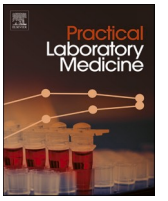
Practical Laboratory Medicine 39 (2024) e00379

Contents lists available at ScienceDirect

Practical Laboratory Medicine

journal homepage: www.elsevier.com/locate/plabm



Compound heterozygous with Hb G-Taipei and Hb Lepore-Boston-Washington: An unexpected finding triggered by HbA_{1c} measurement

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ARTICLE INFO

Keywords:
HbA_{1c}
Hb G-Taipei
Hb Lepore-Boston-Washington
β-thalassemia
Hemoglobin variant

ABSTRACT

Background: Hemoglobin A1c has been widely used to diagnose and monitor diabetes. However, the accuracy of HbA_{1c} analysis can be significantly affected by hemoglobin variants, leading to falsely low or elevated levels and misdiagnosis or inappropriate diabetes management.
Case report: In this study, we present the case of a 23-year-old man with undetectable HbA_{1c} levels during his annual checkup by high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE). To investigate the reason for HbA_{1c} absence, Sanger sequencing, multiplex ligation-dependent probe amplification assay (MLPA), long-read single molecule real-time sequencing (SMRT) and MALDI-TOF mass spectrometry (MS) were performed, and the proband was identified as compound heterozygous of β-thalassemia with Hb G-Taipei (HBB:c.68A > G) and Hb Lepore-Boston-Washington (NG_000007.3:g.63632_71046del).
Conclusion: The combination of these molecular technologies including MLPA, long-read SMRT sequencing and MALDI-TOF MS is beneficial for identifying rare hemoglobin variants. This case also provides essential evidence for uncovering the effect of compound heterozygosity for Hb Lepore-Boston-Washington and Hb G-Taipei on hematological phenotypes and HbA_{1c} analysis.

1. Background

Hemoglobin A1c (HbA_{1c}) is a glycated type of normal adult hemoglobin (Hb A) and is widely used as a diagnostic indicator for diabetes. The formation of HbA_{1c} happens gradually and consistently throughout the lifespan of red blood cells. With increasing glucose concentration in the blood, the production of HbA_{1c} also increases. Therefore, elevated HbA_{1c} levels signify inadequate long-term blood glucose control and are linked to higher risks of diabetes-related complications [1].

The measurement of HbA_{1c} can be influenced by various factors related to the turnover rate of red blood cells, such as vitamin B-12, iron or folate deficiency anemia, asplenia, acute and chronic blood loss, hemolytic anemia and splenomegaly, resulting in prolonged or

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https://doi.org/10.1016/j.plabm.2024.e00379
Received 11 January 2024; Received in revised form 21 February 2024; Accepted 21 February 2024
Available online 22 February 2024

decreased exposure of red blood cells to glucose and affecting HbA_{1c} levels. Furthermore, certain medications and substances like vitamin C, vitamin E, Ribavirin, interferon-α, hypertriglyceridemia, and hyperbilirubinemia have been reported to interfere with HbA_{1c} analysis [2]. Importantly, hemoglobin variants can also impact the accuracy of HbA_{1c} measurements. Hemoglobin variants such as HbS, HbC, HbE, and HbD have been shown to affect HbA_{1c} levels, leading to falsely low or elevated results and potentially leading to misdiagnosis or inappropriate diabetes management [3,4]. Therefore, it is crucial to consider the presence of hemoglobin variants, as they can significantly impact the interpretation of HbA_{1c} results.

In this study, we present a novel compound heterozygous hemoglobin variant that significantly impact the analysis of HbA1c. The proband was 23-year-old man from Shangqiu, Henan Province, visited our hospital for his annual checkup. To our surprise, he had undetectable HbA_{1c} levels using both high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) methods. To understand the reason behind this, we performed a complete blood count (CBC) and hemoglobin variant analysis, which revealed typical thalassemic red cell indices and the presence of Hb variant. We further conducted genetic analysis using Sanger sequencing, multiplex ligation-dependent probe amplification (MLPA) assay, and long-read single molecule real-time (SMRT) sequencing, which identified a heterozygous deletion from the HBD to the HBB gene (NG_000007.3:g.63632_71046del) and a heterozygous missense mutation at the HBB locus (HBB:c.68A > G). These genetic variations resulted in compound heterozygosity for β-thalassemia with Hb G-Taipei and Hb Lepore-Boston-Washington. Additionally, we used MALDI-TOF mass spectrometry (MS) to measure the mass-to-

