Third-Generation Sequencing as a New Comprehensive Technology for Identifying Rare α- and β-Globin Gene Variants in Thalassemia Alleles in the Chinese Population

Jianlong Zhuang, MS; Chunnuan Chen, PhD; Wanyu Fu, BS; Yuanbai Wang, BS; Qianmei Zhuang, MS; Yulin Lu, MS; Tiantian Xie, BS; Ruofan Xu, MS; Shuhong Zeng, BS; Yuying Jiang, BS; Yingjun Xie, PhD; Gaoxiong Wang, BS

• ***Context.***—**Identification of rare thalassemia variants requires a combination of multiple diagnostic technologies.**

Objective.—**To investigate a new approach of comprehensive analysis of thalassemia alleles based on third-generation sequencing (TGS) for identification of α- and β-globin gene variants.**

Design.—**Enrolled in this study were 70 suspected carriers of rare thalassemia variants. Routine gap–polymerase chain reaction and DNA sequencing were used to detect rare thalassemia variants, and TGS technology was performed to identify α- and β-globin gene variants.**

Results.—**Twenty-three cases that carried rare variants in α- and β-globin genes were identified by the routine detection methods. TGS technology yielded a 7.14% (5 of 70) increment of rare α- and β-globin gene variants as compared with the routine methods. Among them, the rare deletional genotype of $-^{THAI}$ was the most common variant. In addition, rare variants of CD15 (G>A) (HBA2:c.46G>A), CD117/118(+TCA) (HBA1:c.354_355insTCA), and β-thalassemia 3.5-kilobase gene deletion were first identified in Fujian Province, China; to the best of our knowledge, this is the second report in the Chinese population. Moreover, HBA1:c.-24C>G, IVS-II-55 (G>T) (HBA1:c.300+55G>T) and hemoglobin (Hb) Maranon (HBA2:c.94A>G) were first identified in the Chinese population. We also identified rare Hb variants of HbC, HbG-Honolulu, Hb Miyashiro, and HbG-Coushatta in this study.**

Conclusions.—**TGS technology can effectively and accurately detect deletional and nondeletional thalassemia variants simultaneously in one experiment. Our study also demonstrated the application value of TGS-based comprehensive analysis of thalassemia alleles in the detection of rare thalassemia gene variants.**

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Thalassemia is a common inherited blood disorder resulting from deficient synthesis of globin chains.[1] According to the deficiency of globin genes, thalassemia can be classified into α, β, δ, and βδ subtypes, among which α- and β-thalassemia subtypes are the most common. Approximately 5% of the global population are carriers for thalassemia mutation, with at least 60 000 severely affected individuals born each year.[2] As there is no effective cure for thalassemia intermedia or major at present, it is of great clinical significance to seek a method for screening and making genetic diagnosis of thalassemia for the sake of preventing and controlling this devastating disease. Conventional thalassemia testing can detect only 23 common types of α- and β-thalassemia in the Chinese population. Fujian Province, China, is a region with high thalassemia incidences, where increased rare thalassemia cases have been reported using DNA sequencing and gap–polymerase chain reaction (Gap-PCR) technology.[3–5] In addition, next-generation sequencing is increasingly used in improving thalassemia detection.[6–8] However, there are still limitations in the investigation of rare structural variations (SVs) of thalassemia genes.

Currently, third-generation sequencing (TGS), represented by Pacific Bioscience (PacBio) single-molecule real-time sequencing (SMRT) and Oxford Nanopore Technologies (ONT), has been used in human genetic analysis, with advantages of long reads, single-molecule resolution, and rapid detection, which can also detect SVs effectively and accurately.[9–11] In addition, it is able to distinguish compound heterozygous mutation (in trans) and describe mutations in

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From the Prenatal Diagnosis Center (J. Zhuang, Fu, Y. Wang, Q. Zhuang, Zeng, Jiang) and the Department of Surgery (G. Wang), Quanzhou Women's and Children's Hospital, Quanzhou, Fujian Province, China; the Department of Neurology, The Second Affiliated Hospital of Fujian Medical University, Quanzhou, Fujian Province, China (Chen); the Third-Generation Sequencing Business Unit, Berry Genomics Corporation, Beijing, China (Lu, T. Xie, Xu); and the Department of Obstetrics and Gynecology, Key Laboratory for Major Obstetric Diseases of Guangdong Province, Key Laboratory of Reproduction and Genetics of Guangdong Higher Education Institutes, The Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, China (Y. Xie). Authors Jiang, Y. Xie and G. Wang are co-lead authors.

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Corresponding author: Yuying Jiang, BS, Prenatal Diagnosis Center, Quanzhou Women's and Children's Hospital, No. 700 Fengze St, Quanzhou, Fujian Province 362000, China (email: 1287194067@qq.com).

