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RESEARCH ARTICLE

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# Detection of hemoglobin H disease by long molecule sequencing

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## Abstract

**Background:** Hemoglobin H (Hb H) disease is a moderate-to-severe form of α-thalassemia (α-thal), and parts of patients may require intermittent transfusion therapy, especially during intercurrent illness. However, rare Hb H diseases remain undetected using routine methods being outside of the testing scope. In this study, we present an approach to detecting Hb H disease by long molecule sequencing (LMS).

**Methods:** A total of 206 known genotype samples were collected and carried to blind detected by LMS on the PacBio Sequel platform. Circular consensus sequencing reads were aligned to the hg19 reference genome using Free-Bayes finished LMS. LMS accuracy would be compared with routine methods, including Gap-PCR and PCR-Reverse dot blot hybridization (PCR–RDB).

**Results:** The assay could detect carriers of both deletion and point mutations. It had an overall accuracy of 100% when compared with routine methods. In addition, LMS detected six mutations based on routine methods and corrected three case results. Hb H diseases were identified using LMS, whether a common or rare genotype, a deletion or non-deletion genotype. However, two cases of Hb H disease were misdiagnosed using routine methods.

**Conclusions:** Long molecule sequencing can be suggested as a rapid and reliable assay to detect probable carriers of hemoglobinopathies. LMS accurately identified the common and rare genotypes of Hb H disease.

**KEYWORDS**
Hb H disease, long molecule sequencing, thalassemia, third-generation sequencing

## 1 | INTRODUCTION

Hemoglobinopathy is one of the most common inherited disorders in southern China, including thalassemia and hemoglobin (Hb) variant.[1–3] α-globin gene cluster on chromosome 16 mainly has two expressed *HBA2* and *HBA1* genes, and mutations cause α-thalassemia (α-thal) in the α-globin gene cluster.[4] Deletion or dysfunction of one gene results in thalassemia silent, and two genes result in thalassemia trait; compound deletion of two genes and deletion or point mutation of one gene cause α-thal intermedia hemoglobin H disease (Hb H disease), while loss of all four genes causes Hb Bart's hydrops fetalis.[5] There are two types of Hb H disease: deletional Hb H (--/-α) and non-deletional Hb H (--/$\alpha^{T}\alpha$). In the southern China, most α-thals are caused by deletions, and the rest are caused by point mutation.[6]

Therefore, there is more deletional Hb H than non-deletion Hb H disease. However, a kit with Chinese FDA approval for the deletional thalassemia test in clinical laboratories is only used to detect --$^{SEA}$, --$^{THAI}$, -$\alpha^{3.7}$, and -$\alpha^{4.2}$ in the Chinese population.[7,8] Predictably, there will be misdiagnosed cases in our routine clinical works if the routine genetic analysis is used. In the high-prevalence area of thalassemia, it is thus important to have a rapid and reliable detection method for the deletional α-thal s test, which avoids misdiagnosing of Hb H disease or Hb Bart's hydrops fetalis.

Third-generation sequencing (TGS), as a current new molecule technique, was described previously to detect the thalassemia and Hb variants on the PacBio Sequel II platform.[9,10] Long molecule sequencing (LMS) is the main characteristic of TGS. In this study, we applied the LMS to test 206 suspected thalassemia samples, compared with the routine genetic analysis. Our results suggested that LMS, the same as routine genetic analysis, could detect deletional mutations and point mutations; moreover, it also detected rare mutations to diagnose Hb H disease. So, it can be an effective and reliable approach for thalassemia screening of suspected individuals with rare mutations or outside of the detection scope.

## 2 | MATERIALS AND METHODS

### 2.1 | Sample

A total of 206 subjects with suspected thalassemia and Hb variants were collected from thalassemia screening in the People's Hospital of Guangxi Zhuang Autonomous Region. Their age ranged from 9-month to 65 years. The study was approved by the People's Hospital of Guangxi Zhuang Autonomous Region ethics committee. All the individuals provided informed written consent. Whole blood samples were collected in tubes with K2-EDTA.

### 2.2 | Complete blood count and hemoglobin analysis

Currently, complete blood count (CBC) and Hb analysis are routinely used as screening tests for thalassemia. Red cell indices were determined with an automated hematology analyzer (Sysmex XT 1800i; Sysmex Corporation). Subjects with a mean corpuscular volume (MCV) of <82 fl or a mean corpuscular (MCH) of <27 pg were suspected of thalassemia. Hb analysis was performed by capillary electrophoresis (CE) (Capillary 2 Flex Piercing; Sebia). When HbA2 was>3.5%, β-thalassemia but not excluded α-thal was considered, whereas when HbA2 was<2.5%, α-thal was suspected (This is the thalassemia screening process recommended by our government policy, but we have to explain that HbA2 value of 2.5%–3.5% cannot rule out the presence of α-thal.). In addition, abnormal peaks of CE are also regarded as positive screening results. All the suspected samples were performed by routine genetic analysis and LMS, respectively.

