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Original Article

Establishment and clinical application of the method for rapidly detecting gene mutation in β Thalassemia based on HRM technique

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Abstract: Objective: This study aimed to establish a rapid screening method for gene mutation of β Thalassemia with HRM technique and to discuss its clinical application value. Methods: Common gene mutation sites of patients with β Thalassemia such as IVS-2-654(C>T) and -28(A>G) in Wenzhou were adopted. TA clone technology was used to construct plasmid DNA as template or genotype control. The method for identifying gene mutation of β Thalassemia with HRM technique was established. A total of 117 suspected β Thalassemia patients were enrolled. DNA of peripheral leukocytes was extracted for IVS-2-654(C>T) and -28(A>G) detections with HRM. The results were compared with the results of bi-directional sequencing. Results: HRM technique could be used to detect IVS-2-654(C>T) and -28(A>G) mutation sites of β Thalassemia. There was no non-specific amplification fragment. Variable coefficient (CV) of melting temperature (Tm) detected by HRM among intra-batch and inter-batch of different genotypes was <0.1%. At least 1000 copies of DNA templates could be tested and mutation with only 10% of variation could even be detected. Among 117 patients, 45 were IVS-2-654(C>T) heterozygous mutant and 9 cases were -28(A>G) heterozygous mutant. Moreover, two locus of homozygous mutant gene were not observed, which were completely inconsistent with direct sequencing. Conclusion: The established HRM technology can be used to screen gene mutation of β Thalassemia with superior specificity and sensitivity. It provides a universal technology platform for detecting other mutation site and SNP.

Keywords: β Thalassemia, gene mutations, TA clone technology, high-resolution melting

Introduction

The β Thalassemia is one of the most common monogenic disorders all over the world. It is caused by a β globin gene mutation, which lead to a lack of β globin gene and hemolytic anemia is formed accordingly. To date, more than 200 types of gene mutation have been reported. Various populations have different mutation spectrum, and all of them consist of the overwhelming common mutations and several rare mutations [1, 2]. In China, 48 types of mutations have been found and seven are most common: CD41-42(-TCTT), IVS-2-654(C>T), CD-17(A>T), -28(A>G), CD71-72(+T), -29(A>G) and CD43(G>T). The β Thalassemia patients in Wenzhou are mainly with IVS-2-654(C>T) and -28(A>G) gene mutations [3]. Although many kinds of molecular biological techniques have been used for the genetic diagnosis of β Thalassemia, such as PCR-reverse dot hybridization, PCR-restriction fragment length polymorphism, PCR-allele specific oligonucleotide probe, PCR-single-strand conformation polymorphism and allele specific PCR [4-8]. All of them have some limitations and cannot be used to analyze gene mutation of β Thalassemia rapidly and accurately. In recent years, high-resolution melting (HRM) has been used to detect BRCA1 gene mutation in patients with breast cancer and analyze IL-28B polymorphism in patients with chronic hepatitis C [9-11]. The results are close to gene sequencing and obtained quickly, so this technique is well suited for mutation

Table 1. Primer sequence on IVS-2-654(C>T) and -28(A>G) sites of β thalassemia analyzed with HRM

Primer	Sequence (5'-3')	Length (bp)	Locus (NG_000007.3)
P1	F: CTAGGGTTGGCCAATCTACTC	163	70459-70621
	R: CTTCTCCTCAGGAGTCAGATGC		
P2	F: TTTCTAATACTTTCCCTAATCTCTT	141	71586-71726
	R: GCAGAAATATTTATATGCAGAGAT		

Notes: P1 was a primer on -28(A>G) mutant site; P2 was a primer on IVS-2-654(C>T) mutant site: F and R represented upstream and downstream primer respectively.

screening and genotyping. This study tries to establish a new method for gene mutation detecting in β Thalassemia, and investigate its value in clinical applications.

