Abstract

Rationale: While thalassemia is a monogenic disease that is relatively common worldwide, there is no recognized radical cure for thalassemia in current medical practice. Prenatal diagnosis is the most important contribution to thalassemia prevention, but due to its technical limitations, rare thalassemia mutations cannot be detected; and the birth of thalassemic babies cannot be completely circumvented. Whole-exome sequencing can, however, compensate for this shortcoming.

Patient concerns: We report the results of whole exon sequencing of amniotic fluid cells in 5 pregnant women with thalassemia.

Diagnosis: Prenatal diagnosis revealed that 4 of them were α-thalassemia carriers and 1 of them was β-thalassemia carrier.

Interventions and Outcomes: We collected amniotic fluid of 5 pregnant women (age range: 25–27 years, Mean ± SD: 28 ± 1.8) with thalassemia. The gestational ages ranged between 16 and 19 weeks. The cells were separated from the amniotic fluid and passaged until a sufficient number of cells were obtained for exome sequencing. We therefore employed whole-exome sequencing of amniotic fluid cells from thalassemic carriers to validate prenatal diagnostic results and to identify novel mutation sites. We found that 4 of 5 samples are SEA which is consistent with the clinical prenatal diagnosis. However, 2 of 5 samples were point mutations in the HBB gene, and were thus different from the clinical prenatal diagnosis.

Conclusion: The identifications from this study showed that prenatal diagnosis has limitations. Whole-exome sequencing can compensate for this shortcoming. And this study would add new insights into understanding of molecular mechanisms in thalassemia.

Abbreviations: CNV = copy number variation, SNP = single-nucleotide polymorphism, WES = whole-exome sequencing.

Keywords: α-thalassemia; β-thalassemia; case report; amniocyte fluid cell; whole-exome sequencing.

1. Introduction

The original term “thalassemia” denoted a Mediterranean anemia as most of the early cases were found in coastal regions of the Mediterranean Sea. However, there are many different types with respect to clinical symptoms, with only anemia and hemolysis as common signs. Therefore, thalassemia is actually a group of disorders, and its etiology entails a deletion or mutation of a globin gene that results in 1 or more defects in globin peptide-chain synthesis. This defect results in a severe imbalance in the ratio of α-globin to β-globin in the hemoglobin molecule, and unbalanced synthetic rates in α- and β-globins then generate anemia.[1–3] Although routine clinical treatments include blood transfusion, iron chelation, and splenectomy, hematopoietic stem cell transplantation has shown to be the most effective method for curing thalassemia.[4–6] As allogeneic hematopoietic stem cell transplantation is limited to patients with a human leukocyte...
antigen-syngeneic donor, matching remains difficult; and sources of bone marrow (hematopoietic stem cells) are rare—with potential graft-versus-host disease severely limiting the promotion of this treatment.\(^4\) \(\gamma\)-globin gene activation is now 1 of the most effective treatments for \(\beta\)-thalassemia: drugs are used to stimulate the effective expression of the \(\gamma\)-globin gene that is repressed after birth, and this improves the imbalance in \(\alpha\)- and non-\(\alpha\)-globin synthesis and increases fetal hemoglobin synthesis to reduce hemolysis and alleviate anemia.\(^7,8\) While numerous drugs can induce \(\gamma\)-globin expression or promote fetal hemoglobin synthesis, many possess deficiencies such as poor efficacy, severely toxic side-effects, and a short half-life.\(^9,10\) Prenatal diagnosis is therefore an effective measure used to prevent the birth of severely thalassemic babies. Invasive prenatal diagnostic methods principally involve the collection of fetal genetic material from the chorion, amniotic fluid, and umbilical cord blood for laboratory testing; and samples that exhibit positive screening results are then used for further genetic analysis and diagnosis. However, this methodology usually only detects common mutation sites in the \(\alpha\)- and \(\beta\)-globin gene clusters, resulting in an inability to distinguish rare thalassemic mutations. As exome sequencing can compensate for this shortcoming, we employed this modality to sequence amniotic fluid cells from

| Sample | Gestational age (wks) | Clinical diagnosis                  | Gene type               |
|--------|-----------------------|-------------------------------------|-------------------------|
| N1     | 16+5                 | \(\alpha\) thalassemia heterozygote | --SEA                  |
| N2     | 18+4                 | \(\alpha\) thalassemia heterozygote | --SEA                  |
| N3     | 16+4                 | \(\alpha\) thalassemia heterozygote | --SEA                  |
| N4     | 16+1                 | \(\alpha\) thalassemia heterozygote | --SEA                  |
| N5     | 18+4                 | \(\beta\) thalassemia heterozygote  | CD27-28 (+C)           |

Figure 1. Pictures of blood smear and amniotic fluid cells. A: The blood smear of normal person (left) and thalassemia (right). B: Photomicrographs of amniotic fluid cells on day 0 and day 14.
thalammasia carriers; and this allowed us to validate prenatal diagnostic results and to uncover novel mutation sites.