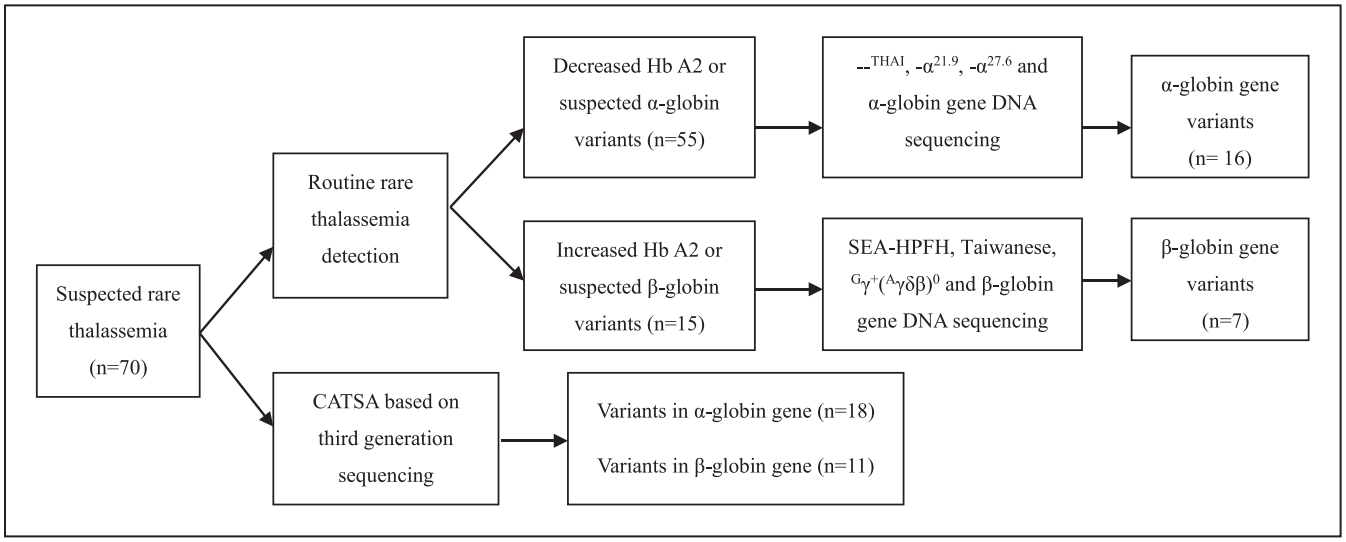

Figure 1. *The flowchart of rare thalassemia detection via the routine detection methods and third-generation sequencing. Abbreviations: CATSA, comprehensive analysis of thalassemia alleles; Hb, hemoglobin.*

the same chromosome (in cis).[12] Although both TGS methods have raw sequencing error rates of approximately 5% to 20%, PacBio takes the advantage of circular consensus sequencing to significantly reduce the errors in raw subreads.[13,14] However, with the increase of coverage depth, both PacBio and ONT platforms can achieve accuracy of more than 99% in the detection of single-nucleotide variants (SNVs) and small insertions and deletions.[13–15] Side-by-side comparison has also shown that the PacBio and ONT platforms have high concordance in SV detection.[16] Recent studies have analyzed the application of PacBio-based TGS technology in the detection of thalassemia genes and proposed the application value of TGS technology in large-scale screening of thalassemia carriers.[12,17] Although it has high potential, the application of ONT on clinical thalassemia testing has not been fully explored so far.

In this study, we enrolled 70 subjects who were suspected to be rare thalassemia variant carriers to perform a comprehensive analysis of thalassemia alleles (CATSA) based on PacBio sequencing technology to identify rare variants in α- and β-thalassemia and hemoglobin (Hb) variants.

MATERIALS AND METHODS

Subjects

A total of 70 subjects who were suspected to be rare thalassemia carriers were recruited at Quanzhou Women's and Children's Hospital (Quanzhou, China). The flowchart of rare thalassemia detection via the routine process and TGS is presented in Figure 1. Among the subjects, 15 were male and 55 were female; they ranged in age from 20 to 47 years. No genetic relationship existed in all samples. All the subjects included in this study signed written informed consent, and the study protocol was approved by the ethics committee of the said hospital (2020 No. 8).

Hematologic Analysis and Common Thalassemia Gene Testing

Routine blood detection was performed in all subjects using an automated cell counter (Sysmex XS-1000i; Sysmex Co, Ltd, Kobe, Japan), and the Hb components were analyzed using an Hb electrophoresis system (Sebia, Evry Cedex, France). A mean corpuscular volume (MCV) of less than 82 fL, and/or a mean corpuscular Hb (MCH) of less than 27 pg, and/or an HbA_2 greater than 3.4% or less than 2.6% or HbF greater than 2.0% was defined as a positive result of thalassemia screening, which was then subjected to thalassemia gene testing.

The genomic DNA of the subjects was extracted via an automatic nucleic acid extractor (Ruibao Biological Co, Ltd, Taiwan) for common α- and β-thalassemia gene detection. The PCR reverse-dot hybridization technique was used to detect the 23 genotypes of α- and β-thalassemia variants (Yaneng Biological Technology Co, Ltd, Shenzhen, China).[18] The experimental procedures were performed strictly according to the manufacturer's protocol.