### 2.3 | Gap-PCR and PCR–Reverse dot blot hybridization (PCR–RDB) (routine genetic analysis/routine methods, RGA)

Gap-PCR and PCR–RDB as routine genetic analysis methods are widely used in the clinical laboratories in China. Gap-PCR was applied to detect the common deletion-type α-thal in the Chinese population, including --$^{SEA}$, --$^{THAI}$, -$\alpha^{3.7}$, -$\alpha^{4.2}$. PCR–RDB was conducted to detect non-deletion α-globin mutations Hb Constant Spring (Hb CS, *HBA2*: c.427T>C, p.Ter143GlnextTer31), Hb Westmead (Hb WS, *HBA2*: c.369C>G, p.His123Gln), Hb Quong Sze (Hb QS, *HBA2*: c.377T>C, p.Leu126Pro). PCR-RDB was also carried out to detect the 17 common β-thalassemia mutations: -32 (*HBB:* c.-82C>A), −30 (*HBB*: c.-80T>C), −29 (*HBB*: c.-79A>G), −28 (*HBB*: c.-78A>G), CDs14-15 (*HBB*: c.45_46insG), CD17 (*HBB*: c.52A>T, p.Lys18Ter), CD26 (*HBB*: c.79G>A, p.Glu27Lys), CDs27-28(*HBB*: c.84_85insC), CD31 (*HBB*: c.94delC), CDs41-42 (*HBB*: c.124_127delTTCT), CD43(*HBB*: c.130G>T, p.Glu44Ter), CDs71-72 (*HBB*: c.216_217insA), IVS-I-1 (*HBB*: c.92 + 1G > T), IVS-1-5 (*HBB*: c.92 + 5G > C), IVS-II-654 (*HBB*: c.316–197C > T), Int (*HBB*: c.2 T > G), and CAP (*HBB*: c.-50A>C). Genomic DNA was extracted from 100 μl whole blood samples using the DNA extraction kit (YanengBio). Gap-PCR (Yishengtang Bio) and PCR–RDB (Yaneng Bio) were done following the kit's instructions.

### 2.4 | Long molecule sequencing and data analysis

As previous studies described,[9,10] genomic DNA samples prepared with the DNA blood mini kit (QIAGEN) were performed multiplex long PCR using an assay Kit (Berry Genomics) after assessing DNA quality. Each PCR product was ligated with a unique identifying barcoded adaptor following purification and end-repair. Single-molecule Real-time (SMRT) bell libraries were prepared using the Sequel Binding and Internal Ctrl Kit 3.0 (Pacific Biosciences). Then the DNA-polymerase complexes were loaded on SMRT cells, and sequencing was performed on the PacBio Sequel platform. In order to obtain aligned subreads, raw reads were processed by the Lima application and the circular consensus sequencing (CCS) software application. The filtered CCS reads were aligned to the human reference genome (hg19), and NGMLR (version 0.2.6) or BLASR and variants called using FreeBayes (version 1.2.0) was selected according to different purposes. Alignments of variant and wild-type molecules were presented by Integrative Genomics Viewer (IGV).

### 2.5 | Confirmation of rare mutations detected by LMS

Rare α-thal deletions were confirmed by Gap-PCR using a rare deletional thalassemia kit (Yaneng Bio). Point mutations of the α-globin gene and β-globin gene were confirmed by Sanger sequencing as we previously described.[11,12]

**TABLE 1** The α-globin and β-globin gene mutation spectrum in this study, excluding Hb H disease

| Mutation type | Number | Detection by RGA | Detection by LMS | Concordance of two methods |
|---|---|---|---|---|
| α-thalassemia/Hb variant | | | | |
| $--^{SEA}/\alpha\alpha$ | 48 | 48 | 48 | Y* |
| $-\alpha^{3.7}/\alpha\alpha$ | 17 | 17 | 17 | Y |
| $\alpha^{CS}\alpha/\alpha\alpha$ (Hb CS) | 8 | 8 | 8 | Y |
| $-\alpha^{4.2}/\alpha\alpha$ | 4 | 4 | 4 | Y |
| $\alpha^{WS}\alpha/\alpha\alpha$ (Hb WS) | 2 | 2 | 2 | Y |
| $\alpha^{QS}\alpha/\alpha\alpha$ (Hb QS) | 1 | 1 | 1 | Y |
| $-\alpha^{3.7}/\alpha^{WS}\alpha$ | 1 | 1 | 1 | Y |
| $-\alpha^{4.2}/\alpha^{CS}\alpha$ | 1 | 1 | 1 | Y |
| $--^{SEA}/--^{SEA}$ | 1 | 1 | 0 | N |
| β-thalassemia/Hb variant | | | | |
| $\beta^{CDs41-42}/\beta^{N}$ | 22 | 22 | 22 | Y* |
| $\beta^{CD17}/\beta^{N}$ | 5 | 5 | 5 | Y |
| $\beta^{-28}/\beta^{N}$ | 4 | 4 | 4 | Y |
| $\beta^{CDs71-72}/\beta^{N}$ | 3 | 3 | 3 | Y |
| $\beta^{IVS-II-654}/\beta^{N}$ | 1 | 1 | 1 | Y |
| $\beta^{CDs27/28}/\beta^{N}$ | 1 | 1 | 1 | Y |
| $\beta^{CD26}/\beta^{N}$ (Hb E) | 1 | 1 | 1 | Y |
| $\beta^{IVS-I-1}/\beta^{N}$ | 1 | 1 | 1 | Y |
| Compound α and β-thalassemia | | | | |
| $\alpha^{CS}\alpha/\alpha\alpha$, $\beta^{CDs41-42}/\beta^{N}$ | 2 | 2 | 2 | Y |
| $-\alpha^{3.7}/\alpha\alpha$, $\beta^{-28}/\beta^{N}$ | 1 | 1 | 1 | Y |
| $-\alpha^{4.2}/\alpha\alpha$, $\beta^{CDs41-42}/\beta^{N}$ | 1 | 1 | 1 | Y* |
| $--^{SEA}/\alpha\alpha$, $\beta^{CDs71-72}/\beta^{N}$ | 1 | 1 | 1 | Y |
| $\alpha^{QS}\alpha/\alpha\alpha$, $\beta^{-28}/\beta^{N}$ | 1 | 1 | 1 | Y |
| $\alpha^{WS}\alpha/\alpha\alpha$, $\beta^{CDs41-42}/\beta^{N}$ | 1 | 1 | 1 | Y |
| $\alpha^{WS}\alpha/\alpha\alpha$, $\beta^{CDs71-72}/\beta^{N}$ | 1 | 1 | 1 | Y |
| $-\alpha^{3.7}/\alpha\alpha$, $\beta^{IVS-II-654}/\beta^{N}$ | 1 | 1 | 1 | Y* |

*Note*: Y*: The detection results of the two methods are consistent, but the LMS has additional detections.

