Prenatal detection of thalassemia by cell-free fetal DNA (cffDNA) in maternal plasma using surface enhanced Raman spectroscopy combined with PCR

XIAOZHOU LI,1,2,5 TIANYUE YANG,1 CAESAR SIQI LI,3 LILI JIN,4 HONG LOU,4 AND YOUTAO SONG2,6

1School of Science, Shenyang Ligong University, Shenyang 110159, China
2College of Environmental Sciences, Liaoning University, Shenyang 110036, China
3College of Medicine, Northeast Ohio Medical University, Rootstown, OH 44272, USA
4School of Life Science, Liaoning University, Shenyang 110036, China
5biophy@163.com
6ysong@lnu.edu.cn

Abstract: Thalassemias are widely occurring genetic hemoglobin disorders; patients with severe thalassemia often require regular blood transfusions for survival. Prenatal detection of thalassemia is currently invasive and carries the risk of miscarriage and infection. A polymerase chain reaction (PCR)-based surface enhanced Raman spectroscopy (SERS) technique was investigated in this paper for the purpose of detecting prenatal α-thalassemia Southeast Asian (SEA) type deletion using maternal plasma. Couples with the same SEA thalassemia (–SEA/αα) were selected, and the quantification of SEA and wild type (WT) alleles in the maternal plasma sample predicted the fetal genotype. PCR was performed using two pairs of fluorescence tag-labeled primers to produce tag-labeled PCR products for both the SEA (labeled with R6G) and WT (labeled with Cy3) alleles. Then, the labeled PCR products containing the two fluorescence tags were measured by SERS. The ratios between the R6G and Cy3 tags were obtained using multiple linear regressions (MLR), and these ratios corresponded with the physical ratio of WT and SEA concentrations in maternal plasma. After verifying this technique on DNA mixtures with known SEA and WT ratios, the plasma from 24 pregnant women was screened. An accuracy of 91.7% was achieved for detecting the fetal genotypes of Hb Bart’s, alpha-trait, and normal trait. The results indicated that the simple PCR-SERS method may be sensitive enough for use on cell free fetal DNA (cffDNA) in maternal plasma for non-invasive prenatal detection (NIPD).

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1. Introduction

Thalassemia is a class of severe inherited single gene blood disorders that occur worldwide. It results in poor production of normal hemoglobin which can lead to anemia and less oxygen delivery through blood [1]. The high-incidence areas of thalassemias include tropical and subtropical regions such as the Mediterranean, the Middle East, and Central and Southeast Asia [2]. Mild thalassemias have been shown to protect against malaria, which helps explain its frequency in those areas [3].

Approximately 56,000 conceptions per year result in a major thalassemia disorder [4]. 9 million pregnant women are carriers annually and 1.33 million pregnancies each year are at risk for a thalassemia major [2]. In China, \(\alpha\) and \(\beta\) are the two most prevalent categories of thalassemia, with especially high prevalence in the Southern provinces of Guandong, Guangxi, and Hainan. 85% of the total thalassemia cases in China are \(\alpha\)-type [5]. Within \(\alpha\)-thalassemia, the Southeast Asian deletion (SEA) is one of the most common variants. For example in Guangdong province, the prevalence of SEA thalassemia is 4.14% (compared with thalassemia \(\beta\) of 2.54%), and single globin gene thalassemia of 4.05%) [6].

Conventional prenatal diagnosis methods require the extraction of fetal DNA from fetal trophoblast or amniotic fluid cells [2]. These invasive procedures can cause risks for the pregnancy. Chorionic villus sampling (CVS) and amniocentesis each result in a miscarriage rate of 0.5-1.0%, or higher if conducted before 15 weeks' gestation [7]. Non-invasive prenatal
detection (NIPD) of α and β thalassemia is an ongoing area of research. The key problem with NIPD is differentiating cell free fetal DNA (cffDNA) from maternal DNA. Depending on the mutations of the parents, three main methods have been used to accomplish this job: exclusion of the paternal mutation type, single-nucleotide polymorphism (SNP) based methods to differentiate the origin of DNA, and relative mutation dosage (RMD) to detect fetal mutations based on the ratio of maternal alleles [8]. RMD methods are often completed using real-time PCR (RT-PCR) by counting the cycle threshold (Ct), which is the number of PCR cycles required to reach a certain amount of PCR product [9,10]. Instead of using Ct, this experiment used surface enhanced Raman spectroscopy (SERS) to quantify the thalassemia alleles.