Routine Detection of Rare α- and β-Thalassemia

The subjects with suspected rare or novel thalassemia mutations were recruited for rare thalassemia gene detection by Gap-PCR and DNA sequencing. Gap-PCR was used for rare α-thalassemia (–THAI, -α27.6, -α21.9) and rare β-thalassemia (Taiwanese, Gγ+(Aγδβ)0, SEA-HPFH) detection (Yaneng BIOScience Technology Co, Ltd, Shenzhen, China). The template DNA concentration was required to be 10 to 100 ng/μL, and purity (A260/A280) was 1.7 to 2.0; 2 μL DNA was added for PCR amplification, the amplified product was subjected to agarose electrophoresis, and the gel imaging system was used to take pictures. Simultaneously, DNA sequencing was performed when the subject was suspected to be a rare α- and β-thalassemia mutation carrier. The primer sequences were as follows: HBA1-F: CCCGTGCTTTTTGCGTCCTGGTGTT; HBA1-R: CCTCCCGCCCCTGCCTTTTCCTACC; HBA2-F: AGTGGCGGGTGGAGGGTGGAGACGT; HBA2-R: TCCCATACT CCCTGCAGTTCTCCCT; HBB-F: CCAAG GACAGGTACGGCTG TCATC; and HBB-R: GCATATGCATCAGGGGCTGTTG. The DNA sequencing of α- and β-globin genes was performed following a previous study.[4,5]

CATSA and Data Analysis

Genomic DNA was extracted from peripheral leukocytes using the QIAamp DNA blood mini kit (Qiagen) according to the manufacturer's instructions. The purified DNA samples were quantified using the Qubit dsDNA BR assay kit (ThermoFisher Scientific) and then sent to an independent laboratory (Berry Genomics, Beijing, China) for CATSA on the PacBio Sequel II platform. The CATSA method was performed as previously described,[17] but with additional primers to further increase the coverage of deletion arrangements. Briefly, genomic DNA samples were subjected to multiplex long-molecule PCR using optimized

primers to generate specific amplicons that encapsulate currently known SV regions (35 kinds of α-thalassemia SVs and 28 kinds of β-thalassemia SVs) and SNVs (903 kinds of mutations in α-globin gene and 1135 in β-globin gene) in the *HBA1*, *HBA2*, and *HBB* genes according to the HbVar, Ithanet, LOVD, and LOVD-China databases. After purification and end repair, double bar code adapters were ligated to the 5′ and 3′ ends and SMRTbell libraries were prepared using the Sequel Binding and Internal Ctrl Kit 3.0 (PacBio). Primed DNA-polymerase complexes were loaded onto SMRT cells (Pacific Biosciences) and sequencing was initiated on the PacBio Sequel II System to generate 10 to 25 subreads per molecule. Following alignment of the subreads, the consensus circular sequence was mapped to the GRCh38 reference and variants were called (FreeBayes software, version 1.2.0). WhatsHap (version 0.18) software was used for linkage analysis (cis or trans) in the long read–based phasing.[19] The Integrative Genomics Viewer (https://software.broadinstitute.org/software/igv/) was used to elicit the alignments of variant and wild-type molecules. The large deletion variants were further confirmed by Gap-PCR using primers spanning the known breakpoints. Mutations in α- and β-globin genes were confirmed by PCR reverse-dot hybridization or DNA sequencing.

RESULTS

A total of 70 subjects who were suspected to be rare *HBA1/2* and *HBB* gene variant carriers were recruited from Quanzhou Women's and Children's Hospital. As shown in Figure 1, routine rare thalassemia identification and TGS were simultaneously conducted. In the routine process, Gap-PCR detection covering the $--^{THAI}$, $-\alpha^{27.6}$, and $-\alpha^{21.9}$ variants and DNA sequencing of the *HBA1/2* gene were performed in the suspected rare α-thalassemia carriers; Gap-PCR detection containing the Taiwanese, $^{G}\gamma^{+}(^{A}\gamma\delta\beta)^{0}$, and SEA-HPFH variants and DNA sequencing of the β-globin gene were performed in patients who were suspected to be rare β-thalassemia carriers. In a blinded manner, the genomics DNA of the subjects was sent to Berry Genomics Laboratory for CATSA based on TGS technology and data analysis.

