

# Raman characterizations of red blood cells with $\beta$ -thalassemia using laser tweezers Raman spectroscopy

Wenguang Jia, MS<sup>a</sup>, Ping Chen, MD<sup>b,\*</sup>, Wenqiang Chen, MD<sup>b</sup>, Yongqing Li, MD<sup>c</sup>

## Abstract

This study aimed to study the differences in Raman spectra of red blood cells (RBCs) among patients with  $\beta$ -thalassemia and controls using laser tweezers Raman spectroscopy (LTRS) system.

A total of 33 patients with  $\beta$ -thalassemia major, 49 with  $\beta$ -thalassemia minor, and 65 controls were studied. Raman spectra of RBCs for each sample were recorded. Principal component analysis (PCA), one-way analysis of variance (ANOVA), and independent-sample *t* test were performed.

The intensities of Raman spectra of  $\beta$ -thalassemia (major and minor) RBCs were lower than those of controls, especially at bands 1546, 1603, and 1619  $\text{cm}^{-1}$ . The intensity ratio of band 1546  $\text{cm}^{-1}$  to band 1448  $\text{cm}^{-1}$  demonstrated that there was a significant difference between the spectra of  $\beta$ -thalassemia major (mostly below 2.15) and those of controls. The spectra of controls could be well distinguished from those of  $\beta$ -thalassemia major using PCA. After normalization, the spectra of two different genotypes with  $\beta^0/\beta^0$  mutations mainly overlapped, while those with  $\beta^+/\beta^+$  mutations had lower intensity at bands 1546, 1603, and 1619  $\text{cm}^{-1}$ .

The present study provided Raman characteristics of RBCs in patients with  $\beta$ -thalassemia major and supported the use of LTRS as a method for screening  $\beta$ -thalassemia major. The recognition rate for  $\beta$ -thalassemia minor needs to be further improved.

**Abbreviations:** HbA = adult hemoglobin, HbF = fetal hemoglobin, LTRS = laser tweezers Raman spectroscopy, MCH = mean corpuscular hemoglobin, MCV = mean corpuscular volume, PCA = principal component analysis, RBCs = red blood cells.

**Keywords:**  $\beta$ -thalassemia, hemoglobin, laser tweezers Raman spectroscopy, Raman spectra, red blood cell

## 1. Introduction

Thalassemia is an inherited blood disorder that may result in the abnormal formation of hemoglobin and is mainly found in the Mediterranean region, the Middle East, South China, and some African countries.<sup>[1]</sup>  $\beta$ -thalassemia, as one of the most common thalassemias, is prevalent in Southeast China.<sup>[2]</sup> It is the consequence of impaired and reduced production of  $\beta$ -globin chains, caused by point mutation in most cases.  $\beta$ -thalassemia is

classified into two types depending on the severity of symptoms: thalassemia major (also known as Cooley's anemia) and thalassemia intermedia.  $\beta$ -thalassemia major is characterized by marked ineffective erythropoiesis and severe hemolysis.<sup>[3]</sup> However, most of them rely on lifelong blood transfusions and iron-chelating drugs for survival.<sup>[4,5]</sup>

Early identification and prevention can reduce the chances of delivering babies with  $\beta$ -thalassemia. That is to say, performing prenatal diagnosis on couples at a high risk of giving birth to a baby with thalassemia major is the best choice while screening  $\beta$ -thalassemia carriers. Recent screening methods for  $\beta$ -thalassemia include complete blood count (typically showing anemia, low mean corpuscular volume [MCV], microcytosis, and hypochromia), cellulose acetate electrophoresis, high-performance liquid chromatography, blood film observation (typically showing target cells and polychromasia), and so on.<sup>[6]</sup> These tests are more complicated, labor-intensive, and rigid for the experimenters. Therefore, a rapid screening method is required.

With the development of laser technique, the combination of laser trapping and Raman spectroscopy (laser tweezers Raman spectroscopy [LTRS])<sup>[7,8]</sup> has been widely used in the research on biological samples, including bacteria and blood cells, providing extensive information on structure and molecular conformations within the particles.<sup>[9–13]</sup> Because of differences in the hemoglobin components of  $\beta$ -thalassemia and normal red blood cells (RBCs), the quaternary structure of hemoglobin has changed, making it possible for LTRS to differentiate between normal and abnormal hemoglobin.<sup>[14–16]</sup> LTRS was used in the present study for differentiating RBCs between controls and patients with  $\beta$ -thalassemia, aiming to perform Raman characterization of  $\beta$ -thalassemia and explore the possibility of LTRS as a potential screening method for thalassemia.

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<sup>a</sup> Department of Pediatrics, <sup>b</sup> Guangxi Key Laboratory of Thalassemia Research, The First Affiliated Hospital, Guangxi Medical University, Nanning, Guangxi Zhuang Autonomous Region, China, <sup>c</sup> Department of Physics, East Carolina University, Greenville, NC.