Abbreviations: LMS, long molecule sequencing; N, No; RGA, routine genetic analysis; Y, Yes.

**TABLE 2** Hematological phenotypes and genotypes of Hb H disease in this study

| Mutation type | Sex | Age (year) | Hb (g/L) | MCV (fl) | MCH (pg) | Hb A (%) | Hb A2 (%) | Hb F (%) | Hb H (%) | Hb Bart's (%) | Detection by RGA | Detection by LMS |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| $--^{SEA}/-\alpha^{3.7}$ | M | 45 | 98 | 59.4 | 16.9 | 97.8 | 1.1 | 0 | 1.1 | 0 | Y | Y |
| $--^{SEA}/-\alpha^{3.7}$ | F | 31 | 89 | 57.3 | 17.4 | 98.0 | 1.3 | 0.2 | 0.5 | 0 | Y | Y |
| $--^{SEA}/-\alpha^{3.7}$ | F | 5 | 89 | 53.8 | 15.5 | 96.0 | 1.5 | 0.3 | 1.7 | 0.5 | Y | Y |
| $--^{SEA}/-\alpha^{3.7}$ | F | 35 | 86 | 65.6 | 18.6 | 96.5 | 0.9 | 0 | 2.4 | 0.2 | Y | Y |
| $--^{SEA}/-\alpha^{4.2}$ | F | 25 | 89 | 52.5 | 16.9 | 98.7 | 1.3 | 0 | 0 | 0 | Y | Y |
| $--^{SEA}/-\alpha^{4.2}$ | F | 9/12 | 88 | 45.0 | 15.7 | 96.1 | 1.6 | 2.3 | 0 | 0 | Y | Y |
| $--^{SEA}/\alpha^{CS}\alpha$ | M | 27 | 82 | 74.2 | 20.1 | 87.6 | 1.1 | 0 | 8.5 | 0 | Y | Y |
| $--^{SEA}/\alpha^{WS}\alpha$ | M | 23 | 122 | 74.8 | 23.1 | 97.8 | 2.2 | 0 | 0 | 0 | Y | Y |
| $--^{SEA}/\alpha^{WS}\alpha$ | M | 26 | 145 | 63.8 | 20.4 | 97.5 | 2.5 | 0 | 0 | 0 | Y | Y |
| $--^{SEA}/-\alpha^{3.7}$ $\beta^{CDs41-42}/\beta^{-28}$ | M | 30 | 105 | 55.6 | 25.1 | 32.7 | 10.9 | 56.4 | 0 | 0 | Y | Y |
| $--^{SEA}/-\alpha^{27.6}$ | F | 1 | 82 | 53.7 | 15.3 | 98.3 | 1.7 | 0 | 0 | 0 | N | Y |
| $--^{SEA}$/Fusion gene | M | 27 | 108 | 75.5 | 21.2 | 67.5 | 0.5 | 0 | 31.0 | 1.0 | N | Y |

Abbreviations: LMS:long molecule sequencing; RGA: routine genetic analysis;

**TABLE 3** Different results detected by LMS and RGA in this study

| Number | Detection by RGA | Detection by LMS | Comment |
|---|---|---|---|
| 1 | $--^{SEA}/\alpha\alpha$ | $--^{SEA}$/fusion gene | Omission diagnosis |
| 2 | $--^{SEA}/--^{SEA}$ | $--^{SEA}/-\alpha^{27.6}$ | Omission diagnosis |
| 3 | $-\alpha^{4.2}/\alpha\alpha$, $\beta^{CDs41-42}/\beta^{N}$ | $-\alpha^{4.2\text{-Q-Thailand}}/\alpha\alpha$, $\beta^{CDs41-42}/\beta^{N}$ | Omission diagnosis |
| 4 | $-\alpha^{3.7}/\alpha\alpha$, $\beta^{IVS-II-654}/\beta^{N}$ | HK$\alpha\alpha/\alpha\alpha$, $\beta^{IVS-II-654}/\beta^{N}$ | Mistake diagnosis |
| 5 | $\alpha\alpha/\alpha\alpha$, $\beta^{N}/\beta^{N}$ | $\alpha\alpha/\alpha\alpha$, $\beta^{CD7}/\beta^{N}$ (Hb G-Siriraj) | Omission diagnosis |
| 6 | $\alpha\alpha/\alpha\alpha$ | $\alpha\alpha/\alpha^{CD13}\alpha$ (Hb Binyang) | Omission diagnosis |
| 7 | $--^{SEA}/\alpha\alpha$, $\beta^{N}/\beta^{N}$ | $--^{SEA}/\alpha\alpha$, $\beta^{-50}/\beta^{N}$ | Omission diagnosis |
| 8 | $\alpha\alpha/\alpha\alpha$ | $\alpha\alpha/\alpha^{CD30}\alpha$ | Omission diagnosis |
| 9 | $\alpha\alpha/\alpha\alpha$, $\beta^{CDs41-42}/\beta^{N}$ | $\alpha\alpha/\alpha^{CD27}\alpha$(HbHekinanII), $\beta^{CDs41-42}/\beta^{N}$ | Omission diagnosis |

Abbreviations: LMS, long molecule sequencing; RGA, routine genetic analysis.