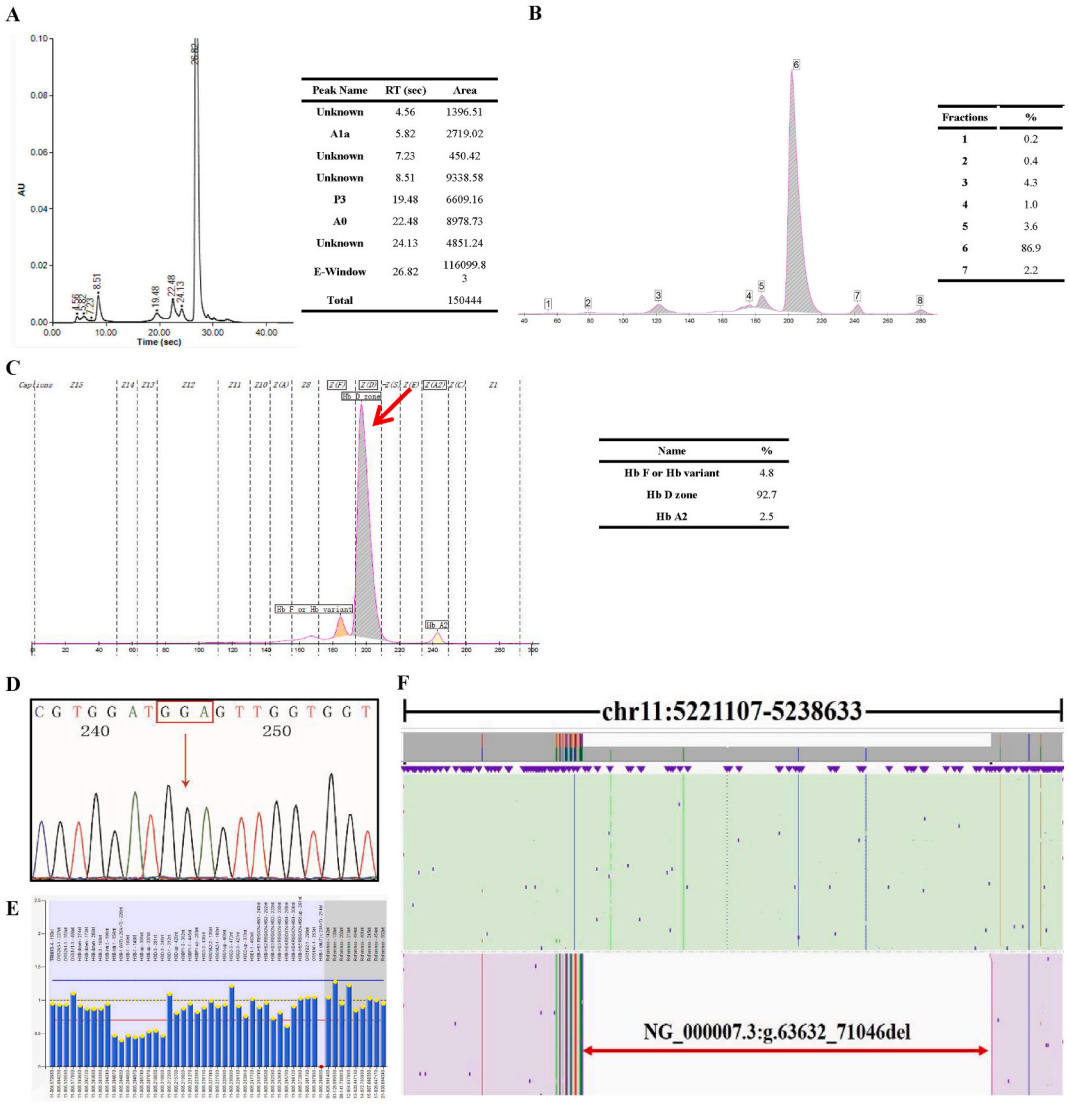

Fig. 1. (A–B). HbA1c levels were measured using an HPLC system (A) and CE system (B). (C). Hb variant analysis was performed using the Hb program on Capillarys 3 TERA. (D). DNA sequencing of the β-globin gene showing an A > G mutation at codon 22. (E). Copy number variations of HBB and HBD gene clusters were analyzed by MLPA. (F). Specific gene breakpoint of globin genes in this patient was validated through long-read SMRT sequencing.