\* Correspondence: Ping Chen, Guangxi Key Laboratory of Thalassemia Research, the First Affiliated Hospital, Guangxi Medical University, Nanning 530021, Guangxi Zhuang Autonomous Region, China (e-mail: chenping@gxmu.edu.cn).

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## 2. Materials and methods

### 2.1. Patients and materials

The study included 82 patients with  $\beta$ -thalassemia (33 major and 49 minor) and 65 controls who admitted to the First Affiliated Hospital of Guangxi Medical University (Nanning, Guangxi province, China). All subjects signed informed consent forms, and the study was approved by the Ethics Committee of Guangxi Medical University. All methods were performed in accordance with the relevant guidelines and regulations.

Complete medical history and family history of each patient were obtained. Then, the complete blood cell count with RBC indices, blood smear, hemoglobin electrophoresis, and high-performance liquid chromatography were performed. Patients manifested with microcytosis (MCV < 80 fL), hypochromia (mean corpuscular hemoglobin (MCH) < 27 pg), hemoglobin A<sub>2</sub> (HbA<sub>2</sub>)  $\geq$  3.5%, and fetal hemoglobin (HbF) 0.1% to 5% were regarded as  $\beta$ -thalassemia minors. Patients manifested with moderate-to-severe anemia HbF  $\geq$  40% were considered as  $\beta$ -thalassemia majors. DNA analysis for  $\beta$ -globin mutations was performed to confirm the selections.<sup>[17,18]</sup> The controls with normal aforementioned tests were selected for continuous collection of LTRS spectra of subjects, which consecutively enrolled over the same period. For all subjects, infection, iron deficiency anemia, leukemia, and  $\alpha$ -thalassemia were excluded.

Of these 33 patients with  $\beta$ -thalassemia major, 10 received blood transfusion at several weeks' intervals, and the rest were first diagnosed with no blood transfusion history. Patients with  $\beta$ -thalassemia minor had some clinical manifestations.

### 2.2. Preparation of RBCs

Blood (2 mL) was taken by venipuncture from each patient with  $\beta$ -thalassemia major when the patient was first diagnosed, or before he/she received blood transfusions. All blood samples were anti-coagulated using anticoagulant citrate dextrose solution pipes. For preparing RBCs, every blood sample was centrifuged, and the plasma was discarded. The RBCs were washed twice with 0.9% saline solution and stored at 4°C. The RBCs of controls were collected using the same method.

### 2.3. Recording Raman spectra by LTRS

For every sample, 1  $\mu$ L aliquots of RBCs and 200  $\mu$ L of 0.9% saline solution were placed in the LTRS system.<sup>[19]</sup> The RBCs drifted at a speed of a few micrometers per second in saline solution before they stuck to the bottom of the container. Once a cell was captured by the laser tweezers, it was trapped within a

small space and the Brownian motion was controlled. Then, the Raman spectrum of this cell was recorded at the exposure time of 20 seconds using a 780.24 nm laser. The power of the laser was 14.5 mW. For each sample, Raman spectra of 42 RBCs were recorded.

### 2.4. Statistical analysis

After subtracting background and smoothing, the average spectra of 42 cells were obtained to represent the spectrum of each sample. Origin (OriginLab Corp., Northampton, MA) and SPSS (IBM, NY) software were used to process the Raman spectroscopic data. The principal component analysis (PCA) was performed for distinguishing spectra of RBCs from those of patients with  $\beta$ -thalassemia and controls. In this analysis, a data matrix consisting of 147 spectra (33 major, 49 minor, and 65 normal) in the bands 692 to 1834  $\text{cm}^{-1}$  was analyzed. Band 1448  $\text{cm}^{-1}$  was chosen as the normalization point. Furthermore, the intensity ratio of band 1546  $\text{cm}^{-1}$  to band 1448  $\text{cm}^{-1}$  was investigated. No information about the disease statements of patients was provided to the algorithm. Only the processed raw data of spectra were used for this classification. A one-way analysis of variance (ANOVA) was used to evaluate the intensities of characteristic bands. A *P* value less than .05 was statistically considered significant.

## 3. Results

### 3.1. Hematological data

The hematological data are shown in Table 1. No age difference was observed among the 3 groups. Also, no statistical difference was found in the MCH concentration between  $\beta$ -thalassemia major and  $\beta$ -thalassemia minor. Besides, no statistical difference in RBCs was found between normal and  $\beta$ -thalassemia minor. However, other parameters had statistically significant differences.