2. Case report

We collected amniotic fluid from 5 pregnant women who were thalassemia carriers at gestational ages between 16 and 19 weeks (i.e., mid-second trimester) (Table 1). Hypochromic and microcytic red blood cells and target cells were shown in the peripheral blood smear of patients with thalassemia (Fig. 1A). We used gradient centrifugation to isolate fetal fibroblasts for in vitro culture. After 14 days of culturing fetal fibroblasts to allow proliferation (Fig. 1B), we extracted genomic DNA from the cells for Whole-exome sequencing (WES). Of the 5 samples, the clinical prenatal diagnostic results showed 4 amniotic fluid samples from Southeast Asian deletion (--SEA) \( \alpha \)-thalassemia carriers and 1 from a CD27-28 (+C, frameshift mutation) \( \beta \)-thalassemia carrier (Table 1).

By exploiting the East Asian 1000 Genomes database, we compared the normal copy number of chromosomal genes in the HGNC database to obtain the copy number variation (CNV) for genes on every chromosome (data were not shown), and noted that most \( \alpha \)-thalassemia cases were due to fragment deletion. As we observed numerous CNVs for the \( \alpha \)-globin gene, we screened for globin-gene copy number in our exome-sequencing data. We determined that the copy number for the \( \alpha \)-globin genes HBA1 and HBA2 on chromosome 16 in 4 samples (N1, N2, N3, N4) was 1, and that there was copy number loss (the normal copy number for \( \alpha \)-globin genes HBA1 and HBA2 is 2). Only the N5 sample manifested a copy number of 2 for HBA1 and HBA2 (Fig. 2). These results were consistent with

![Figure 2](image)

**Figure 2.** Statistical analysis of HBA1 and HBA2 gene copy number in the 5 samples. A: HBA1 gene copy number in the 5 samples. B: HBA2 gene copy number in the 5 samples. The yellow dots represent insert (INS), blue dots represent delete (DEL), and red dots represent normal.

| Sample | Diagnostic genotype | Whole-exome sequencing |
|--------|---------------------|------------------------|
| N1     | --SEA               | Both HBA1 and HBA2 only have 1 copy/HBB gene mutation (rs10768683: G > C) |
| N2     | --SEA               | Both HBA1 and HBA2 only have 1 copy |
| N3     | --SEA               | Both HBA1 and HBA2 only have 1 copy |
| N4     | --SEA               | Both HBA1 and HBA2 only have 1 copy |
| N5     | CD27-28 (+C)        | HBB gene mutation (rs10768683: G > C) |

![Table 2](image)

**Table 2**

Clinical prenatal diagnosis and whole-exon sequencing results of the 5 samples.

![Figure 3](image)

**Figure 3.** \( \beta \)-globin-mutation screening. A: HBB gene-mutation sites in N1 and N5 samples. WT, normal human gene type; MUT, mutation; B, \( \beta \)-globin point mutation (G > C) in N1 and N5 samples.

| Sample | Number of SNPs | Number of InDels |
|--------|----------------|------------------|
| N1     | 47347          | 4053             |
| N2     | 47792          | 4097             |
| N3     | 47869          | 4068             |
| N4     | 47697          | 3969             |
| N5     | 48190          | 4048             |

SNP = single-nucleotide polymorphism.
the clinical prenatal diagnosis that the N1, N2, N3, and N4 samples were from --SEA α-thalassemia carriers (Table 2).

A majority of β-thalassemia cases are due to point mutations, and, therefore, single-nucleotide polymorphism (SNP) and small insertion and deletion (InDel) mutation annotations can be used for screening. The β-globin (HBB) gene is located on chromosome 11, and we located a point mutation (rs10768683: G > C) on the β-globin gene (ENSG00000244734) in the N1 and N5 samples; these were non-synonymous SNP mutations that resulted in a single amino acid substitution (A-P) (Fig. 3). This outcome was thus significantly different from the clinical prenatal diagnosis that revealed that only the N5 sample reflected a CD27-28 (+C, frameshift mutation) mutation in the HBB gene (Table 2).

There are abundant mutation sites in the human genome, and some mutations are extremely common and proven not to alter normal human survival rates. However, some mutations are highly correlated with human disease, and we applied GATK software for statistical analysis of SNP and InDel mutations (Table 3). In order to identify significant disease mutations, we used ANNOVAR software for annotation analysis of the detected SNP and InDel mutations combined with external databases to confirm the genome location, mutation frequency, protein harmfulness, genotype heterozygosity, and functional pathway information.[12] GO-enrichment analysis was executed on the common mutation sites in the 5 samples and 26 terms were significantly enriched based upon the false-discovery rate-corrected P value (i.e., q value) of .05 as the upper limit. The Gene Ontology (GO) terms included plasma membrane, actin cytoskeleton, extracellular matrix structural constituent, calcium ion binding, and transmembrane signaling receptor activity (Fig. 4). When we used Kyoto Encyclopedia of Genes and Genomes-enrichment analysis for the common mutation sites, we noted 13 signaling pathways as significantly enriched—including sugar metabolism, β-alanine metabolism, ECM receptor interaction, and complement and coagulation cascades (Fig. 5).