Patients and methods

Patients

A total of 117 suspected β Thalassemia patients in Yuying Children Hospital and Second Affiliated Hospital of Wenzhou Medical University from February 2014 to January 2015 were enrolled. Their male/female ratio was 61/56 and the age was 22 (1-74) years. Inclusion criteria for suspected β Thalassemia patients [12]: mean cellular volume (MCV) ≤80 fL and (or) mean corpuscular hemoglobin (MCH) <27 pg; heamoglobinA2 (HbA2) ≥4%; heamoglobin F (HbF) is normal or rises up but ≤3.5%. Microcytic hypochromic anemia resulted by iron-deficiency anemia and other chronic diseases were excluded.

Instruments and reagents

PCR amplifier (German Eppendorf Company), Incubator shaker (Harbin Donglian Electronic Technology Development Co., Ltd), Gel imaging system (Beijing Liuyi Instrument factory), ultraviolet spectrophotometer (American Beckman Coulter Company), Fluorescent quantitative instrument-Roche Lightcycler 480 model (German Roche Group). Whole blood high purity genome DNA extraction kit, PCR kit, Gel clean-up kit, all pMD18-T simple carriers were offered by Takara (Dalian) Engineering Co., Ltd, plasmid mini kit was provided by Beyotime Institute of Biotechnology; and Type-it® HRM™ kit was supplied by German Qiagen Company.

Sampling

Peripheral blood (2.0 mL) was extracted with EDTA-K_2 anti-coagulation. DNA of peripheral blood leukocytes were obtained with the conventional method. The specific procedure was preceded based on the instruction of kit. Moreover, concentration of genome DNA was adjusted to 10 mg/L finally.

Plasmid with wild and mutant type of IVS-2-654(C>T) and -28(A>G) sites

Peripheral blood genome DNA of healthy people (has been stored in our laboratory) was used for template. Plasmid involved wild type gene fragments was established by adopting TA clone following high fidelity PCR amplification. After that, the constructed wild type plasmid DNA was used as template, and the method of overlap extension PCR site directed mutagenesis was involved. Then the needed mutation site was introduced with two cycles of PCR amplification. In the end, plasmid involved IVS-2-654(C>T) and -28(A>G) mutant genetic segments was constructed after TA clone. Afterwards, plasmid DNA was extracted with plasmid mini kit. The detailed procedure should be done following kit instruction.

Determination of copy numbers of plasmid template

Plasmid DNA was diluted to the ratio of 1:50, and distilled water was thought as blank to test absorbance (*A*) value at 260 nm and 280 nm. After that, purity of nucleic acid was evaluated on the basis of A_{260}/A_{280} and DNA copy numbers were calculated through formula:

Copy numbers = (amount × 6.022 × 10^{23})/(length × 1 × 10^9 × 650) (1) and the amount represented DNA concentration and the length represented the length of DNA.

Detection of IVS-2-654(C>T) and -28(A>G) locus by HRM

HRM primers for IVS-2-654(C>T) and -28(A>G) were designed by software Primer Premier5.0 respectively. The sequence of each primer and its position on β protein reference sequence

(ref|NG_000007.3 GI: 28380636 HBB) were described in **Table 1**. All primers were synthesized by Takara (Dalian) Engineering Co., Ltd. HRM reactive system: 1 × HRM Master MIX (including NTPs, EvaGreen, Type-it HRM PCR Buffer, HotStarTaq Plus DNA Polymerase and Q-Solution), forward and reverse primers with concentration being of 0.7 μmol/L, 10 ng of genome DNA, and 25 μl was supplemented by Ranse-free water. The two sites were detected with Lightcycler 480 fluorescent quantitative instrument according to the following loop parameters. Amplification conditions: 5 min of pre-denaturation was preceded under 95°C; and then 10 s of denaturation was conducted under the same temperature; afterwards, 30 s of annealing was carried under 55°C, followed by 10 s of extension under 72°C and 45 circulations. HRM was executed directly after amplification. The detailed conditions were: 95°C one min, 40°C one min, melting curve data was collected from 65°C to 95°C; climbing speed of temperature was 0.02°C/s and 25 times of data acquisition being carried out for each 0.02°C increase. After all, software Roche LC480 1.5.0 was used to analyze the data.