### 3.2. Raman spectra of RBCs

Averaged Raman spectra of RBCs trapped in 0.9% saline solution from the three groups are shown in Figure 1. The bands of the Raman signal from the RBCs were assigned as described in previous studies.<sup>[20,21]</sup>

Raman spectra showed the skeleton of hemoglobin in RBCs (Fig. 1). Several prominent scattering bands were observed: 750  $\text{cm}^{-1}$  resulting from the porphyrin breathing vibration mode, and 1000  $\text{cm}^{-1}$  resulting from the symmetric ring breathing mode of

**Table 1**

**Baseline characteristics of patients with  $\beta$ -thalassemia and controls.**

	$\beta$ -thalassemia major (n=33)		$\beta$ -thalassemia minor (n=49)		Control (n=65)	
	Mean	SD	Mean	SD	Mean	SD
Hemoglobin (g/L)	60.73	2.92	103.66	2.02	139.24	2.50
RBCs ( $10^{12}/L$ )	2.83	0.16	5.07	0.11	4.64	0.08
MCV (fl)	69.47	1.04	64.17	0.084	90.36	0.70
MCH (pg)	22.06	0.46	20.43	0.25	30.06	0.24
MCHC, g/L	317.4	5.09	318.02	4.10	333.78	2.41
HbF (%)	79.19	3.88	1.94	0.20	1.46	0.11
HbA <sub>2</sub> (%)	11.02	3.37	5.39	0.08	2.41	0.03

HbA<sub>2</sub> = hemoglobin A<sub>2</sub>, HbF = fetal hemoglobin, MCH = mean corpuscular hemoglobin, MCHC = mean corpuscular hemoglobin concentration, MCV = erythrocyte mean corpuscular volume, RBCs = red blood cells, SD = standard deviation.

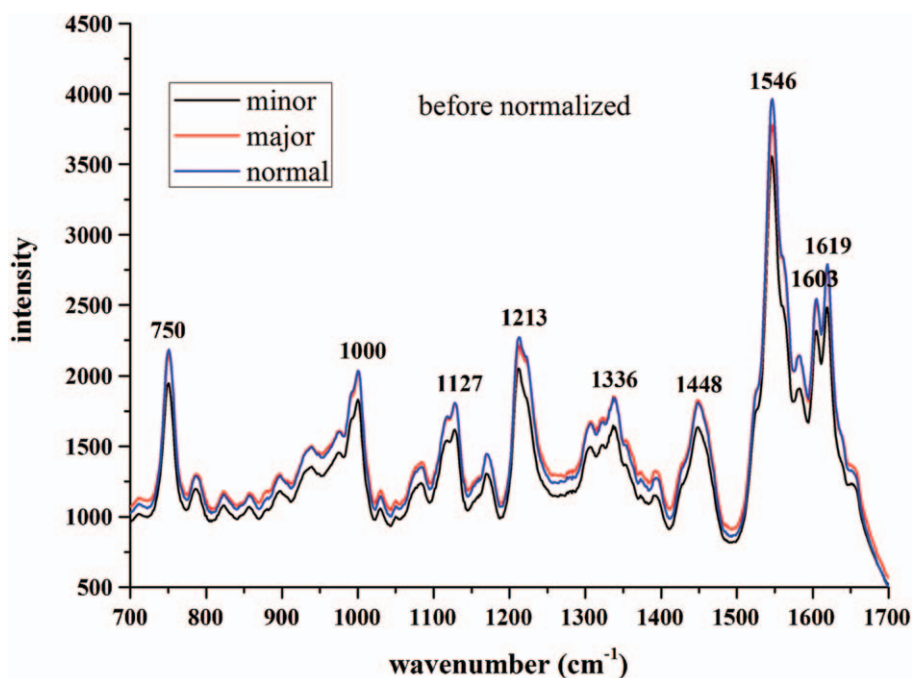


Figure 1. Average spectra of  $\beta$ -thalassemia major,  $\beta$ -thalassemia minor, and normal RBCs (before normalization). RBCs=red blood cells.

phenylalanine. The bands at 1448 and 1546  $\text{cm}^{-1}$  originated from bending vibrations of  $-\text{CH}_2$  and C-C (the amide II). Bands 1603 and 1619  $\text{cm}^{-1}$  stood for the C=C plane bending mode of phenylalanine/tyrosine and the C=C stretching mode of tyrosine/tryptophan, respectively.<sup>[20]</sup> Before normalization (Fig. 1), the normal spectral intensity was higher than those in other 2 groups,

especially at bands 750, 1000, 1127, 1213, 1546, 1603, and 1619  $\text{cm}^{-1}$ .

Control spectra were the highest at band 1546  $\text{cm}^{-1}$  compared with the spectra for  $\beta$ -thalassemia minor and major (Fig. 2) ( $P = .01$ ).