Figure 4. The significantly enriched biological functions of the common SNP and Indel using GO are illustrated in bar chart. GO = Gene Ontology, SNP = single-nucleotide polymorphism.
3. Discussion

Thalassemia is a monogenic disorder that exhibits a high global incidence. Even though improvements have been made in thalassemia advocacy and prenatal diagnosis, there remain many patients who are afflicted but not yet identified. We performed exome sequencing amniotic fluid cells from 5 pregnant women who were thalassemia carriers, and CNV results showed that α-globin gene-copy loss was present in 4 samples, which was congruent with clinical prenatal results of α-thalassemia-carrier status in 4 corresponding samples. SNP and InDel mutation annotation screening, however, revealed that non-synonymous mutation in the HBB gene was found in the N1 and N5 samples, which differed significantly from the clinical prenatal results that showed that only the N5 sample contained a CD27-28 (+C, frameshift mutation) in the HBB gene. These data show that exome sequencing can complement the short-comings of prenatal clinical diagnosis, and that it is an effective tool for identifying novel thalassemia-mutation sites.

GO-enrichment analysis of the mutated genes showed that 26 terms were significantly enriched, including plasma membrane, actin cytoskeleton, and extracellular matrix structural constituent. Athanasiou et al employed micropipette aspiration to measure the elastic shear modulus in erythrocytes from both a thalassemic mouse model and from patients with thalassemia, and demonstrated that their erythrocyte stiffness was significantly greater than in normal erythrocytes.[13] Investigators reported that a diminution in erythrocyte deformability and membrane stability induced the destruction of erythrocytes as they passed through the medullary cavity, splenic sinuses, and capillary network; and that it shortened both erythrocyte lifespan in blood and an affected individual’s lifespan.[14] We subsequently carried out Kyoto Encyclopedia of Genes and Genomes-enrichment analysis on the mutated genes, and ascertained that 13 signaling pathways were significantly enriched—including sugar metabolism and complement and coagulation cascades. Chen et al also demonstrated that thalassemia was correlated with complications of diabetes mellitus, as type 2 diabetics exhibited higher levels of serum ferritin relative to non-diabetic individuals.[15] Luo Y. et al conducted a study on 79 moderately thalassemic patients (114 of whom had HbH and 18 with HbE/β), and reported that 33 were hypoglycemic, 25 showed impaired glucose tolerance, and 4 manifested diabetes. Impaired glucose tolerance in symptomatic diabetes mellitus was substantiated for thalassemic patients.[16] An excess of deformed globin chains in thalassemic erythrocytes are deposited on the erythrocyte membrane, leading to membrane protein aggregation and binding to autoantibodies and complement, and this in turn promotes the rapid clearance of erythrocytes in the circulation.[17] Abnormalities of the innate immune system associated with thalassemia were reported including reduced absorption and phagocytic ability of polymorphonuclear neutrophils, and altered intracellular metabolic processes.[18,19] Complement is a part of the innate immune system which plays an essential role in defense against pathogens and intinsome homeostasis.[20] Hemolytic diseases are often accompanied by dysregulation and overactivation of the complement system, which may be induced by free extracellular heme.[21-23] In our study, we uncovered complement-related immune abnormalities in thalassemic fetuses.

YeeHo et al used pyrosequencing to detect beta-thalassemia mutations in prenatal samples (chorionic villus biopsies and cultured and uncultured amniocytes). However, pyrosequencing has strict requirements on the concentration of DNA template, and the presence of maternal cell contamination can interfere with interpretation of results, particularly when the fetus carries the same mutation as the mother.[24] Murad et al used molecular screening and direct DNA sequencing to evaluate a prenatal diagnosis of β-thalassemia by collecting 55 amniotic fluid samples in Syria.[25] In another study fetal cells were isolated using micromanipulators at 8 weeks of gestation in women with high-risk pregnancies and nested polymerase chain reactions were performed.[26] That study demonstrated that prenatal diagnosis of β-thalassemia may be feasible at an early stage of pregnancy. In our study, we performed WES to analyze amniotic fluid cells from 5 pregnant women who were thalassemia carriers, and noted WES as an effective tool for identifying novel thalassemia-mutation sites. However, since exome sequencing requires high DNA concentrations, the process requires 10 to 14 days of cell culture in vitro; and the high sequencing costs also limit its use in clinical prenatal diagnosis. The obstetricians/gynaecologists were most concerned that ordering an expensive test could lead to overtreatment, higher cost of care and may increase parental anxiety.

4. Conclusion

In conclusion, we found that the use of exome sequencing enables a more comprehensive analysis of mutation sites. As the costs of sequencing fall, and bioinformatic and analytic capabilities improve, this will improve diagnostic capabilities because of improved ability to detect a wider range of genomic abnormalities, including non-coding variants, copy number changes and larger rearrangements. We will further accumulate the number of samples, in order to provide reliable evidence for improving the accuracy and sensitivity of clinical diagnosis of thalassemia.

Author contributions

JMN, YL and JZ designed research; XKL, XH, XZ performed research; WZ, XKL, XXW analyzed data; WZ, XKL and XXW wrote the paper.

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