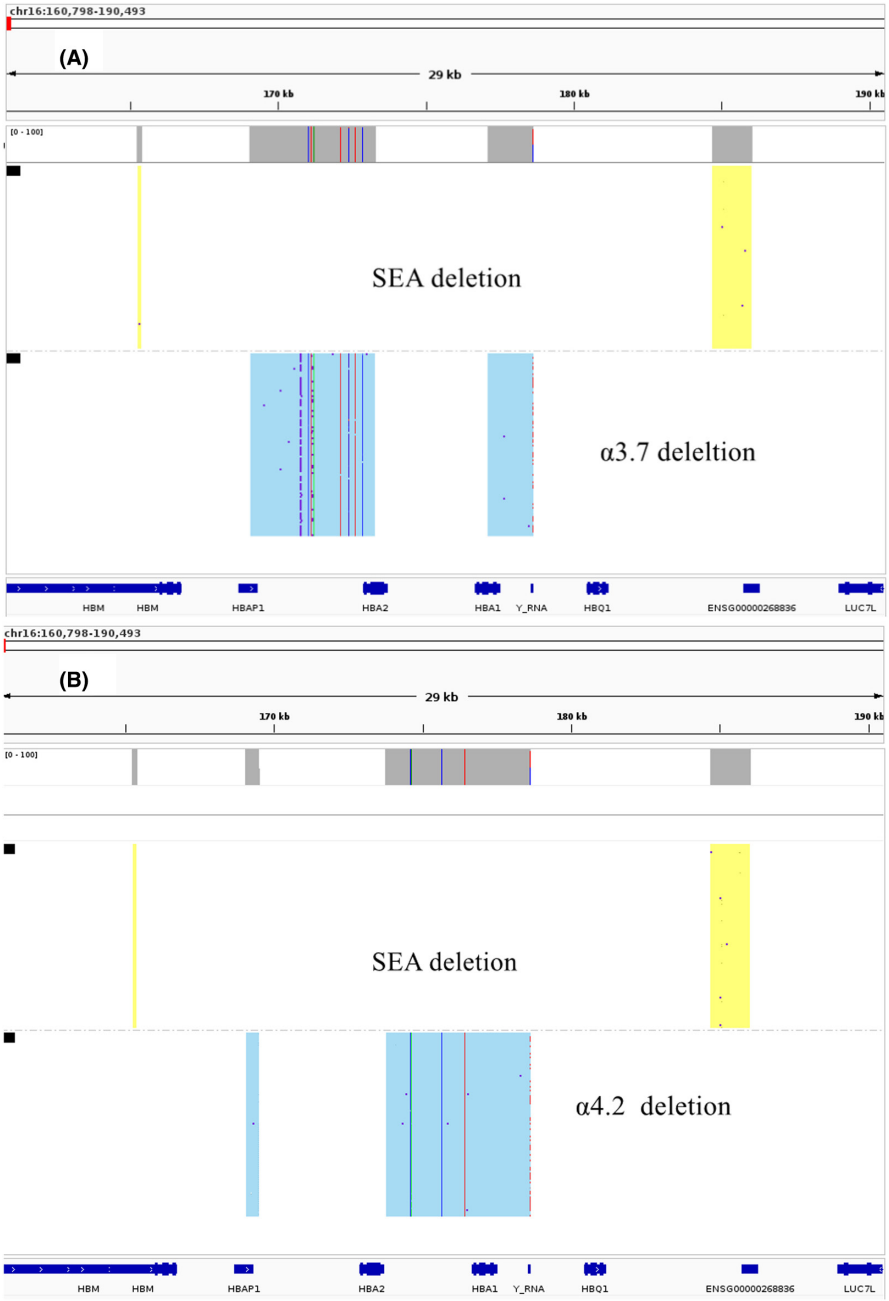


**FIGURE 1** Integrative Genomics Viewer (IGV) plots of common deletional Hb H disease, including $--^{SEA}/-\alpha^{3.7}$ (A) and $--^{SEA}/-\alpha^{4.2}$ (B). IGV showed the $--^{SEA}$ deletion in the yellow area, the $-\alpha^{3.7}$ deletion in the blue area (A), and the $-\alpha^{4.2}$ deletion in the blue area (B).

## 3 | RESULTS

### 3.1 | Gap-PCR and PCR–RDB

Of the 206 suspected cases, 140 subjects were diagnosed with thalassemia and Hb variants using routine Gap-PCR and PCR–RDB methods. Of these thalassemias and Hb variants, the $--^{SEA}/\alpha\alpha$ mutation (34.3%) was the most common α-thal genotype, followed by $-\alpha^{3.7}/\alpha\alpha$ (12.1%) and $\alpha^{CS}\alpha/\alpha\alpha$ (5.7%); the most common β-thalassemia mutation was $\beta^{CDs41-42}/\beta^{N}$ (15.7%), followed by $\beta^{CD17}/\beta^{N}$ (3.6%) and $\beta^{-28}/\beta^{N}$ (2.9%) (Table 1). Besides, 9 cases were diagnosed with compound α and β-thalassemia, including eight genotypes (Table 1).