charge ratio (m/z) of the variant globin chains, which confirmed the presence of variant globin chains with m/z 15795 (Hb G-Taipei) and 15865 (Hb Lepore-Boston-Washington), but no normal β-globin chain ($m/z = 15868$). Our study provides important evidence of the impact of compound heterozygosity for Hb Lepore-Boston-Washington and Hb G-Taipei on HbA_{1c} analysis and hematological phenotypes.

2. Material and methods

2.1. Patients

The proband, a 28-year-old man, visited Peking University Shenzhen Hospital for his annual checkup. Venous blood was collected in an EDTA-K_2 tube for the following analysis. The hematological parameters were analyzed by Sysmex automatic hematology analyzer (XN-9000). This study was approved by the Ethics Committee of Peking University Shenzhen Hospital.

2.2. HbA_{1c} and hemoglobin analysis

HbA_{1c} levels were measured using an HPLC system (Bio-Rad D-100, USA) and CE system (HbA_{1c} program; Capillarys 3 TERA, Sebia, France). Hb variant analysis was performed using the Hb program on Capillarys 3 TERA, and MALDI-TOF MS system (QuanTOF, Intelligene Biosystems, China).

2.3. Routine thalassemia gene analysis

Common α-thalassemia gene deletions ($-^{SEA}$, $-\alpha^{3.7}$ and $-\alpha^{4.2}$) and β-thalassemia gene non-deletion alterations ($\alpha^{CS}\alpha$, $\alpha^{QS}\alpha$, $\alpha^{WS}\alpha$, $\beta^{CD41/42}$, $\beta^{IVS-II-654}$,β^{-28}, $\beta^{CD71/72}$, β^{CD17}, β^{-29}, β^{CD43}, β^{CD26}, β^{-30}, β^{-32}, $\beta^{CD14/15}$, β^{CD31}, $\beta^{27/28}$,$\beta^{IVS-I-1}$, $\beta^{IVS-I-5}$, $\beta^{Cap+40-43}$, and $\beta^{Int\ CD}$) were detected using gap-PCR (Shenzhen Yilifang biotechnology) and reverse dot blot (Shenzhen Yaneng Bioscience) kit.

2.4. Sanger sequencing and MLPA

Genomic DNA was extracted using Genomic DNA Isolation Kit (Zeesan Biotech). PCR amplification and Sanger sequencing were performed to analyze the HBA1, HBA2, and HBB gene sequences. Copy number variations of HBB and HBD gene clusters were analyzed using the P102-HBB MLPA kit and Coffalyser MLPA-DAT software (MRC Holland). A 50% decrease or increase in peak area ratio compared to control samples indicated a heterozygous deletion or replication, respectively.

2.5. Long-read SMRT sequencing and validation

The purified DNA was used to construct a library with the Sequel Binding and Internal Ctrl Kit 3.0 (PacBio) and sequenced on the PacBio Sequel II system. Structural variations, single-nucleotide variations, and indels were identified using Freebayes (Biomatters).

Table 1
Hematological and molecular characteristics of these Hb variants.

Hb variant	Compound heterozygous with Hb G-Taipei and Hb Lepore-Boston-Washington	Hb G-Taipei heterozygotes	Hb Lepore-Boston-Washington heterozygotes
Clinical phenotype	Normal	Normal	Anemia
Age (y)	28	26	–
Sex	Male	Female	–
RBC (10^{12}/L)	6.32	–	5.57
HGB (g/L)	143	123	124
MCV (fL)	68.2	88.5	72.5
MCH (pg)	22.6	29.7	22.3
MCHC (g/L)	332	335	–
Hb A2 (%)	2.5	–	2.4
Hb F (%)	4.8	–	3.4
Hb G-Taipei (%)	77.25	36–40	–
Hb Lepore-Boston-Washington (%)	15.45	–	9.9
Serum ferritin (mg/mL)	122.0	–	–
Effect on HbA1c analysis (HPLC and CE)	Yes (Failure to give results)	No [5]	Not reported
HBB genotype	$\beta^{G-Taipei}/\beta^{Lepore-Boston-Washington}$	$\beta^{G-Taipei}/\beta$	$\beta/\beta^{Lepore-Boston-Washington}$
References	This study	Landman et al. [6]	Xu et al. [7]