Band 1448  $\text{cm}^{-1}$  was chosen as the normalization point. After normalization, the spectra of  $\beta$ -thalassemia minor and controls

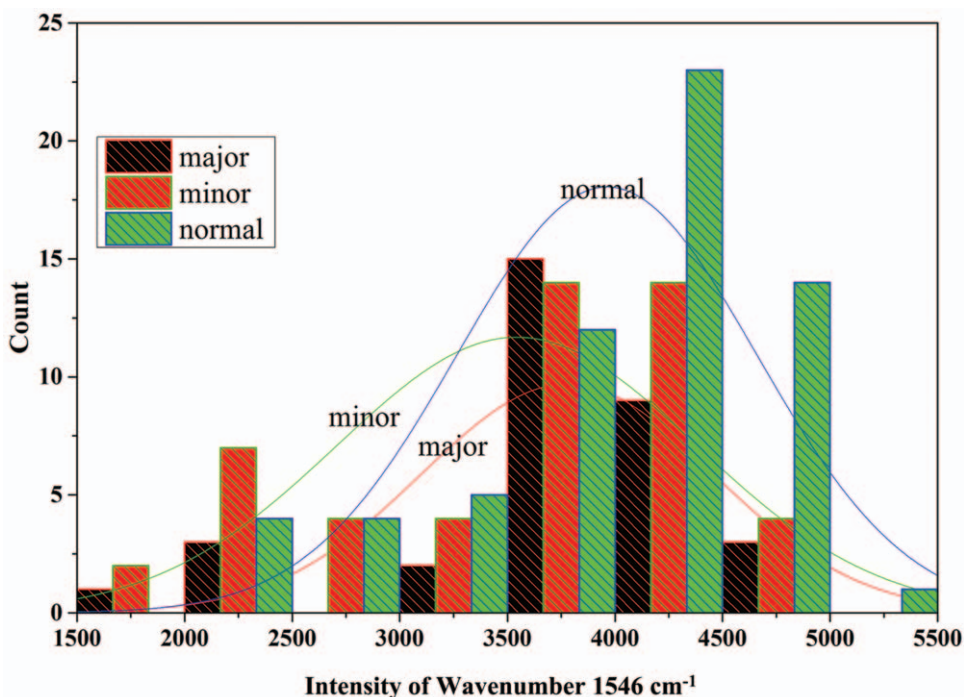
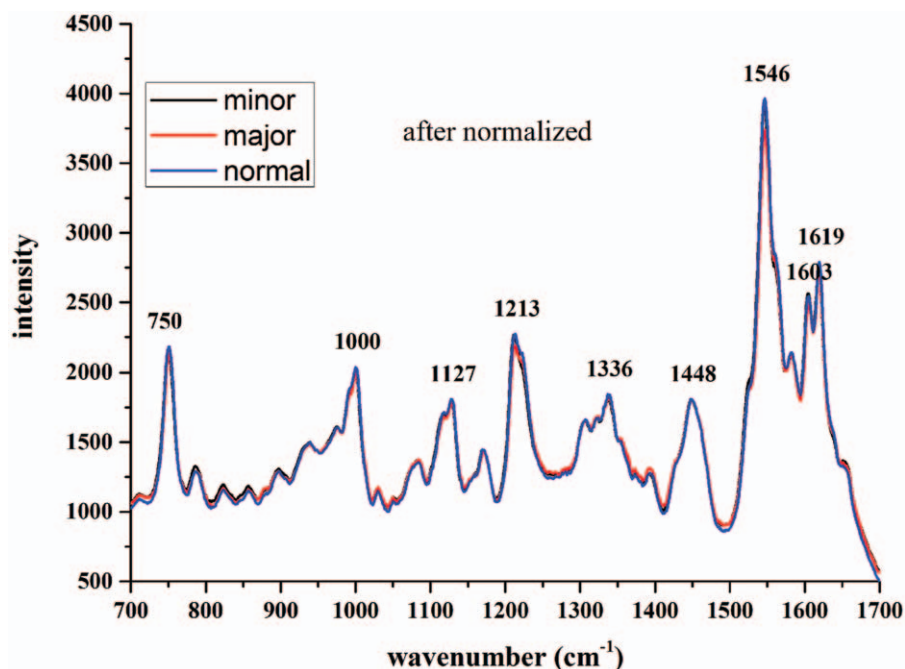


Figure 2. Intensities of wave number 1546  $\text{cm}^{-1}$  among the three groups (one-way ANOVA,  $F = 4.37$ ,  $P = .01$ ). ANOVA=analysis of variance.



**Figure 3.** Average spectra of  $\beta$ -thalassemia major,  $\beta$ -thalassemia minor, and normal RBCs (after normalization). RBCs=red blood cells.

basically overlapped (Fig. 3). Decreased intensities at bands 750, 1213, 1546, 1603, and 1619  $\text{cm}^{-1}$  (Fig. 4A, C, and D) and increased intensities at 890 to 945  $\text{cm}^{-1}$ , 945 to 960  $\text{cm}^{-1}$ , 1230 to 1240  $\text{cm}^{-1}$ , 1240 to 1260  $\text{cm}^{-1}$ , and 1260 to 1300  $\text{cm}^{-1}$  were observed in patients with  $\beta$ -thalassemia major (Fig. 4B and C).