SERS is a scattering spectroscopy method that involves inelastic vibration of the secondary structure of molecules. It has the sensitivity to detect even single molecules [11]. The high sensitivity is due to the plasmonic effects that occur when target molecules adhere on noble metal surfaces [12]. SERS can detect DNA sequences either through direct measurement or by indirect measurement of Raman-active tags labeled to target sequences [13].

Approximately 3-20% of maternal plasma DNA is composed of trophoblastic cell derived cffDNA, and this cffDNA has shorter sequences than maternal DNA [14]. Thus, certain amplification and treatment steps are required for the detection of cffDNA in plasma. PCR methods has been previously used for the simultaneous detection of 3 target strands extracted from epizootic pathogens [15]. For detecting mutations, mutation-specific PCR [16] and PCR-like methods such as exponential strand displacement amplification (SDA) [17] and ligase detection reaction (LDR) [18] have been introduced before SERS. In those processes, only mutated sequences are amplified and then detected by SERS. The features of multiplex PCR with fluorescence-labeled primers, which is used in this paper, include the fact that both the SEA mutation and wild type (WT) alleles can be amplified simultaneously. This is useful for comparing the ratios between SEA and WT, and thus this method was selected to pretreat the maternal plasma prior to SERS measurement.

In this paper, a NIPD method for α-thalassemia detection based on PCR and SERS was investigated. The aforementioned PCR-SERS method was verified on DNA mixtures simulating maternal DNA with cffDNA fractions of 2%, 5%, 10%, 15%, and 20% for all three gene types: Hb Bart's, alpha-trait and normal, respectively. Then, actual maternal plasma was measured using the PCR-SERS method to determine the ratio between SEA and WT alleles. Twenty-four pregnant women participated in our study. For all SERS spectra, the SEA/WT ratios were calculated using multiple linear regression (MLR). Finally, multi-class ROC analysis was applied on the obtained SEA/WT ratios determine the prediction ability of those ratios for each of the three fetal genotypes (Hb Bart's, alpha-trait and normal).

2. Materials and methods

2.1 Samples

DNA mixtures with known SEA/WT (–SEA/αα) ratios were prepared first for the verification of the suggested PCR-SERS method. CffDNA comprises about 3-20% of the total DNA in maternal plasma. To simulate that, DNA solutions with the three possible fetal genotypes (–SEA/–SEA, –SEA/αα, and αα/αα) were added to DNA solutions with the maternal genotype (–SEA/αα) in concentrations of 2%, 5%, 10%, 15%, and 20%. The total concentration of the final DNA mixtures was $5 \times 10^{-11}$ M.

Twenty-four couples that were both –SEA/αα were selected in this experiment. The probabilities of each pregnancy resulting in the three genotypes are 25% for Hb Bart’s (–SEA/–SEA), 50% for alpha-trait (–SEA/αα) and 25% for normal (αα/αα), respectively. The average age of the pregnant women was 28 years, and blood samples were collected at gestation weeks 13-23. All pregnant women were screened by abdominal amniocentesis to
ensure the thalassemia statuses of the fetuses were known. Peripheral blood samples (3 mL) were collected from each patient between 7:00 and 8:00 a.m. after a 12 hour overnight fast. The collected blood was mixed with EDTA anticoagulant and was centrifuged at 5,000 rotations/min for 10 min at 4°C to remove blood cells. The resulting plasma was stored in a 1.5 mL Eppendorf tube at −80°C. Plasma DNA was extracted with a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations. The extracted DNA was stored in a refrigerator at −20°C.

2.2 PCR

Multiplex PCR targeting both SEA and WT alleles was conducted with fluorescence tag-labeled primers. Primer sequences and PCR conditions were as described in [10]. Fluorescence tags and primer sequences are listed in Table 1. PCR reactions were performed in a total volume of 25 μL containing 1x PCR buffer, 5 μL extracted DNA, 0.3 μM of each primer, 2.5 mM MgCl₂, 0.3 mM dNTP, and 1.25 units DNA polymerase. Thermal cycling conditions involved a 5 minute initial denaturation at 94°C, a 45-cycle amplification step (95°C for 15 seconds, 55°C for 50 seconds, and 72°C for 45 seconds), and a final extension step at 72°C for 10 minutes. The resulting PCR products were then purified using the PCR purification kit (Qiagen, USA) to remove unincorporated primers.