3. Results

3.1. HbA_{1c} and hemoglobin variant analysis

A 23-year-old man from Shangqiu, Henan Province, visited our hospital for his annual checkup. Interestingly, the HbA_{1c} peak was not detected in this patient by HPLC (Fig. 1A). In order to exclude the limitations of detection methods, capillary electrophoresis (CE) was performed to analyze HbA_{1c}, result also showed the absence of HbA_{1c} (Fig. 1B). To understand the reason behind this, complete blood count (CBC) was conducted and the patient's hematological parameters were as follows: red blood cell number (RBC) 6.32×10^{12} (NV: 4.3×10^{12} -5.8×10^{12}), hemoglobin (Hb) 143 g/L (NV: 130–175 g/L), mean corpuscular volume (MCV) 68.2 fL (NV: 82.0–100.0 fL), mean corpuscular hemoglobin (MCH) 22.6 pg (NV: 27.0–34.0 pg), and mean corpuscular hemoglobin concentration (MCHC) 332 g/L (NV: 316–354 g/L) (Table 1). He was asymptomatic and showed a normal level serum ferritin (122.0 mg/mL), but by taking the decreased levels of MCV and MCH into consideration, we further analyzed the hemoglobin variants. As is shown in Fig. 1C, hemoglobin electrophoresis revealed markedly increased abnormal Hb variant levels (92.7%) (Fig. 1C) and required to be identified.