In this study, 10 cases were detected with Hb H disease. Among them, 6 cases were diagnosed with deletional Hb disease ($--^{SEA}/-\alpha^{3.7}$ and $--^{SEA}/-\alpha^{4.2}$), while 3 cases were non-deletional Hb H disease ($--^{SEA}/\alpha^{CS}\alpha$ and $--^{SEA}/\alpha^{WS}\alpha$). A case of compound Hb H and β-thalassemia intermedia was accidentally detected. As Table 2 illustrates, 8 out of 10 Hb H diseases showed anemia, and the remaining two cases of $--^{SEA}/\alpha^{WS}\alpha$ had no anemia but decreased MCV and MCH values.

### 3.2 | LMS analysis

After the raw data was processed, we obtained high-quality sequencing data. LMS identified 81 carriers of α-thal or α-chain Hb variants, 38 carriers of β-thalassemia, and 11 carriers of compound α-thal (α-chain Hb variants) and β-thalassemia. All of the mutations detected by routine genetic analysis could also be detected by LMS (Table 1). LMS additionally identified four rare Hb variants, including Hb Hekinan II (*HBA1*:c.84G>T), Hb G-Siriraj (*HBB*: c.22G>A), Hb Binyang (*HBA2*:c.40G>T), and Hb Q-Thailand (*HBA1*:c.223G>C), which were not detected by routine genetic analysis. A case genotype was Hong Kong αα by LMS, but the routine genetic analysis showed a 3.7 deletion heterozygote (Table 3).

In addition, LMS detected 12 cases with Hb H disease (Figures 1 and 2), two more cases than routine genetic analysis. In other words, 2 cases with Hb H disease were misdiagnosed using the routine genetic analysis method (Figure 3). One case was detected as a SEA heterozygote ($--^{SEA}/\alpha\alpha$) by routine genetic analysis, but compound SEA heterozygote and ψα1–α2 fusion gene ($--^{SEA}$/Fusion gene) by LMS (Table 3). The other case was identified as a SEA homozygote

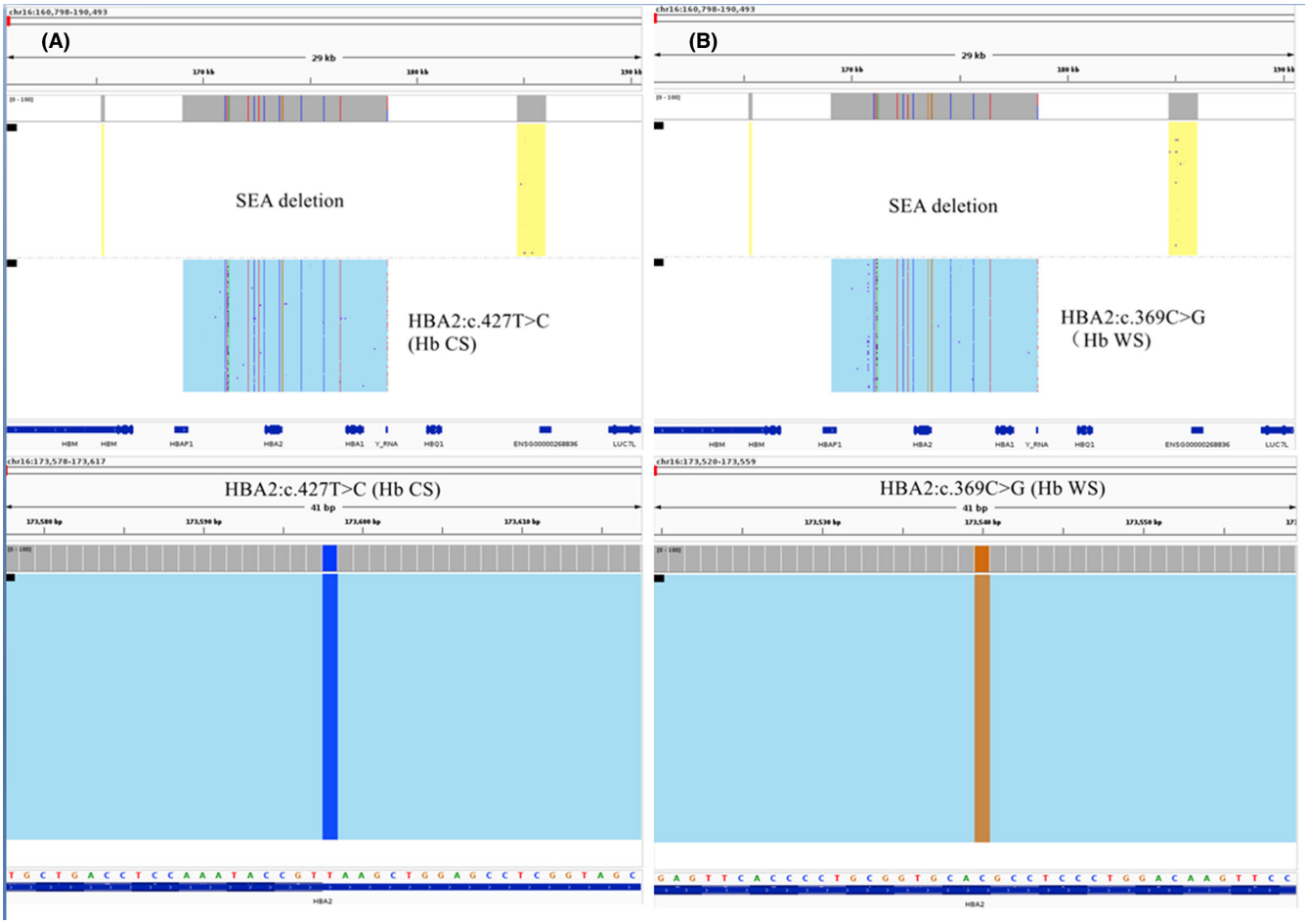


FIGURE 2 Integrative Genomics Viewer (IGV) plots of common non-deletional Hb H disease, including $--^{SEA}/\alpha^{CS}\alpha$ (A) and $--^{SEA}/\alpha^{WS}\alpha$ (B). IGV of (A) showed the $--^{SEA}$ deletion in the yellow area and one allele with $\alpha^{CS}$ mutation in the blue area. (B) revealed the $--^{SEA}$ deletion in the yellow area and one allele with $\alpha^{WS}$ mutation in the blue area.