In this study, DNA sequencing and Gap-PCR technology were conducted as the routine procedure for detection of rare α- and β-globin variants in the 70 patients, including 55 who were suspected to be rare α-globin gene variant carriers and 15 who were suspected to be rare β-globin gene variant carriers. As delineated in Figure 1 and the Table, 15 rare α-thalassemia gene variants and 1 Hb variant were identified in the 55 patients who were suspected to be rare α-globin gene variant carriers, among whom 6 were carriers of $--^{THAI}/\alpha\alpha$, which was the most common of the rare α-globin gene variants. In the remaining 9 patients who harbored α-thalassemia gene variants, IVS-II-55 (T>G) (HBA2:c.300+55T>G) and $-\alpha^{27.6}$ were first identified in the Quanzhou region; CD15 (G>A) (HBA2:c.46G>A) and CD117/118 (+TCA) (HBA1:c.354_355insTCA) were first reported in Fujian Province, China. Hb Maranon (AGG>GGG) (HBA2:c.94A>G), IVS-II-55 (G>T) (HBA1:c.300+55G>T), and Cap+14 (C>G) [HBA1:c.-24C>G(or HBA2)] were first identified in the Chinese population (Table). In the 15 patients who were suspected to be rare β-globin gene variant carriers, 7 patients harbored rare β-globin variants, including rare β-thalassemia variants in 4 patients and rare Hb variants in 3 patients (Table). To our knowledge, the mutation of CD6 (GAG>AAG) (HBB:c.19G>A) had never been described in Fujian Province before.

TGS-based CATSA covering 2101 variants was also performed in a blinded manner to reveal the molecular characterization of α- and β-globin genes in the enrolled subjects. As shown in the Table, 29 subjects were detected as harboring α- or β-globin gene variants, including 18 carrying rare α-globin gene variants and 11 carrying β-globin gene variants. TGS yielded an 8.57% (6 of 70) increment of rare α- and β-globin gene variants as compared with the traditional procedures. The examples of SVs including $--^{THAI}$, $--^{FIL}$, $-\alpha^{27.6}$, and $\alpha\alpha\alpha^{anti3.7}$ were displayed in Integrative Genomics Viewer to share the variants (Figure 2, A through D). Among them, α-globin gene variants $--^{FIL}$ and $\alpha\alpha\alpha^{anti3.7}$ were detected by TGS but missed by the conventional rare thalassemia detection process (Figure 2, B and D). As for β-globin gene variants, 4 cases including $\beta^{3.5kb}/\beta^{N}$, $\beta^{IVS\text{-}II\text{-}672}/\beta^{N}$, or $\beta^{IVS\text{-}II\text{-}806}/\beta^{N}$ were identified by TGS but missed by routine rare thalassemia detection (Table). Two subjects who harbored β-thalassemia 3.5-kilobase (kb) gene deletion were first identified in Fujian Province (Figure 2, E), which, to the best of our knowledge, was also the second report in the Chinese population.

SNVs and small insertions and deletions in α-globin genes were identified by TGS in suspected cases of rare α-thalassemia that exhibited a decreased level of HbA_2 and were without common α-globin gene variants; these are displayed in Figure 3, A through F. As shown in Figure 3, A, the variant of HBA1:c.-24C>G was first identified in the Chinese population. HBA1:c.354_355insTCA led to in-frame insertion of Ser and Hb Phnom Penh (Wexham) (Figure 3, B). HBA2:c.300+55T>G was a polymorphic SNV that may lead to α-thalassemia silent (Figure 3, C). A patient with heterozygous HBA1:c.300+55G>T mutation exhibited mild anemia and high levels of MCV and MCH, accompanied with megaloblastic anemia (Figure 3, D). The mutation

Variants of Rare α- and β-Globin Genes Detected by Third-Generation Sequencing (TGS) and the Traditional Method

Types	Variants	TGS	Traditional Process
Variants in α-globin gene	$--^{THAI}/\alpha\alpha$	6	6
	$\alpha\alpha^{IVS\text{-}II\text{-}55(T>G)\ in\ \alpha2}/\alpha\alpha$	2	2
	$\alpha\alpha^{IVS\text{-}II\text{-}55(G>T)\ in\ \alpha1}/\alpha\alpha$	1	1
	$\alpha\alpha^{CD117/118}/\alpha\alpha$	1	1
	$\alpha\alpha^{HBA1:c.-24C>G}/\alpha\alpha$	1	1
	$\alpha\alpha^{Maranon}/\alpha\alpha$	1	1
	$\alpha\alpha^{Owari}/\alpha\alpha$	1	1
	$\alpha\alpha^{CD15}/\alpha\alpha$	1	1
	$\alpha\alpha\alpha^{anti3.7}/\alpha\alpha$	1	0
	$--^{FIL}/\alpha\alpha$	1	0
	$-\alpha^{27.6}/--^{SEA}$	1	1
	Hb G-Honolulu	1[b]	1
Variants in β-globin gene	$\beta^{3.5kb}/\beta^{N}$	2	0
	$\beta^{IVS\text{-}II\text{-}672}/\beta^{N}$	2	1
	$\beta^{IVS\text{-}II\text{-}806}/\beta^{N}$	1	0
	$\beta^{CD54\text{-}58}/\beta^{N}$	1	1
	$\beta^{IVS\text{-}I\text{-}130}/\beta^{IVS\text{-}II\text{-}81}$	1	1
	$\beta^{CD39,IVS\text{-}II\text{-}81}/\beta^{IVS\text{-}II\text{-}806}$	1[a]	1
	HbC	1	1
	HbG-Coushatta	1	1
	Hb Miyashiro	1[b]	1
Total		**29**	**23**

[a] In this case, $--^{SEA}/\alpha\alpha$ was compounded.
[b] In this case, $-\alpha^{4.2}/\alpha\alpha$ was compounded.