Table 1. Primer sequences for multiplex PCR targeting SEA and WT

| Primer sequences                        |
|----------------------------------------|
| Common forward primer: 5’-TCG GTC GTC CCC ACT GT |
| Reverse primer for SEA: 5’-R6G-CAG CCT TGA ACT CCT GGA CTT AA |
| Reverse primer for WT: 5’-Cy3-GGA CTG CTC CGC TCC AC |

2.3 SERS

SERS spectra were recorded on a Renishaw Raman microspectrometer system (Gloucestershire, UK) equipped with a He-Ne laser (λ = 632 nm, beam diameter: 1.5 μm) and an electrically cooled CCD detector. The laser power at the sample was 4 mW. Exposure time of the CCD was 10 s.

Spermine coated Ag colloids were made in accordance with the published method [19]. Briefly, 10 mL AgNO₃ (1 mM) and 5 µL spermine hydrochloride (0.1 M) was mixed under vigorous stirring, then 25 µL of NaBH₄ (0.1 M) was added to the solution and stirred for 20 min. Spermine functioned as an aggregating and DNA backbone neutralizing agent in this experiment [20]. SERS was measured by mixing 60 µL of the nanoparticles and 10 µL of the PCR products. The measurement was conducted within one min after the mixing.

2.4 Statistical analysis

Statistical analysis was performed in the R language environment (https://www.r-project.org). MLR was used on the SERS spectra to decompose it into combining spectra of tags (R6G and Cy3). In the MLR, the SERS spectra of pure R6G and Cy3 (which representing SEA and WT, respectively) were used as reference spectra. The SEA/WT (−SEA/αα) ratio as indicated by the R6G/Cy3 ratio was used for the prediction of fetal thalassemia type. This is possible because the three fetal genotypes of Hb Bart’s (−SEA/−SEA), alpha-trait (−SEA/αα) and normal (αα/αα) can either increase (>1), not change (= 1), or decrease (<1) the −SEA/αα ratio of DNA from the maternal plasma (−SEA/αα). Finally, one-vs-rest receiver operating characteristic (ROC) curves were used to determine the prediction abilities of the SEA/WT ratios for each of the three fetal genotypes - Hb Bart’s, alpha-trait and normal.
3. Results

3.1 Verification of PCR-SERS

First, the SERS spectra of the tags R6G and Cy3, which were labeled to the SEA and WT alleles respectively, were measured. There were differences in both peak positions and intensities between the two SERS spectra [Fig. 1]. Six peaks were found in the R6G spectra and 10 peaks in Cy3. The major peaks of R6G were located at 1129, 1183, 1314, 1365, 1510, 1574, and 1652 cm$^{-1}$, while the major peaks of Cy3 were located at 556, 612, 796, 927, 1121, 1184, 1271, 1398, 1471, and 1590 cm$^{-1}$. For R6G, the highest peak occurred at 1314 cm$^{-1}$, and the lowest peak occurred at 1365 cm$^{-1}$. While for Cy3, the highest peak occurred at 1471 cm$^{-1}$, and the lowest peak occurred at 1121 cm$^{-1}$. Those differences enable the deconvolution of PCR product SERS spectra by MLR into the spectra of different tags feasible.

![Fig. 1. SERS spectra of R6G and Cy3 tagged to primers targeting SEA and WT alleles.](image)