Methodological evaluation for HRM detection

IVS-2-654(C>T) mutation was taken as methodological research site to make series of assessment for constructed HRM system. This included (1) specificity: five normal genomes DNA, two genomes DNA of Japanese rabbits and one deionized water were selected randomly as blank control to execute HRM detection. Melting curve analysis was done by adopting Roche LC480 1.5.0 software after the instrument running out. The variation between melting peak curve and melting temperature (Tm) were then observed. PCR products (5 μL) were fetched to make 1% agarose gel electrophoresis (AGE) verification. Amplified fragments and expected PCR products were sent to Beijing Liuhe genomics technology Co., Ltd, Shanghai branch to make bio-directional sequencing, so that, specificity of detection method can thus be evaluated. (2) Repeatability: 1 wild type, 1 hybrid subtype (wild type plasmid DNA mixed with homozygous mutant type according to 1:1 proportion; both of them had same concentration) and 1 homozygous mutant type plasmid DNA with the same concentration were selected to make HRM detection. Each sample was repeated for 10 tubes in order to compute Tm value mean (*m*), standard deviation (s) and variable coefficient (*CV*) of different genotype plasmid DNA. By this way, repeatability within the batch could thus be evaluated. After that, 1 wild type, 1 hybrid subtype and 1 homozygous mutant type plasmid DNA with the same concentration were taken to do HRM detection; once per day with continuous 10 days. In the next step, *m*, s and *CV* of Tm value in plasmid DNA with various genotypes were calculated to assess the repeatability among batches. (3) Sensitivity: 10 times of gradient dilution was made for plasmid DNA to do HRM detection. Template amount added in each gradient were, in order: 10^9, 10^8, 10^7, 10^6, 10^5, 10^4 and 10^3 copies. After the detection was over, reactive products (5 μL) were fetched to make 1% agarose gel electrophoresis (AGE) verification. (4) Evaluation for detecting mutation hybrid ratio: the total concentration of genome was kept constant, and wild type plasmid DNA was mixed with homozygous mutant type (all of them were at the same concentration) to prepare the template with different mutant proportion, and the mutant proportions from high to low were: 100%, 80%, 50%, 30%, 10%, 5%, 1% and 0; and then HRM detection was carried out to evaluate the range of detecting mutation hybrid ratio.

Detection of suspected β Thalassemia patients by HRM

Plasmid DNA of wild type, hybrid type and homozygous mutant type gene fragments with IVS-2-654(C>T) and -28(A>G) sites contained in were regarded as genotyping control respectively to do HRM test of 2 sites for 117 cases of genome DNA samples from clinically suspected β Thalassemia patients to confirm their genotype.

Coincidence rates when comparing with bi-directional sequencing

The HRM products of genome DNA samples in 117 suspected β Thalassemia patients were sent to Beijing Liuhe genomics technology Co., Ltd, Shanghai branch to make bi-directional sequencing. After that, the testing result was analyzed by Chromas 2.22 software and NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST). Furthermore, the genotype obtained by HRM detection was contrasted to genotype result gotten by sequencing to judge their coincidence rates.