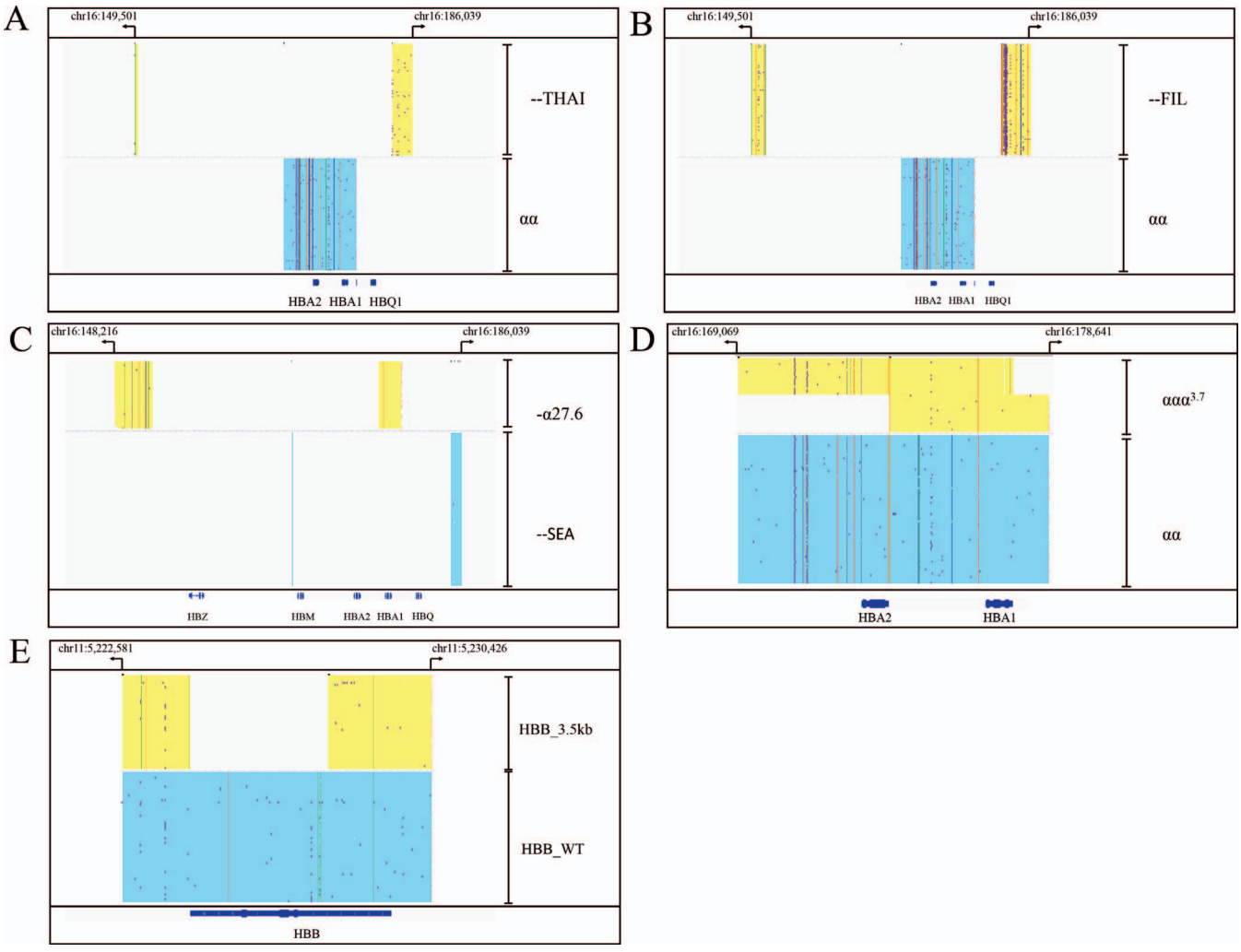

Figure 2. *The structural variants in α- and β-thalassemia detected by third-generation sequencing. A, Rare deletional α-thalassemia of –^{THAI}/αα. B, Rare deletional α-thalassemia of –^{FIL}/αα. C, Rare deletional α-thalassemia of -α$^{27.6}$/–^{SEA}. D, α-globin gene tristrain of ααα$^{anti3.7}$/αα. E, Rare β-thalassemia 3.5-kb gene deletion.*

HBA2:c.46G>A involved a GGT to AGT change at codon 16 within exon 1 of the *HBA2* gene, resulting in a Gly to Ser replacement (Figure 3, E). Mutation of HBA2:c.94A>G generated a premature termination codon and made hyperunstable Hb variant Hb Maranon, which was also the first report in the Chinese population (Figure 3, F).