The intensity ratio with bands 1546 to 1448  $\text{cm}^{-1}$  (I1546/I1448) was studied, suggesting a significant difference ( $P=.00$ ) between controls and  $\beta$ -thalassemia major (the ratio for majority of patients with  $\beta$ -thalassemia major  $< 2.15$ ; Fig. 5). However, no significant differences were found between  $\beta$ -thalassemia minor and controls.

PCA was performed to gain further insight of these spectra among the three groups.  $\beta$ -thalassemia major and normal RBCs were displayed in a relatively independent space in a scatter plot (Fig. 6). The same result was not confirmed for  $\beta$ -thalassemia minor and controls.

Of the 33 cases with  $\beta$ -thalassemia major, 8 cases had homozygous for codon 41–42 M/41–42 M ( $\beta^0/\beta^0$ ) mutations, 5 cases had double heterozygous for codon 41–42 M/17 M ( $\beta^0/\beta^0$ ) mutations, and 2 cases had homozygous for codon -28 M/-28 M ( $\beta^+/\beta^+$ ). The results showed that the spectra of codon 41–42 M/41–42 M ( $\beta^0/\beta^0$ ) and 41–42 M/17 M ( $\beta^0/\beta^0$ ) often overlapped after normalization, while the spectra of codon -28 M/-28 M ( $\beta^+/\beta^+$ ) showed lower intensity at bands 1546, 1603, and 1619  $\text{cm}^{-1}$  (Fig. 7).

#### 4. Discussion

$\beta$ -thalassemia, one single-gene genetic disease, is a worldwide health problem. Approximately 200 mutations or small insertions/deletions interfere with the expression of  $\beta$ -globin, resulting in the birth of a number of babies with  $\beta$ -thalassemia every year.<sup>[22]</sup> Prevention focuses on screening the carriers. Traditional screening methods include hemolysis, hemoglobin solution, and electrophoresis based on the analysis of different

compositions and quantity of hemoglobin. These complex and labor-intensive methods may be burdensome for the countries with a high prevalence of  $\beta$ -thalassemia.

Initially, the laser technologies for hemoglobin research needed hemolysates. In 2003, Liu et al<sup>[23]</sup> compared infrared absorption spectra and found decreased  $\alpha$ -helix, increased tyrosine ring, and cysteine Cys-SH in patients with  $\beta$ -thalassemia. These changes could be detected using infrared spectroscopy and might be useful for screening  $\beta$ -thalassemia carriers. It was feasible to analyze the composition at the cellular level with the development of laser technology.<sup>[9,24–26]</sup> Using the technique of LTRS in  $\alpha$ -thalassemia research, Chen X et al<sup>[16]</sup> found that the RBCs of thalassemia HbH-CS were easy to oxygenate, but difficult to deoxygenate, providing a spectral vision on the oxygenation of thalassemia HbH-CS. De Luca et al<sup>[27]</sup> found a decrease in the oxygenation capability of  $\beta$ -thalassemic RBCs after comparing the Raman spectra of six patients with  $\beta$ -thalassemia minor with controls.<sup>[27]</sup>

The Raman spectra of RBCs derived from patients with  $\beta$ -thalassemia major in this study, using the LTRS system with little labor intensity, revealed the characterization of hemoglobin structure compared with those of age-matched non-thalassemic individuals. The major Raman features included decreased lactam II, increased number of  $\beta$ -sheets and  $\beta$ -turns, and change in the proportion of globin chain (especially decreased intensity ratio of band 1546  $\text{cm}^{-1}$  to band 1448  $\text{cm}^{-1}$ ). The possible reasons for these results were greater differences in hemoglobin composition in patients with  $\beta$ -thalassemia major (mainly HbF) and normal individuals (mainly adult hemoglobin [HbA]), and excessive deposition of alpha chain in thalassemic RBCs.

Different genotypes of  $\beta^0/\beta^0$  mutations with  $\beta$ -thalassemia major may have the same Raman characterization. Theoretically, no differences were observed in the hemoglobin component among these different genotypes of patients with  $\beta$ -thalassemia major. Therefore, it is inferred that their Raman spectra are similar to each other. However, spectra of  $\beta^+/\beta^+$  mutations having lower Raman intensity may possibly attribute to relatively