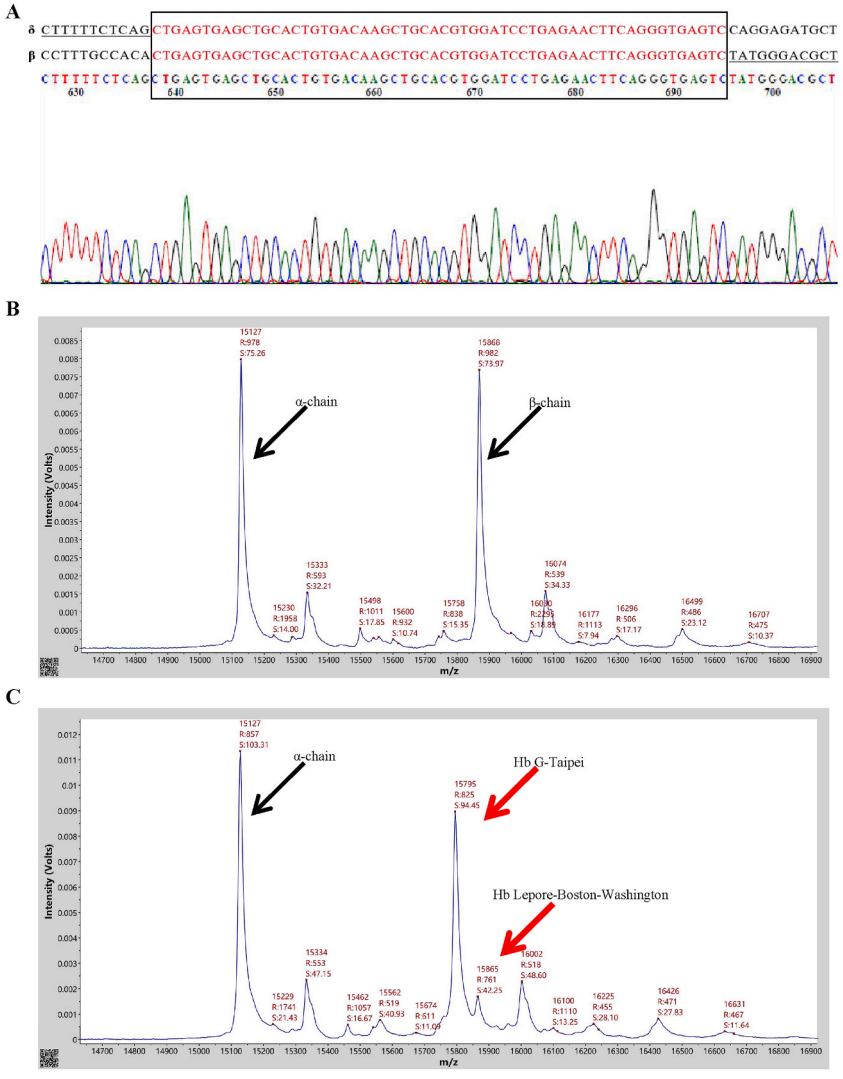

Fig. 2. (A). In-frame fusion of the δβ-globin genes was verified by Sanger sequencing. The DNA sequence highlight in red indicates homologous regions of the δ-globin gene and β-globin gene. (B–C). Hemoglobin chains of normal control (B) and the proband (C) were analyzed by MALDI-TOF MS. Arrows indicate the corresponding globin chains. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2. Identification of Hb G-Taipei by Sanger sequencing

To identify the mutation responsible for the abnormal Hb variant, the patient's DNA was analyzed for 23 common thalassemia gene variations using gap-PCR and reverse dot blot (RBD). No common deletions or mutations associated with thalassemia were detected in this patient. However, Sanger sequencing of the α- and β-globin genes revealed a mutation from GAA to GGA at codon 22 on the β-globin gene (Fig. 1D). This mutation results in the replacement of glutamate by glycine (Glu > Gly) and the formation of Hb G-Taipei. Interestingly, only one signal peak was identified at codon 22 in the Sanger sequencing data. To determine if the GAA > GGA mutation was in a homozygous or compound heterozygous state, MLPA was performed to address the situation. The MLPA results showed a 50% decrease in seven P102-HBB probe (HBB-intron 1-154nt, HBB-exon 1-189nt, HBB-exon 1-148nt, HBB-upstream-365nt, HBB-upstream-337nt, HBD-exon3-391nt and HBD-exon3-346nt) signal peaks (Fig. 1E), suggesting a heterozygous deletion from the upstream of HBD exon3 to the downstream of HBB intron 1 at the hemoglobin gene clusters.

3.3. Compound heterozygosity for Hb G-Taipei/Hb Lepore-Boston-Washington of this case

The specific gene breakpoint in this patient was further validated through long-read SMRT sequencing. A deletion of approximately 7.4 kb was identified from the δ-globin to the β-globin gene (NG_000007.3:g.63632_71046del) (Fig. 1F). This deletion resulted in an in-frame fusion of the δβ-globin genes and the formation the Hb Lepore-Boston-Washington variant. The precise breakpoint location was further determined through Sanger sequencing (Fig. 2A). Consistent with previous studies [8,9], Hb Lepore-Boston-Washington is formed by unique crossing-over events between the homologous regions of the δ-globin and β-globin gene, creating a fusion gene between exon 1/2 of δ-globin and exon 3 of β-globin. These findings indicate that the patient is a compound heterozygote for Hb G-Taipei/Hb Lepore-Boston-Washington.

3.4. MALDI-TOF MS analysis of Hb G-Taipei and Hb Lepore-Boston-Washington

Previous studies have shown that both Hb G-Taipei and Hb Lepore-Boston-Washington migrate to the "D zone" in electrophoresis [7,10]. Interestingly, our CE analysis only revealed a single variant peak in the "D zone", suggesting an overlap between Hb G-Taipei and Hb Lepore-Boston-Washington. To accurately identify the Hb G-Taipei and Hb Lepore-Boston-Washington, we performed MALDI-TOF MS to analysis the mass-to-charge ratio (m/z) of these two variant globin chains. As is shown in Fig. 2B and C, MALDI-TOF MS confirmed the presence of two variant globin chains with m/z values of 15795 for Hb G-Taipei and 15865 for Hb Lepore-Boston-Washington (Fig. 2B and C), which coherent with the molecular weight computed by Compute pI/Mw tool [11,12].