As for the allele mutation of globin gene in cis or trans, the TGS technology could effectively conduct single-nucleotide polymorphism linkage analysis in the long read–based phasing. In this study, the rare compound heterozygous mutation β$^{IVS-I-130}$/β$^{IVS-II-81}$ was detected by the traditional process in a subject who exhibited an increased level of HbA_2 and normal common thalassemia detection results; then, the TGS technology detection result verified the allele mutation in trans. In addition, 3 β-thalassemia gene variants, including CD39 (C>T) (HBB:c.118C>T) (Figure 4, A), IVS-II-81 (C>T) (HBB:c.315+81C>T) (Figure 4, B), and IVS-II-806 (G>C) (HBB:c.316-45G>C) (Figure 4, C), compounded with a heterozygous –^{SEA} variant (Figure 4, D) were identified in a patient with high level of HbA_2 by conventional methods. But it was impossible to determine whether the mutations were located in the same (cis configuration) or a different chromosome (trans configuration) using the conventional methods. Subsequently, the result of TGS detection confirmed the *HBB* variants were in trans, among which HBB:c.118C>T and HBB:c.315+81C>T mutations were in cis (Figure 4, A and B). The result of TGS-based CATSA was consistent with the subsequent family analysis and verification.

DISCUSSION

Thalassemia intermedia or major causes a heavy burden on families and society because no effective treatment is available at present.[20,21] In China, the traditional 3-level screening for thalassemia through hematology and thalassemia genetic testing can detect most thalassemia carriers. However, conventional thalassemia gene testing targets only the common mutations in the Chinese population, and therefore rare thalassemia gene mutations are often misdiagnosed. If one member of a couple is diagnosed as a thalassemia gene carrier, their baby is more likely to be born with thalassemia intermedia or major. Previous studies have shown diverse and complex genotypes of thalassemia in Fujian Province, China, and an increased number of rare thalassemia variants have been reported,[3–5] which were generally detected by DNA sequencing or next-generation