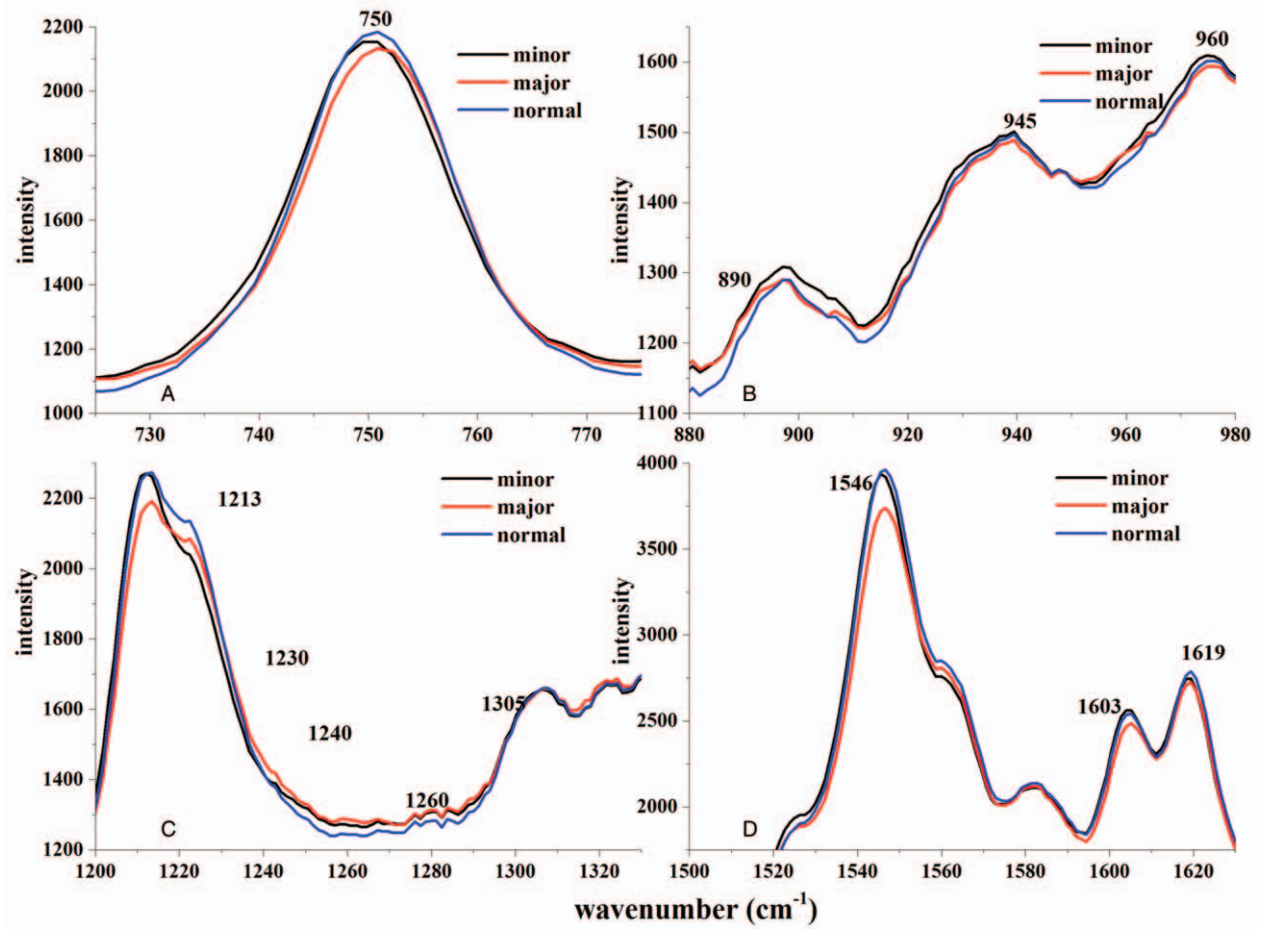


Figure 4. Specific bands area of  $\beta$ -thalassemia major,  $\beta$ -thalassemia minor, and normal RBCs. RBCs=red blood cells.