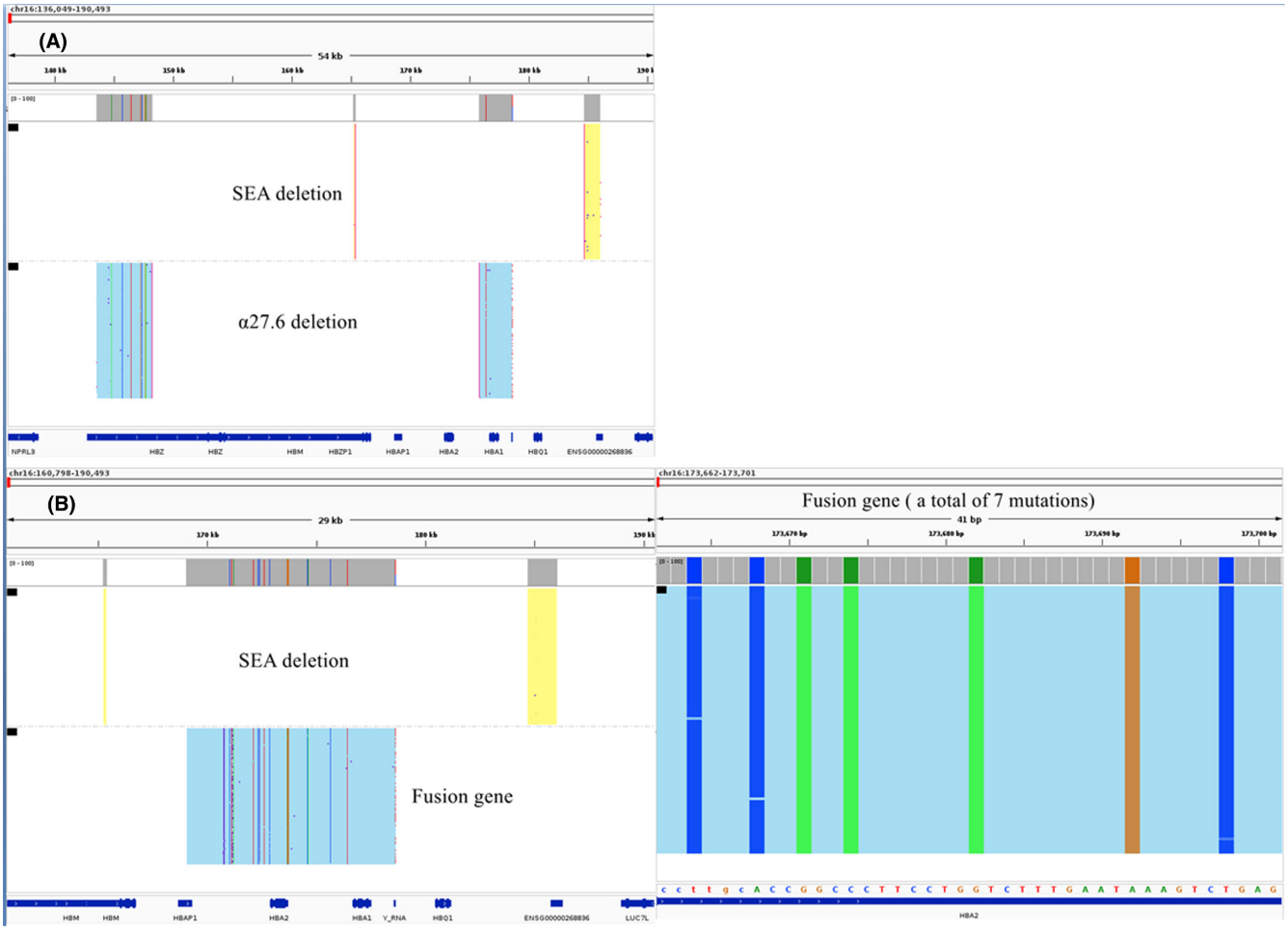


FIGURE 3 Integrative Genomics Viewer (IGV) plots of rare deletional Hb H disease, including --$^{SEA}$/-$\alpha^{27.6}$ (A) and --$^{SEA}$/Fusion gene (B). (A) Displayed the --$^{SEA}$ deletion in the yellow area and the -$\alpha^{27.6}$ deletion in the blue area. (B) Presented the --$^{SEA}$ deletion in the yellow area and the other allele with the ψα1–α2 fusion gene.

(--$^{SEA}$/--$^{SEA}$) by routine genetic analysis, but LMS presented compound SEA deletion and $\alpha^{27.6}$ deletion (--$^{SEA}$/-$\alpha^{27.6}$) (Table 3).