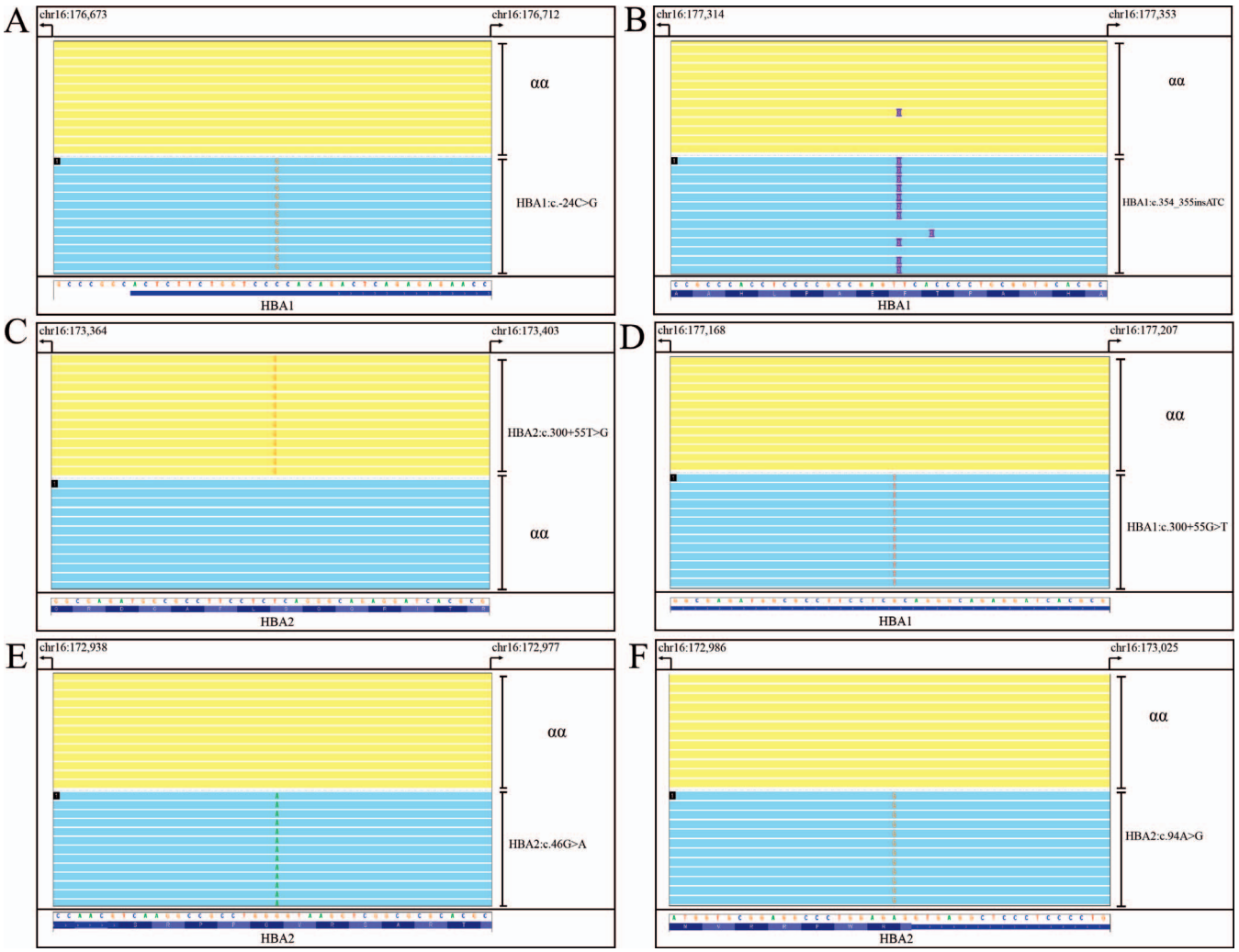

Figure 3. *Rare mutations of α- and β-thalassemia detected by third-generation sequencing. A, Mutation of Cap+14 (C>G) (HBA1:c.-24C>G) observed in* HBA1 *gene. B, Rare mutation of CD117/118 (+TCA) (HBA1:c.354_355insTCA) in* HBA1 *gene. C, IVS-II-55 (T>G) (HBA2:c.300+55T>G) mutation in* HBA2 *gene. D, IVS-II-55 (G>T) (HBA1:c.300+55G>T) mutation in* HBA1 *gene. E, Rare mutation of CD15 (G>A) (HBA2:c.46G>A) present in* HBA2 *gene. F, Mutation of Hb Maranon (AGG>GGG) (HBA2:c.94A>G) in* HBA2 *gene.*

sequencing combined with Gap-PCR technology.[22–24] In the current study, we used a new TGS-based CATSA method and conventional DNA sequencing and Gap-PCR to detect thalassemia gene variants cis or trans in a blinded manner. The results showed that TGS technology could effectively and accurately detect some deletional and nondeletional thalassemia variants that the conventional methods could not detect.

A recent double-blind parallel study[12] showed that TGS technology could accurately detect all variants of thalassemia with an accuracy rate of 100%, as compared with the traditional thalassemia gene detection process. In a large-scale prospective study covering 1759 samples for thalassemia variant investigation by TGS-based CATSA, Liang et al[17] reported that there was no false-negative result with the TGS technology compared with the standard thalassemia detection methods. They believed that TGS could serve as an effective carrier screening for couples at risk. In the present study, we found a 7.14% incremental yield in rare α- and β-globin gene variants by TGS technology as compared with the conventional detection methods. Rare variants of α-globin gene, $--^{FIL}$ and $\alpha\alpha\alpha^{anti3.7}$, were detected by TGS but missed by the routine methods. The $--^{FIL}$ variation was beyond the detection range of the traditional methods, and $\alpha\alpha\alpha^{anti3.7}$ exhibited an increased level (6.5%) of HbA2, suggesting that rare β-globin gene variants may exist; also, a duplication α-globin gene was identified by TGS technology. The α-globin gene tristrain is known to aggravate the symptoms of anemia and cause β-thalassemia intermedia when it combines with β-thalassemia. We therefore should pay attention to both α- and β-globin gene variants in the increased level of HbA_2. Also, it is important to further understand the hematologic phenotypes of rare thalassemia variants and choose appropriate detection methods, especially in regions of high thalassemia prevalence. With respect to β-globin gene variants, we identified 2 cases of $\beta^{3.5kb}/\beta^{N}$, 1 case of $\beta^{IVS\text{-}II\text{-}672}/\beta^{N}$, and 1 case of $\beta^{IVS\text{-}II\text{-}806}/\beta^{N}$ using the TGS technology, but they were missed by the traditional methods. The 3.5-kb deletion (NC_000011.10: g.5224302-5227791del3490bp) leading to β^{0}-thalassemia, which removes the β-globin gene promoter and the whole β-globin gene, has occasionally been identified in Thailand.[25–27] In 2018, He et al[28] first described this variant in the Chinese population. Our study identified 2 subjects who