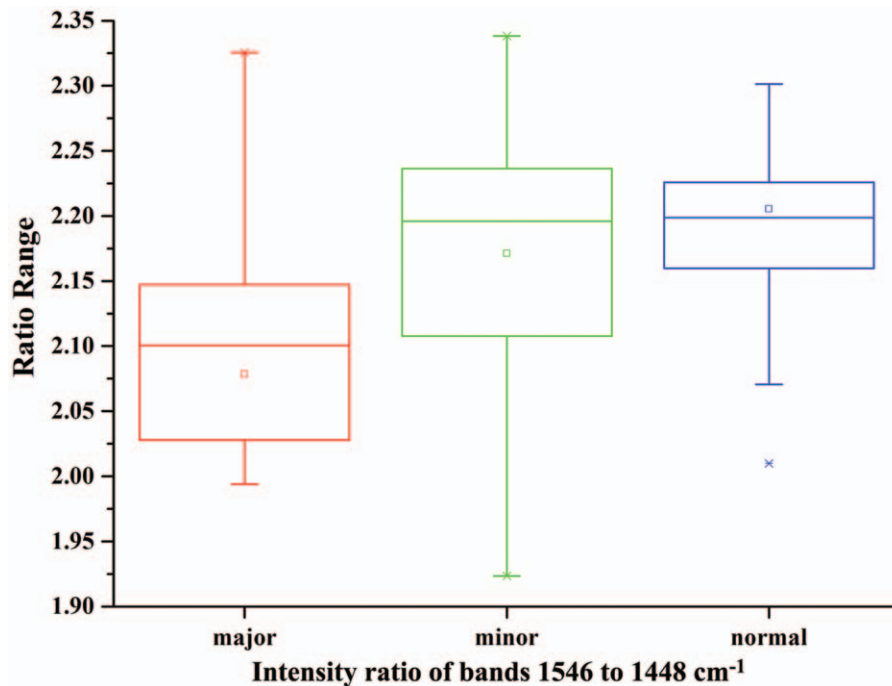
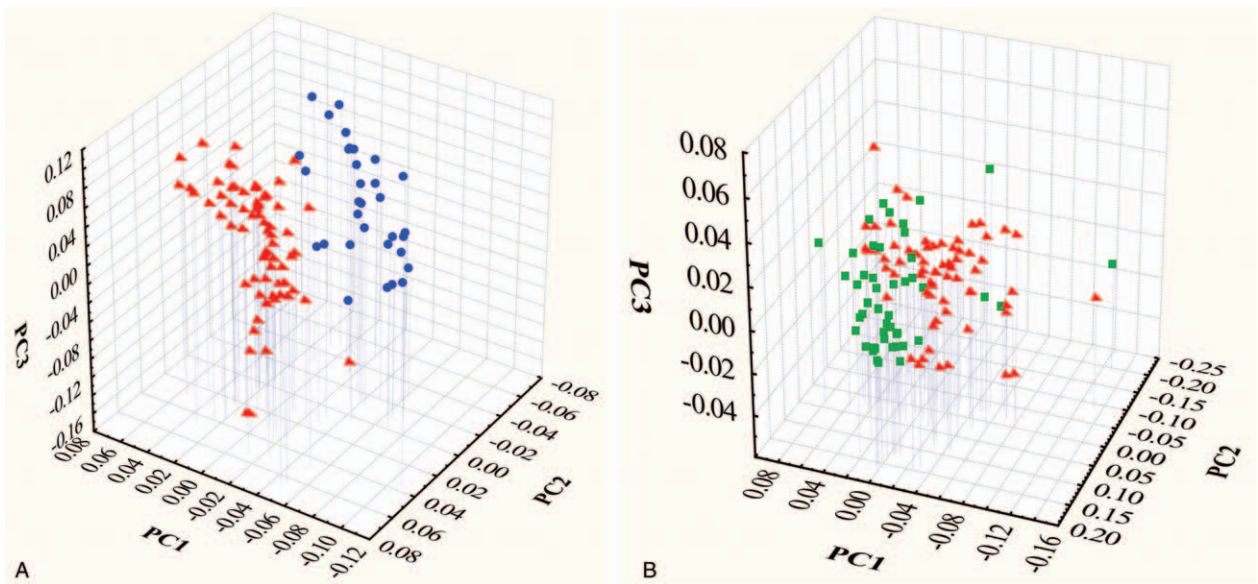


Figure 5. Intensity ratio with bands 1546 to 1448  $\text{cm}^{-1}$  (1546/1448) of normal RBCs,  $\beta$ -thalassemia minor, and  $\beta$ -thalassemia major. RBCs=red blood cells.



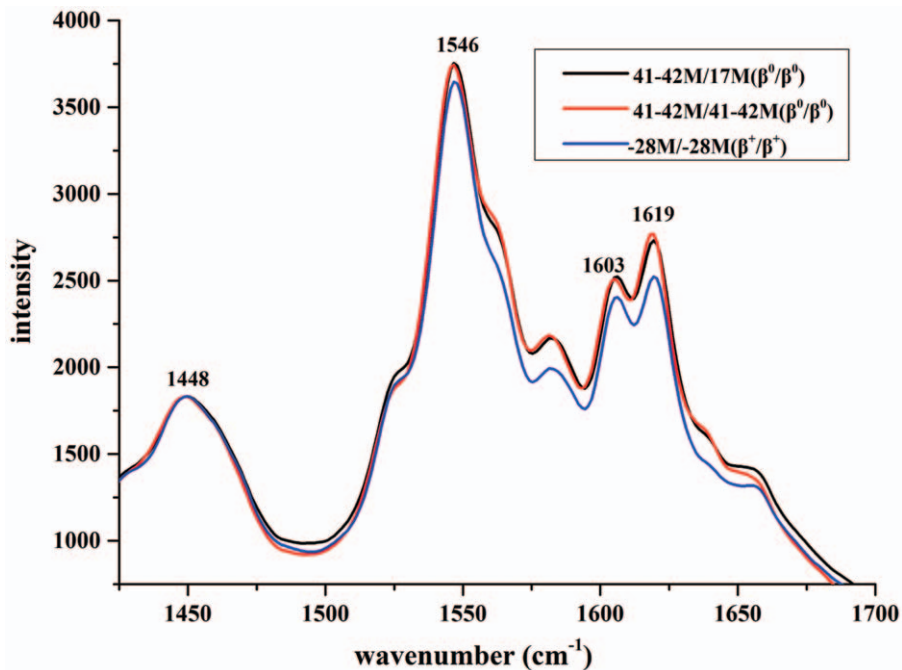
**Figure 6.** Three-dimensional (3D) distribution of PCA results of  $\beta$ -thalassemia major and controls (A), and  $\beta$ -thalassemia minor and controls (B). Red triangle stands for controls, blue cycle for  $\beta$ -thalassemia major, and green square for minor  $\beta$ -thalassemia. PCA=principal component analysis.