## 4 | DISCUSSION

In the present study, LMS, as well as the routine genetic analysis, detected 140 carriers with deletion or point mutations. In addition, nine mutations were detected, which were not detected by routine genetic analysis. The thalassemia routine kits are only used to identify the 24 common thalassemias and variants in the Chinese population, so misdiagnosis frequently occurs. However, LMS performs long fragment amplification, including entire *HBA1/2* and *HBB* genes, and then mutations can be easily viewed with the IGV tool after finished bioinformatics analysis. Therefore, since the complete gene regions are analyzed, the assay can detect mutations outside the scope or difficult to detect using routine genetic analysis.

The deletional Hb H (--/-α) disease is caused by compound heterozygote of $\alpha^{0}$- and $\alpha^{+}$-thalassemia (-α/) alleles. The common genotype of deletional Hb H diseases are --$^{SEA}$/-$\alpha^{3.7}$ and --$^{SEA}$/-$\alpha^{4.2}$, and a few are --$^{THAI}$/-$\alpha^{3.7}$ and --$^{THAI}$/-$\alpha^{4.2}$ in southern China.[13,14] Our study showed that LMS identified 6 cases of common deletional Hb H disease and a case of Hb H combined with β-thalassemia intermedia as routine methods. The difference was that 1 case was identified as SEA homozygote (--$^{SEA}$/--$^{SEA}$, Hb Bart's hydrops fetalis) by routine genetic analysis, while LMS was identified as (--$^{SEA}$/-$\alpha^{27.6}$, Hb H disease) (Figure 3A). The -$\alpha^{27.6}$ is a rare deletional mutation type and was first reported in a Chinese family, outside the routine method's test scope.[15] In fact, there were some occasional reports of similarly rare large deletion of α-chain.[16–18] Since the normal control primers of the routine genetic analysis are designed on the *HBA2* gene, it will be detected as a SEA homozygote if combined with a SEA heterozygote as long as the large fragment deletional sample involves this region. However, we considered Hb H disease combined with the comprehensive screening results. Based on the results described earlier, we recommend that LMS operate as a reliable and rapid approach for detecting these similar rare Hb H diseases as --$^{SEA}$/-$\alpha^{27.6}$.

As shown in Table 3, although most Hb H diseases were deletional types, there are also non-deletional types. The common

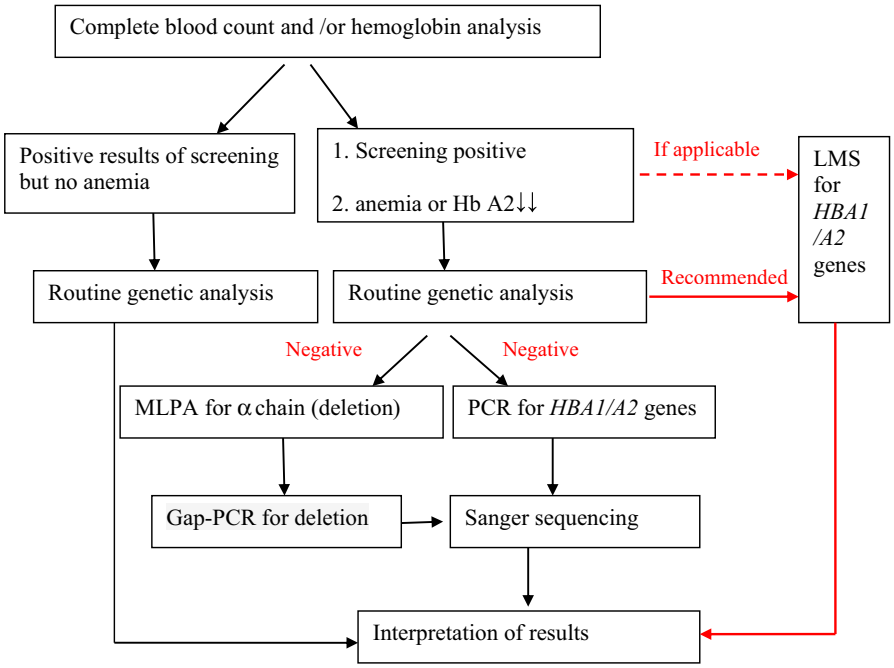


**FIGURE 4** Work flow of Hb H disease diagnosis by routine genetic analysis and LMS.

non-deletional Hb H diseases were identified either by the routine genetic analysis or LMS, such as $--^{SEA}/\alpha^{CS}\alpha$ and $--^{SEA}/\alpha^{WS}\alpha$ (Figure 2 A,B). However, we need to clarify that the phenotype of $--^{SEA}/\alpha^{WS}\alpha$ is similar to SEA heterozygotes, which is not classified as Hb H disease, but the Hb WS is a mutation that is routinely detected in China. Surprisingly, LMS detected a case of Hb H disease with compound SEA heterozygote and ψα1–α2 fusion gene, but it was displayed as a SEA heterozygote using routine genetic analysis (Figure 3B). The fusion gene is a point mutation type involving seven alleles (*HBA2*:c.*64T>C, *HBA2*:c.*68A>C, *HBA2*:c.*71G>A, *HBA2*:c.*74C>A, *HBA2*:c.*82G>A, *HBA2*:c.*92A>G, and *HBA2*:c.*98T>C), and compound SEA heterozygote and ψα1–α2 fusion gene is a rare type of Hb H disease.[19,20] This suggested that detecting non-deletional Hb H disease using LMS had high accuracy.