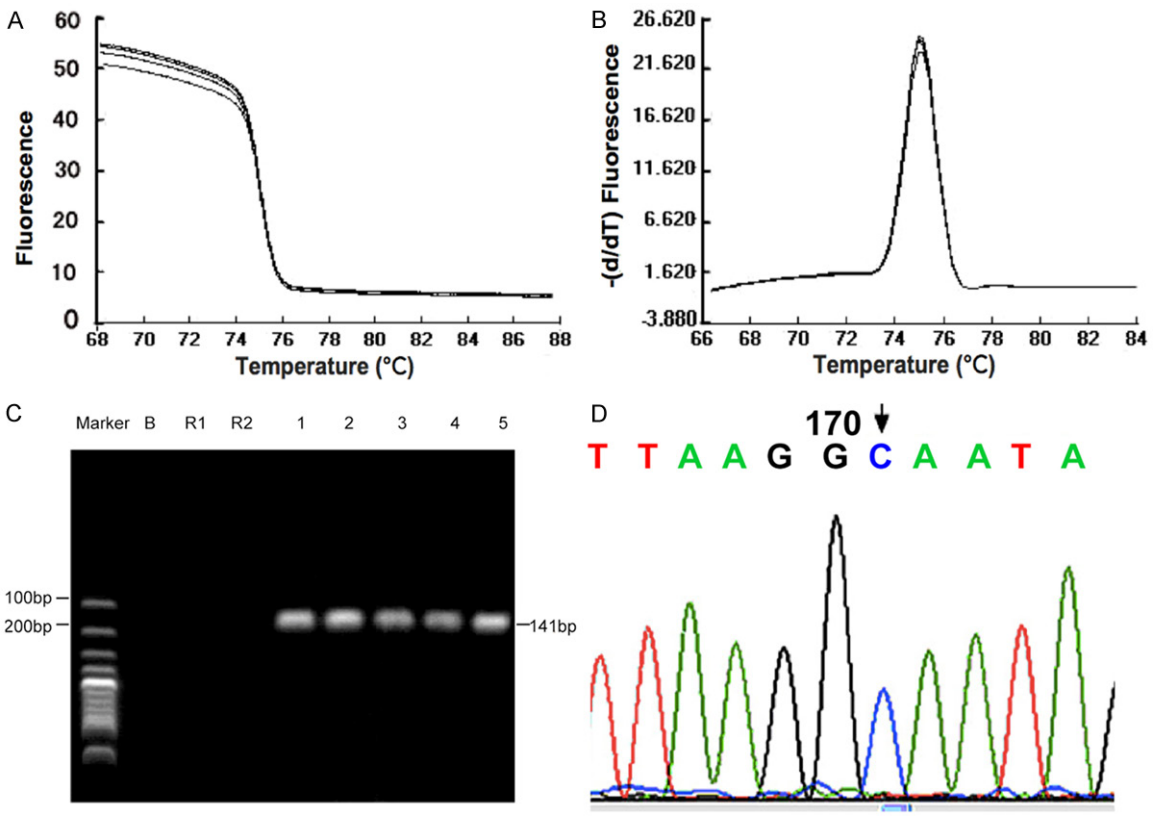

Figure 1. Specificity evaluating results for HRM detecting system.

Table 2. Repeatability of plasmid DNA with different genotypes detected by HRM

Genotype	Repeat time	Intra-bath Tm (°C, $m \pm s$)	Intra-bath *CV* (%)	Inter-bath Tm (°C, $m \pm s$)	Inter-bath *CV* (%)
Wild type	10	75.65±0.024	0.03	75.64±0.055	0.07
Heterozygous type	10	75.26±0.025	0.03	75.25±0.051	0.07
Homozygous mutant type	10	75.03±0.031	0.04	75.04±0.047	0.06

Results

Methodological evaluation

Specificity: Correct fluorescence signals were achieved among five human genome DNA samples. Moreover, they showed well amplification efficiency. In addition, disturbance peak or abnormal broadening of main peak was not found through melting curve analysis. Besides, they had small variation of Tm values (*CV* was 0.05%); and electrophoresis result of PCR products indicated that amplified fragment length was in light of expectation. It was confirmed to be the target sequence through bi-directional sequencing. Except that, fluorescence signals were not observed in two cases of genome DNA in Japanese rabbits and one case in blank control tube. Besides, the band was not seen in electrophoresis results of PCR products, which suggested that amplification of this reactive system was specific (**Figure 1**).

The "A" represented normal melting curve diagram of amplification fragments from human genome DNA; "B" represented melting peak diagram of amplification fragments from human genome DNA; "C" represented electrophoretogram of PCR products (M and B represented standard band and blank control, respectively; R1 and R2 were genome DNA of Japanese rabbits; 1-5 were human genome DNA specimens); D represented sequencing peak diagram (arrow pointed to IVS-2-654 site).