4. Discussion

Thalassemia is a significant cause of mortality and morbidity in inherited monogenic diseases worldwide, with α-thalassemia and β-thalassemia being the most common types. While α-thalassemia is predominantly caused by α-globin gene deletions, nondeletion mutations including single nucleotide substitutions and small oligonucleotide insertions or deletions are responsible for the majority of β-thalassemia cases. Over 900 different mutation types of the β-globin gene have been identified in β-thalassemia patients [13]. Both Hb G-Taipei and Hb Lepore-Boston-Washington are rare hemoglobin variant. The Hb G-Taipei variant has been reported in only one case out of 189,414 subjects in southeastern China and one case out of 9,731 candidates in the Shaokwan region [14,15]. Hb Lepore-Boston-Washington, on the other hand, occurs at low frequencies in various ethnic groups, mainly in Mediterranean countries [16–19]. Previous studies have reported compound heterozygosity for Hb Lepore-Boston-Washington with other Hb variants such as Hb Peterborough [20], Hb Johnstown [21] and Hb E [22]. However, compound heterozygosity for Hb Lepore-Boston-Washington and Hb G-Taipei has not been identified until now, making this case report significant in filling this gap in knowledge.

Based on the available literature, the clinical and hematological phenotype of Hb G-Taipei carriers seems to be asymptomatic. Studies have shown that the hematological parameters of individuals with heterozygous mutations for Hb G-Taipei are essentially normal (Hb 12.3 g/dl, MCV 88.5 fl, MCH 29.7 pg, MCHC 33.5 g/dl) [6] (Table 1). Xu et al. [23] also reported normal erythrocyte parameters in Hb G-Taipei variant heterozygotes. On the other hand, individuals with heterozygous mutations for Hb Lepore-Boston-Washington have been reported to exhibit typical thalassemic red cell indices and symptoms of anemia [7,24] (Table 1). However, in the case presented in this study, a 28-year-old male with compound heterozygosity for Hb G-Taipei and Hb Lepore-Boston-Washington, showed decreased levels of MCV and MCH. However, compensatory increased red blood cell (RBC) production was observed, and the proband did not exhibit symptoms of anemia. Compared with Hb G-Taipei carriers, the proband with compound heterozygosity for Hb G-Taipei and Hb Lepore-Boston-Washington had undetectable HbA_{1c} levels using both high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE) methods. In addition, the effect of Hb Lepore-Boston-Washington variant on HbA_{1c} analysis remains unclear and requires further investigation (Table 1).

It has been reported that the Hb G-Taipei variant accounted for 36–40% of the total Hb in a Hong Kong proband with a heterozygous mutation [6]. Similarly, in a study by Zeng et al. [25], three individuals with a heterozygous missense mutation had Hb G-Taipei variant levels of 27.0%, 32.2%, and 36.5%. In the case presented in this study, hemoglobin electrophoresis revealed that the levels of Hb G-Taipei and Hb Lepore-Boston-Washington were 92.7%. Additionally, the level of Hb G-Taipei was five times higher than that of Hb Lepore-Boston-Washington, as detected by MALDI-TOF MS. Based on these findings, it can be inferred that the Hb G-Taipei variant accounted for 77.25% of the total Hb in this proband, which is significantly higher than what is typically observed in individuals with heterozygous Hb G-Taipei.

5. Conclusion

This study is the first to describe a case of compound heterozygosity for Hb Lepore-Boston-Washington and Hb G-Taipei and its impact on HbA_{1c} analysis. Furthermore, we also analyzed the molecular weight of these two hemoglobin variants by MALDI-TOF MS. This report provides essential evidence for uncovering the effects of compound heterozygosity for Hb Lepore-Boston-Washington and Hb G-Taipei on hematological and clinical phenotypes.

CRediT authorship contribution statement

Yu Li: Writing – original draft, Investigation, Formal analysis, Data curation. **Anping Xu:** Writing – original draft, Resources, Investigation. **Juan He:** Validation, Funding acquisition. **Limin Huang:** Software, Formal analysis. **Li Lin:** Conceptualization. **Jie Li:** Validation, Software. **Yong Xia:** Resources, Funding acquisition, Conceptualization. **Ling Ji:** Supervision, Resources, Project administration, Funding acquisition.

Declaration of competing interest

None.

Acknowledgements

This work was supported by Key-Area Research and Development Program of Guangdong Province [grant number 2020B1111160001]; “San-ming” Project of Medicine in Shenzhen [grant number SZSM201812088]; and Shenzhen Science and Technology Program [grant number JCYJ20220531093816038 and JCYJ20210324110011031].

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