Hemoglobin H disease is heterogeneous, and the clinical manifestations vary widely from mild asymptomatic anemia to a severely anemic state. An accurate diagnosis of thalassemia genotype is important for couples who have pregnancies affected with Hb H disease or Hb Bart's hydrops fetail syndrome or anemia patients who need to be treated. Currently, diagnosis of Hb H disease needs several tests by routine methods, which are required for non-deletion and deletion mutations in separate tests. In addition, the test mutations of the routine kits are limited. If a rare or novel large fragment deletion is suspected, multiplex ligation-dependent probe amplification (MLPA) screening is first required, followed by Gap-PCR amplification, and then Sanger sequencing determines the break sites.[21] However, as presented in this study, LMS provided a rapid and more effective method for identifying the common Hb H genotype and enabled the detection of the rare Hb H genotype. It can avoid the time-consuming and laborious of MLPA method and possible misdiagnosis. So, for anemia patients with iron deficiency anemia excluded, or couples of childbearing age with suspicious thalassemia screening results, LMS can be considered a suitable method when the routine genetic analysis cannot be detected (Figure 4).

## AUTHOR CONTRIBUTIONS

All the authors have accepted responsibility for the entire content of this manuscript and approved its submission.

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## CONFLICT OF INTEREST

Authors state no conflict of interest.

## DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author, [Youqiong Li], upon reasonable request.

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## REFERENCES

1. Zhang J, He J, Mao X, et al. Haematological and electrophoretic characterisation of β-thalassaemia in Yunnan province of south-western China. *BMJ Open*. 2017;7(1):e013367.
2. Xiong F, Sun M, Zhang X, et al. Molecular epidemiological survey of haemoglobinopathies in the Guangxi Zhuang autonomous region of southern China. *Clin Genet*. 2010;78(2):139-148.
3. Yin A, Li B, Luo M, et al. The prevalence and molecular spectrum of α- and β-globin gene mutations in 14,332 families of Guangdong Province, China. *PLoS One*. 2014;9(2):e89855.
4. Hu L, Shang X, Yi S, et al. Two novel copy number variations involving the α-globin gene cluster on chromosome 16 cause thalassemia in two Chinese families. *Mol Genet Genomics*. 2016;291(3):1443-1450.

5. Fucharoen S, Viprakasit V. Hb H disease: clinical course and disease modifiers. *Hematology Am Soc Hematol Educ Program*. 2009;26-34:26-34.
6. Luo S, Chen X, Chen L, et al. Analysis of Hb levels and degree of anemia in relation to genotype in 615 patients with hemoglobin H disease. *Expert Rev Hematol*. 2020;13(9):1027-1033.
7. Li YQ, Li YW, Liang L, Meng MH, Zhang XQ. First detection of Hb Cenxi [β46(CD5)Gly→Arg (GGG>CGG), HBB: c.139G>C] by capillary electrophoresis. *Hemoglobin*. 2021;45(4):262-264.
8. Wang M, Zhang X, Zhao Y, Lu Z, Xiao M. Prevalence and genetic analysis of thalassemia in childbearing age population of Hainan, the free Trade Island in southern China. *J Clin Lab Anal*. 2022;36(3):e24260.
9. Xu L, Mao A, Liu H, et al. Long-molecule sequencing: a new approach for identification of clinically significant DNA variants in α-thalassemia and β-thalassemia carriers. *J Mol Diagn*. 2020;22(8):1087-1095.
10. Liang Q, Gu W, Chen P, et al. A more universal approach to comprehensive analysis of thalassemia alleles (CATSA). *J Mol Diagn*. 2021;23(9):1195-1204.
11. Li Y, Huang H, Chen Z, et al. Comparison of capillary electrophoresis and high performance liquid chromatography for detection and quantification of hemoglobin New York. *Clin Chem Lab Med*. 2016;54(1):91-95.
12. Li Y, Tian M, Qin T, Wan L. Capillary electrophoresis resolves inconclusive HPLC analysis for hemoglobin variants: a study of two cases. *Clin Lab*. 2018;64(7):1305-1309.
13. He S, Li J, Li DM, et al. Molecular characterization of α- and β-thalassemia in the Yulin region of southern China. *Gene*. 2018;655:61-64.
14. Wang Z, Sun W, Chen H, et al. Prevalence and molecular spectrum of α- and β-globin gene mutations in Hainan. *China Int J Hematol*. 2021;114(3):307-318.
15. Wei X, Shang X, He D, et al. Molecular characterization of a novel 27.6-kb deletion causing α(+) thalassemia in a Chinese family. *Ann Hematol*. 2011;90(1):17-22.
16. Mo Z, Yu C, Hu Z, Feng W. A novel deletion of −2.8 kb removing the entire alpha 2-globin gene observed in a Chinese patient with Hb H. *Clin Lab*. 2012;58(11–12):1309-1312.
17. Hottentot QP, de Meijer E, Buermans HPJ, et al. Breakpoint characterization of a rare alpha0 -thalassemia deletion using targeted locus amplification on genomic DNA. *Int J Lab Hematol*. 2021;43(6):1628-1634.
18. Brieghel C, Birgens H, Frederiksen H, Hertz JM, Steenhof M, Petersen J. Novel 31.2 kb α0 deletion in a Palestinian family with α-thalassemia. *Hemoglobin*. 2015;39(5):346-349.
19. Ju A, Jiang F, Li J, et al. Detection of an α-globin fusion gene using real-time polymerase chain reaction-based multicolor melting curve. *Hemoglobin*. 2020;44(6):427-431.
20. Huang J, Shang X, Zhao Y, et al. A novel fusion gene and a common α(0)-thalassemia deletion cause hemoglobin H disease in a Chinese family. *Blood Cells Mol Dis*. 2013;51(1):31-34.
21. Moore JA, Pullon BM, Drake KM, Brennan SO. Novel α0-thalassemia deletion identified in an Indian infant with Hb H disease. *Hemoglobin*. 2020;44(4):297-301.


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