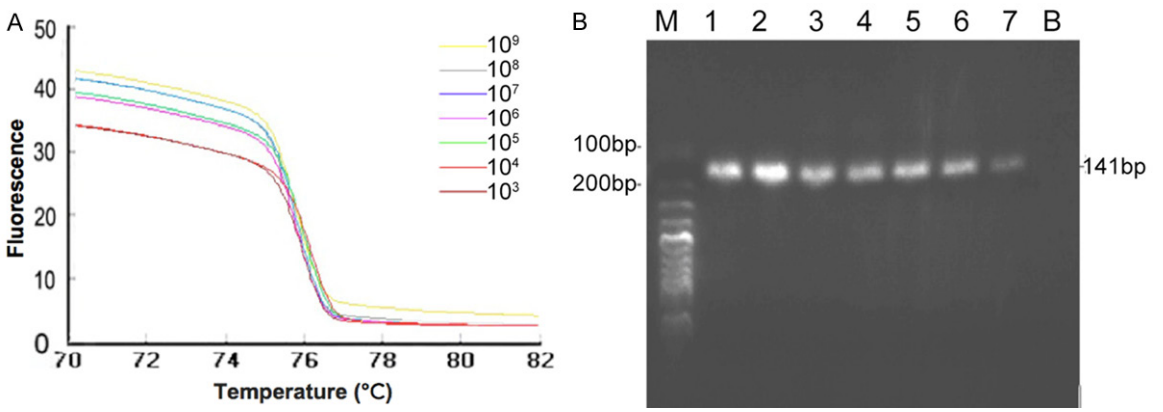

Figure 2. Sensitivity evaluating result of HRM detection system. A. Represented HRM detection melting curve for plasmid DNA with different concentration; B. Represented electrophoresis validation diagram for PCR amplified product of plasmid DNA with different concentration (M and B represented standard band and blank control res; 1-7 were plasmid DNA samples with 10^9-10^3 copies in turn).

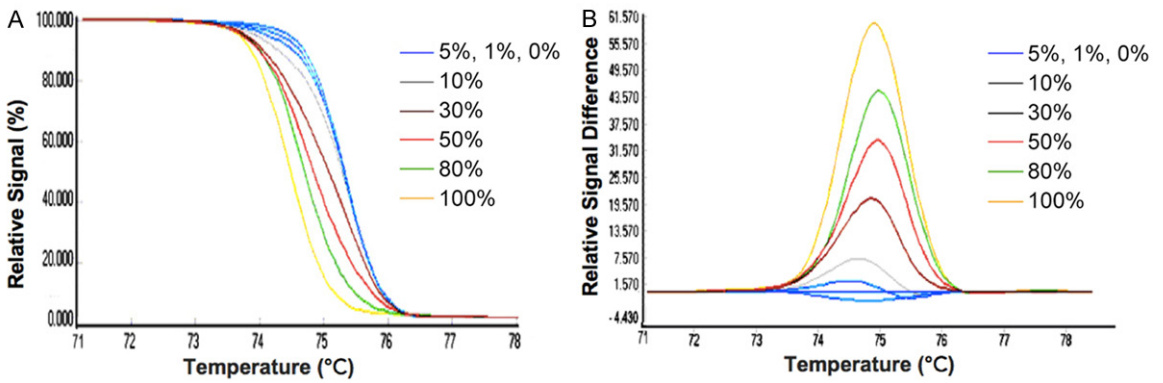

Figure 3. Detection result of plasmid DNA with distinct hybrid ratio by HRM system. A. Was HRM analysis normal melting curve for plasmid DNA samples with different hybrid ratio; B. Was relative signal difference diagram by taking hybrid ratio 5%, 1% and 0% as basic line.

Repeatability: Both intra-batch and inter-batch *CV* of Tm values of plasmid DNA with respect wild type, heterozygous type and homozygous mutant type were <0.1% (**Table 2**).