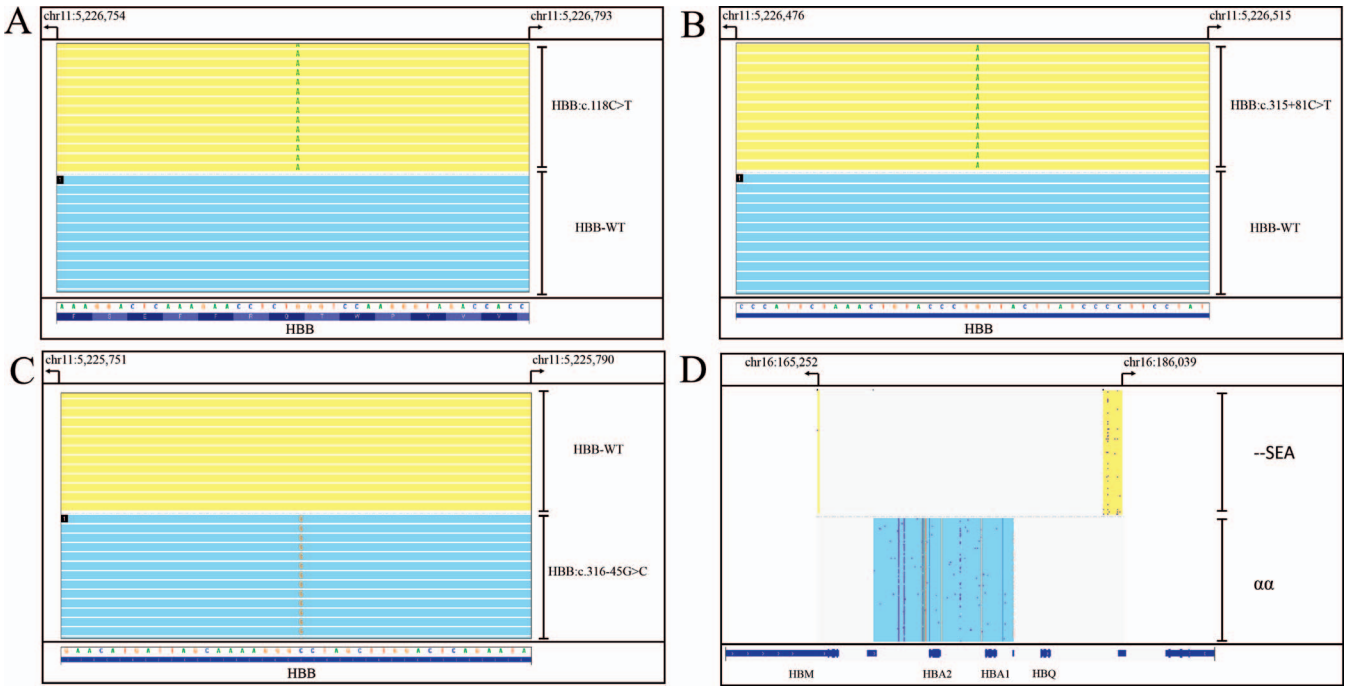

Figure 4. *The variants of thalassemia in cis or trans were effectively distinguished by third-generation sequencing in a case with several variants. A through C, Mutation of CD39 (C>T) (HBB:c.118C>T) and IVS-II-81 (C>T) (HBB:c.315+81C>T) was observed in a linked single-nucleotide polymorphism (in cis), and mutation of IVS-II-806 (G>C) (HBB:c.316-45G>C) was listed in trans. D, A deletional α-thalassemia of $--^{SEA}/\alpha\alpha$ was also combined in the case.*

carried the rare β-thalassemia 3.5-kb gene deletion; this is also the second report in the Chinese population. Additionally, missed detection of the mutation of IVS-II-672 (A>C) and IVS-II-806 (G>C) by the traditional methods may be due to diversity of the hematologic parameters, which may exhibit normal or low levels of HbA_2.

Of the 70 subjects included in this study, 29 cases with rare α- and β-globin gene variants were identified, with a detection rate of 41.43%. Our study is the first to our knowledge to describe in the Chinese population the Hb Maranon (AGG>GGG) (HBA2:c.94A>G) mutation, which has been reported to be an unstable Hb variant occurring in codon 31 of the *HBA2* gene that produces the α-thalassemia phenotype.[29] The patient in our study who carried this mutation leading to α^+-thalassemia exhibited a normal MCV level (84 fL) and Hb value (113×10^{12} g/L), and a slightly low level of MCH (26.5 pg) without iron-deficiency anemia, which is consistent with the previous study.[29] In addition, the HBA1:c.300+55G>T mutation has never, to our knowledge, been described before. It was found in our study that the patient exhibited mild anemia (90×10^{12} g/L) and high levels of MCV and MCH, accompanied with megaloblastic anemia. It was for the first time that we identified the rare HBA1:c.-24C>G variant in the Chinese population. The Cap+14 (C>G) [HBA1:c.-24C>G (or HBA2)] mutation reported in the literature typically occurred on the *HBA2* gene. This HBA1:c.-24C>G variant was first reported in 2020.[30] Our study is the first to describe the variants of HBA2:c.46G>A and HBA1:c.354_355insTCA in Fujian Province. Previous studies first reported the novel variant of HBA2:c.46G>A in the Chinese population and found a GGT→AGT in the *HBA2* gene and a Gly→Ser replacement at α15(A13).[31,32] Hb Wexham [a117(GH5) and 118(H1) insertion Ser (HBA1:c.354_355insTCA)], which occurs at CD117/118 in exon 3 of the α1-globin gene, leading to α^+-thalassemia, has rarely been reported in the literature.[33,34]

Our study has demonstrated the overt advantages of TGS technology in the diagnosis of thalassemia gene variants, including cis-trans configuration, α-globin gene tristrain, and homologous gene mutation analysis.

CONCLUSIONS

Our research has initially demonstrated the application value of TGS-based CATSA in detecting rare thalassemia. In addition, we identified several rare globin gene variants, which may enrich the mutation spectrum of thalassemia in China. It is proposed that CATSA testing be performed in individuals with positive hematologic screening results.

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