented as mean \pm standard deviation (SD). Reference range values are shown as a dotted line. Asterisks indicate statistically significant differences: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

often misdiagnosed. Hence, routine screening of this nucleotide substitution and NGS are recommended for genetic counseling of suspected cases, especially in populations with a significantly high frequency.

Acknowledgements. We are grateful to our patients for participating in this study.

Ethic statement. The study protocol was approved by the Medical Ethics Committee of the 923rd Hospital of

the Joint Logistics Support Force of the People's Liberation Army and the First People's Hospital of Zigong. All patients provided written informed consent.

Funding. This study was supported by the Scientific Research Project of Guangxi Zhuang Autonomous Region Health Committee (Z20201266) and Key Science and Technology Project of Zigong (2020YXY04).

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