Sensitivity: Plasmid DNA within a wider range of concentration gradient (10^3~10^9 copies) could get right fluorescence signals. After gradient heating, melting curve of fluorescence intensity (variation with the temperature) had single, apparent inflection point with fixed location (**Figure 2A**). Electrophoresis validation was made for DNA amplified products with different copy numbers, bright fluorescent strip could be seen among all products (**Figure 2B**).

Capacity for detecting mutant and hybrid ratio

Figure 3 stated the evaluation results of ability for detecting mutant and hybrid ratio by HRM. Various types of melting curve could be seen when mutant and hybrid ratios were both among 10% to 100%. When the ratio was 0, 5% and 10%, melting curve was the same type, which revealed that this method could identify the existence of mutation as low as 10%.

Clinical verification results

With the HRM, there were 45 cases with IVS-2-654(C>T) heterozygous mutant types and nine with -28(A>G) heterozygous mutant types in the DNA samples of suspected β Thalassemia patients. Homozygous mutant gene of two sites was not observed. This result was completely consistent with genotype results obtained by bi-directional sequencing. HRM results were shown in **Figures 4** and **5**. Sequencing results of each genotype (local sequencing peak dia-

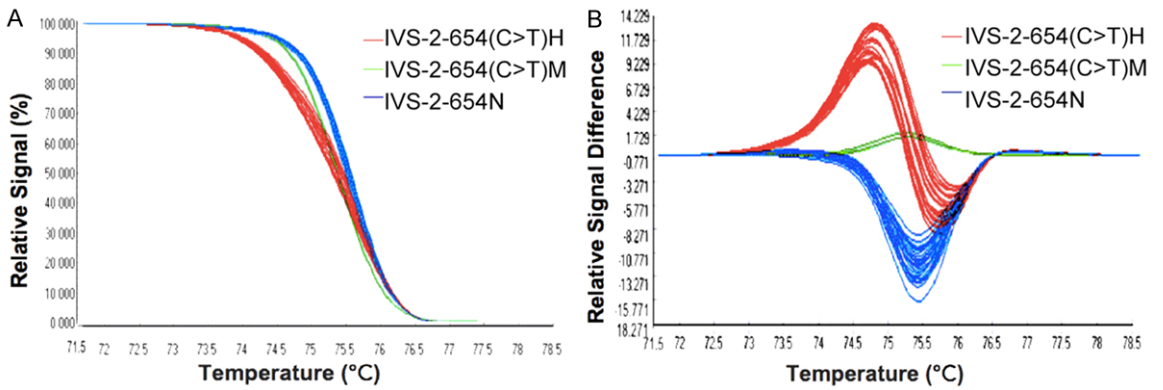

Figure 4. HRM diagram of IVS-2-654 site in suspected β Thalassemia patients. M represented homozygous the mutant genotype; H was hybrid type; and N represented the wild genotype; A. Represented normal melting curve diagram; B. Represented relative signal difference diagram by considering IVS-2-654(C>T) homozygous mutant type as the base line.