DNA mixtures were then created to verify the PCR-SERS technique. Fetal DNA with known genotypes (Hb Bart’s (–SEA/–SEA), alpha-trait (–SEA/αα) and normal (αα/αα)) were mixed with reference maternal DNA in different percentages (2%, 5%, 10%, 15%, and 20%) and the solutions were tagged and measured by the suggested PCR-SERS method. For each fetal gene type, the SERS spectra were similar in both peak positions and peak heights [Fig. 2]. The peak heights did not show any obvious change when the fetal DNA percentages decreased (2%-20%). As the SERS spectra are contributed to by the fluorescence tags labeled to primers - R6G and Cy3, they contain feature peaks from both of the two tags. The trend plots [Figs. 2(B), 2(D), and 2(F)] show more clearly the peak height changes. Four peaks were selected for the comparison: two major peaks of R6G located at 1314 and 1652 cm$^{-1}$, and two major peaks of Cy3 located at 1398 and 1471 cm$^{-1}$. When the fetal genotype added was –SEA/–SEA, the two peaks contributed to by R6G tagged to SEA had an upward trend, but they decreased when αα/αα was added instead. The situation is reversed as expected for the two peaks contributed to by Cy3. When the fetal thalassemia type was –SEA/αα, the peak heights of all four peaks remained stable.
Fig. 2. SERS of PCR products of five DNA mixtures with manmade fetal DNA percentages of 2%, 5%, 10%, 15%, and 20% for fetal gene types of Hb Bart’s (–SEA/–SEA) (A), alpha-trait (–SEA/αα) (C) and normal (αα/αα) (E). Peak height changes of the four major peaks of R6G (red) and Cy3 (blue) for the three fetal gene types Hb Bart’s (B), alpha-trait (D), and normal (F).

Figure 3(A) shows the SEA/WT ratios obtained by MLR for the standard –SEA/–SEA, –SEA/αα, and αα/αα mixtures, respectively. Due to the fact that –SEA/αα maternal DNA accounts for more than 80% of the total DNA, the SEA/WT ratios for the –SEA/αα mixtures should be approximately 1. The ratio for the fetal type of –SEA/αα appeared constant regardless of the fetal DNA percentages. For fetal types of –SEA/–SEA and αα/αα, the SEA/WT ratios will deviate from 1 with the increase of fetal DNA percentages. For all the three types of fetal thalassemia types, a scatter plot based on actual and calculated SEA/WT ratios was drawn. The calculated SEA/αα ratio showed good concordance with the actual ones (R² = 0.91) [Fig. 3(B)].
3.2 Application on maternal plasma

PCR-SERS was then applied on the plasma taken from 24 pregnant women. Couples with the same thalassemia type –SEA/αα were selected so that the fetus could have the possible genotypes of –SEA/–SEA, –SEA/αα, and αα/αα with the probability ratios of 1:2:1. As Fig. 4 indicates, the SERS spectra of the fetal thalassemia types of –SEA/–SEA, –SEA/αα, and αα/αα had trivial differences in peak heights. Peaks at 1314 and 1471 cm⁻¹ (R6G and Cy3) showed larger differences, while peaks at 612 and 1574 cm⁻¹ (Cy3 and R6G) showed smaller differences.
Fig. 4. Representative SERS spectra of three types of fetal thalassemia: –SEA/–SEA, –SEA/αα, and αα/αα.

The SEA/WT ratios of the 24 samples were then calculated by MLR. Figure 5 is the scatter plot drawn using the SEA/WT ratios. The spots are grouped according to the actual fetal genotypes as determined by abdominal amniocentesis. As expected, the SEA/WT ratios of the –SEA/–SEA group were higher than that of –SEA/αα, and the ratios of –SEA/αα were higher than that of αα/αα. The observed trends were similar to those of the known DNA mixtures.

Fig. 5. Scatter plot of the SEA/WT ratios of the three fetal types –SEA/–SEA (red), –SEA/αα (green), and αα/αα (blue) marked with mean and standard errors.
ROC was then utilized to see the prediction performance of the SEA/WT ratios. For the three groups in this experiment, a one-vs-rest ROC method was used. Sensitivity, specificity, and accuracy ranged from 75% to 100% for the three groups of –SEA/–SEA-vs-rest, –SEA/aa-vs-rest, and aa/aa-vs-rest (Table 2). Since the SEA/WT ratios of all three groups had a general relationship of –SEA/–SEA >–SEA/aa > aa/aa, the optimal cutpoints of –SEA/–SEA-vs-rest, and aa/aa-vs-rest were used for the discrimination of all the three groups. The total accuracy obtained using the two cutpoints was 91.7% [Fig. 6].