reduced synthesis of hemoglobin compared with that of  $\beta^0/\beta^0$ . This hypothesis was confirmed by the present study, although further analysis with the increased number of samples is also required to confirm this hypothesis.

Our results suggest that there is no significant spectra difference between the  $\beta$ -thalassemia major and the normal control. We considered that it is mainly attributable to the fact that LTRS gave us a mixed spectrum of all hemoglobin components in RBCs (i.e., HbA [ $\alpha_2\beta_2$ ], HbA<sub>2</sub> [ $\alpha_2\delta_2$ ], and HbF [ $\alpha_2\gamma_2$ ]). The main hemoglobin components in normal and

$\beta$ -thalassemia minor erythrocytes are HbA (> 90%), while slightly elevated HbA<sub>2</sub> and HbF are not recognized by current spectral processing methods. For these reasons, our next work is to isolate a single hemoglobin component (HbA, HbA<sub>2</sub>, and HbF) by hemoglobin electrophoresis, to obtain their respective Raman characterization, and to design a method for optimizing the spectra data process.

$\beta$ -thalassemia intermedia is a highly clinical concern with no single method for identification.<sup>[17]</sup> In our future work, we will attempt to apply LTRS system for identification of  $\beta$ -thalassemia



**Figure 7.** Raman spectra of different genotypes with  $\beta$ -thalassemia major.

intermedia in the following aspects: the first one is the spectral intensity. Our study has confirmed that the intensity of band  $1546\text{cm}^{-1}$  is directly proportional to the hemoglobin level. However,  $\beta$ -thalassemia intermedia (hemoglobin 7–9 g/dl) and  $\beta$ -thalassemia major (less than 6–7 g/dl) all have different hemoglobin levels. Therefore, it can be preliminarily differentiated through the intensity of band  $1546\text{cm}^{-1}$  whether that is a  $\beta$ -thalassemia intermedia or  $\beta$ -thalassemia major. The second aspect is related to the characteristics of HbA<sub>2</sub>. For  $\beta$ -thalassemia major, the HbA<sub>2</sub> level is lower than 4%. This may be helpful for identification of  $\beta$ -thalassemia intermedia and  $\beta$ -thalassemia major. The third aspect is I1546/I1448 which can reflect the relative proportion of different hemoglobins. Though the HbF level in  $\beta$ -thalassemia intermedia is not necessarily different to that of  $\beta$ -thalassemia major, the relatively lower proportion of HbF for most  $\beta$ -thalassemia intermedia individuals may be reflected in I1546/I1448.

Although several methods were proposed before introducing LTRS system to identify different kinds of thalassemia, however, this technique has several advantages:

- (1) compared to classic testing (such as complete blood cell count (CBC) index, blood smear and electrophoresis), it is reagent-free, without any need for stain and fixatives;
- (2) compared with infrared absorption spectra, it is performed under physiological conditions, without any need for hemolysates;
- (3) it uses only a low volume of RBCs, leaving blood material for other clinical purposes; and
- (4) it can be easily performed without rigid demands for expertise. Microfluidic devices are introduced with LTRS for simplification, thereby reducing the cost and shortening the time.

This is the first study on  $\beta$ -thalassemia major by using LTRS system in mainland China. The present study provided Raman characteristics of RBCs in patients with  $\beta$ -thalassemia major and supported the use of LTRS as a method for screening  $\beta$ -thalassemia major. However, the maneuverability of LTRS for screening  $\beta$ -thalassemia minor requires further improvement.

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## Author contributions

CP and LYQ conceived and designed the experiments. JWJ and CWQ performed the experiments. JWJ analyzed the data. JWJ drafted the manuscript. JWJ and CP revised the manuscript. JWJ and CWQ were involved in reference collection and data management.

**Conceptualization:** Ping Chen, Yongqing Li.

**Data curation:** Wenguang Jia, Wenqiang Chen.

**Formal analysis:** Wenguang Jia, Ping Chen, Yongqing Li.

**Funding acquisition:** Ping Chen.

**Writing – original draft:** Wenguang Jia.

**Writing – review & editing:** Ping Chen, Wenguang Jia.

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