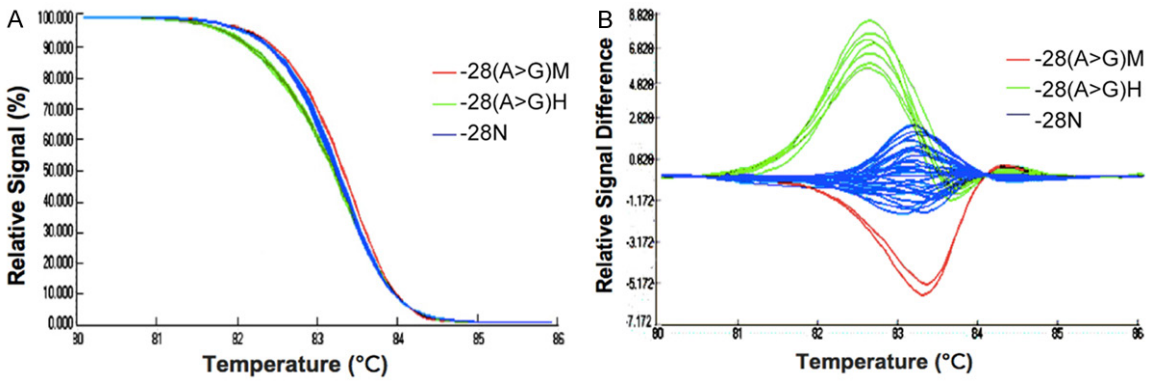

Figure 5. HRM diagram of -28(A>G) site in suspected β Thalassemia patients. M represented homozygous the mutant genotype; H was hybrid type; and N represented the wild genotype; A. Represented normal melting curve diagram; B. Represented relative signal difference diagram by considering -28(A>G) wild genotype as the base line.

gram with mutant sites included) were shown in **Figure 6**.

Discussion

The β Thalassemia is one kind of monogenic diseases with the highest morbidity in southern China, which affects the quality of the newborn significantly. However, to date there is no specific treatment method for β Thalassemia, population surveillance and prenatal genetic screening by adopting molecular diagnostic techniques are the most effective way to reduce prevalence rate. HRM technology, which has emerged recently, is a novel molecular diagnostic technique used for genetic mutant detection and genotyping. With this technology, the products are executed high-resolution melting directly after finishing real-time fluorescence PCR. Then melting process of nucleic acid is monitored by new type of saturated fluorescent dyes (such as SYTO 9 and Eva Green) with stronger DNA combining capacity and lower inhibiting effect. Melting curve is generated according to variation of Tm values, so that different genotypes are distinguished. In addition, the sensitivity and specificity of this technology are significantly higher than those of common mutant detecting techniques. Besides, with HRM technology both known and unknown site mutant can be identified quickly and cost-effectively [13-15]. Based on mentioned HRM characters, this study builds up the method of detecting β Thalassemia gene mutation according to HRM technique through experimental improvement and reactive system optimization. Furthermore, the methodological evaluation and primary clinical application research are also involved.

Through primer design and system optimization, the experimental results indicate that