Table 2. Prediction ability of ROC for the three fetal genotypes: –SEA/–SEA, –SEA/aa, and aa/aa

|                | –SEA/–SEA | –SEA/aa | aa/aa |
|----------------|-----------|---------|-------|
| Sensitivity    | 100%      | 100%    | 80%   |
| Specificity    | 100%      | 89%     | 100%  |
| Accuracy       | 100%      | 75%     | 100%  |

4. Discussion

This paper utilized a PCR-SERS technique to detect α-thalassemia in maternalcffDNA. A key step in this process was RMD, a common method to detect single gene disorders when the parents are both heterozygotes [21]. The three batches of standard DNA containing fetal genotypes –SEA/–SEA, –SEA/aa, and aa/aa in 2%, 5%, 10%, 15%, and 20% fractions was first used to verify the PCR-SERS method. The obtained spectra of PCR products labeled with R6G and Cy3 tags allowed for analysis of key feature peaks. The SEA/WT (–SEA/aa) ratios were calculated by MLR analysis, which deconvoluted the obtained spectra into composing spectra of fluorescent primer-attached R6G and Cy3. When the fetal DNA percentages increased in the maternal background of –SEA/aa, SEA/WT ratios showed the expected changes towards the genotype of fetus. When the 24 experimental samples were tested using this process, The SEA/WT ratios for –SEA/aa was 1 as expected. While the SEA/WT ratios were higher and lower than 1 for the other two groups –SEA/–SEA and
αα/αα, respectively. An accuracy of 91.7% was achieved using ROC analysis for the three fetal genotypes of Hb Bart’s (–SEA/–SEA), alpha-trait (–SEA/αα) and normal (αα/αα).

For mutation detection, other PCR-like methods have been implemented for the pretreatment of gene solutions prior to undergoing SERS. Those methods include exponential strand displacement amplification (SDA) [17] and ligase detection reaction (LDR) [18], which amplifies the target mutation sequences specifically. In comparison, the PCR method used in this experiment was much easier to implement and is ready for the simultaneous amplification of multiple gene alleles [16]. This method was first conducted for the multiplex genotyping of three genes using Raman-active dyes [20]. The fluorescence tags selected in this experiment were found to have equally good differentiation abilities for multiplex detection [22]. Additionally, in comparison with peak heights which are commonly used for identification, MRL in this experiment proved to be a useful deconvolution method with high quantitative accuracy. The main method for the detection of α and β thalassemia by means of RMS was real time quantitative PCR (RT-PCR). The prediction accuracy were 100% (13/13) [10], 86.7% (137/158) [9], and 66.7% (26/39) [23], separately. In comparison, the prediction accuracy of the suggested PCR-SERS methods in this paper was 91.7% (22/24). In RT-PCR, the quantification of PCR products is done using differences in the cycle threshold (ΔCt). While the SERS method makes the quantification of PCR products simpler and direct by comparison of feature peaks.

Fetal DNA of any genotype will cause slight but detectable changes in the maternal DNA. This fact will allow the aforementioned PCR-SERS method to be extended for the detection of fetal thalassemia genotypes from couples with other thalassemia combinations. The development and inclusion of other PCR or SERS technologies can also further increase the prediction accuracy. For example, multiplex detection of mutations techniques such as TaqMan [24] can be incorporated at the PCR step, and magnetic nanoparticles (MNPs) [15] can be used to aggregate target sequences at the SERS step.

5. Conclusion

In conclusion, the PCR-SERS method demonstrated in this manuscript is a potential NIPD technique that uses cffDNA for the detection of SEA thalassemia. The strategy of this method is to first use PCR to get fluorescence-labeled target sequences and then use SERS to measure the fluorescence tags, for which the SEA/WT ratios are calculated using MLR. DNA mixtures containing three different fetal genotypes (–SEA/–SEA, –SEA/αα, and αα/αα) in increasing percentages were used to verify the PCR-SERS method. The obtained SEA/WT ratios were as expected. The method was then applied on the maternal plasma samples from 24 -SEA/αα type couples. The ROC prediction accuracy was 91.7% for all the possible fetal genotypes. In conclusion, the multiplex PCR method proved to be simple and flexible for amplifying multiple alleles simultaneously, and the subsequent SERS and MLR method effectively detected and then deconvoluted the fluorescence tags labeled to PCR products, respectively.

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Disclosures

The authors declare that there are no conflicts of interest related to this article.