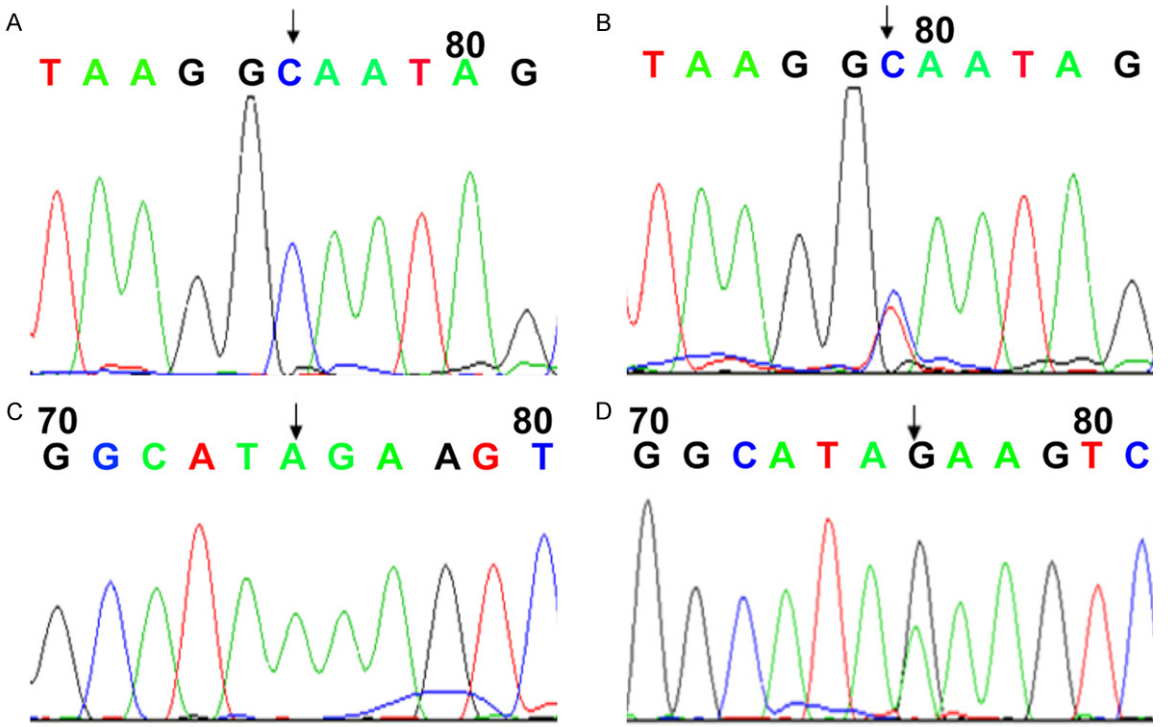

Figure 6. Sequencing diagram of IVS-2-654(C>T) and -28(A>G) sites in suspected β Thalassemia patients. A, B. Represented wild type and hybrid-mutant type gene of IVS-2-654 site respectively; C, D. Represented wild type and hybrid-mutant type gene at -28 site respectively; the arrow indicated position of each detecting site.

except human DNA product peak, neither disturbance peak nor abnormal broadening of major peak appears in melting curve diagram. The length of product fragment achieved the ideal result, which indicates high specificity of HRM detecting system. The *CV* of Intra-batch and inter-batch Tm value with different genotypes detected by HRM are <0.1%, which suggested the superior accuracy and stability of this method. Moreover, this method has superior sensitivity, which can identify DNA with the copy numbers being as low as 1000 copies. Therefore, this method has relatively low requirement for specimen. Human genome DNA with various sources such as blood, saliva, hair and amniotic cells can be used for mutation screening and detection. A total of 117 specimens in suspected β Thalassemia patients were tested in our study with this method. The results showed that 45 cases were IVS-2-654 (C>T) heterozygous mutant type and nine cases were -28(A>G) heterozygous mutant types. And homozygous mutant genes of two sites were not found, which was as same as the results of bi-directional sequencing. The accuracy of HRM detection has been fully verified. Besides, 96 or 384 pores detection can be made simultaneously with our method, which could reduce experimental cost significantly. Furthermore, not any isolation and purification are needed for this method. HRM analysis can be executed directly after finishing PCR amplification, which is not only control cross contamination but also achieve the truly tube close operation, and the working efficiency is enhanced accordingly. On the other hand, through testing condition optimization, our study makes the HRM detection of two sites IVS-2-654(C>T) and -28(A>G) can be realized under the same experimental conditions (both annealing temperature). Thus, the plan that multiple mutant sites can be detected by this method is implemented primarily, which lays experimental foundation for further studying the high-throughput test of this method.

In addition to that, there are only two conditions including homozygous and hybrid mutation (mutation ratio is 50%) for β Thalassemia gene mutation. If HRM technology is used for somatic mutation detection, resistant micro-organisms mutation detection, methylation detection, non-invasive prenatal diagnosis (include lots of genome DNA from parent source) and so on, few mutants will be needed to be tested under large number of wild background. In this circumstance, proportion occupied by mutation

is often low. Hence, in this study, plasmid DNA with different mutant ratio are prepared, whether plasmid DNA with lower hybrid proportion can be tested by HRM technology is studied. All the results indicate that this method can detect the existence of mutation with the ratio as low as 10%. In contrast to gold standard (sanger sequencing method, generally speaking, the detected mutant-hybrid ratio is only up to 25%) for mutation detection, the capacity of HRM method for testing mutant and hybrid proportion is much higher [16].

Overall, the built method for detecting β Thalassemia gene mutation on the basis of HRM technology in this research is easy and quick with strong specificity, high sensitivity and accurate result. Hence, it can be used for genetic diagnosis of β Thalassemia. Meanwhile, it also offers a universal technical platform for testing other mutant sites of β Thalassemia and genome single nucleotide polymorphism genotype.

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Disclosure of conflict of interest

None.

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