INTRODUCTION

The hemoglobinopathies are endemic in many world populations, and have genetic carrier rates exceeding 40% in Africa and Southeast Asia (Modell & Darlison, 2008). These disorders include mutations in globin coding sequences that lead to structural changes in encoded proteins, such as the sickle hemoglobin, and mutations that alter the expression of the α- and β-globin genes such as the thalassemias. Although the etiology of β-thalassemia is well understood, the disease is highly heterogeneous at both the molecular and clinical levels. Approximately 200 β-thalassemia alleles have been characterized and archived in the Var database (Patrinos et al., 2004). Unlike α-thalassemia, in which the vast majority of mutations are large deletions in the α-globin gene cluster, β-thalassemia is usually caused by mutations involving...
one or a few nucleotides in β-globin gene (HBB) or its immediate flanking regions. Mutations that completely abolish expression of the HBB are designated β0 alleles, while other mutations in HBB cause varying degrees of quantitative reduction in β-globin expression and are classified as β+ or β++ alleles (Thein, 2005). An imbalance between α and β chains causes accumulation of aberrant β4 tetramers in α-thalassemia, or the toxic aggregation of α-globin in β-thalassemia (Khandros, Thom, D’Souza, & Weiss, 2012), both result in anemia due to hemolysis compounded by a cellular stress response and ineffective erythropoiesis (Ribeil et al., 2013). Hemolysis leads to physiological iron overload, particularly in thalassemia major. The elevated stores of iron may be a key factor in increased inflammation and susceptibility to infection (Wanachiwanawin, 2000). Infection is among the most common causes of death in individuals with β-thalassemia (Miller, Dykes, & Polesky, 1988).

Currently, individuals with β-thalassemia are assessed clinically by measurement of blood indices, electrophoresis of hemoglobin (Hb) molecules in peripheral blood, and physical examination. A better understanding of the disease could develop through the study of patterns of transcription in blood cells of individuals with β-thalassemia, as these findings could indicate the major areas of compensatory or aberrant regulation that accompany the primary DNA sequence defects. Here, we studied transcriptome profiles of two members of a family with β-thalassemia carrying a novel mutation in the HBB. This is the first use of RNA sequencing to investigate broadly the blood cell gene expression in β-thalassemia in humans. We compare our findings with published results of RNA-sequencing analysis of sickle cell disease (SCD) and erythroblasts from a KLF1-null neonate with hydrops fetalis, and recognize similarities and differences in their transcriptional expression patterns.

2 | MATERIALS AND METHODS

2.1 | Editorial policies and ethical considerations

The collection of samples for this study was approved by the Pasteur Institute of Iran, application number 6266. Consent to participate in the study was contained in the written form signed by family members or guardians. A written informed consent for publication of personal information, including clinical descriptions and test results was obtained for each family member at the Pasteur Institute of Iran.

2.2 | Case description and clinical examination

The subjects were a 5-year-old girl and her parents. Both parents were clinically asymptomatic for β-thalassemia, but clinical examination of their daughter showed anemia, pallor, jaundice, and splenomegaly. She also had a history of transfusion dependence but her most recent transfusions were in the years before this study was undertaken, so the transfused cells are not expected to interfere with the present study. A complete blood count and Hb analysis were carried out for the family according to standard methods. Molecular studies were undertaken on both genomic DNA and RNA isolated from family members, after obtaining informed written consent. The mother's samples were labeled “M”, the daughter's samples were labeled “D”, and control samples were labeled “N”.

2.3 | Mutation detection in the β-globin gene

Following the isolation of genomic DNA using a standard salting-out procedure (Miller et al., 1988), the entire HBB gene was polymerase chain reaction (PCR)-amplified and the nucleotide sequence was determined, using two primer sets encompassing exons 1 and 2 (fragment A: Beta1F 5’-GGG CCA AGA GAT ATC TCT TAG-3’, Beta1R 5’-AATGACATGAACCTTACCATAG-3’) and another set encompassing exon 3 and the 3’UTR (fragment B: Beta2F 5’-GCA CCA TTC TAA AGA ACA ATA CAG-3’, Beta2R 5’-GTT TGA ACT AGC TCT TCA TTT C-3’). The PCR product was purified using a QIAquick PCR Purification Kit (Qiagen), and it was subjected to sequencing by the chain termination method on an ABI 3730 XL sequencer (Applied Biosystems, Foster City, CA, USA). The nucleotide numbering is based on GenBank accession number U01317. Investigations of the hemoglobin A (HBA) genes were carried out by both DNA sequencing and by screening for common α-globin gene deletions (−α3.7, −α4.2, −α20.5, −Mβ) using a standard Gap-PCR method (Chong, Boehm, Higgs, & Cutting, 2000).

2.4 | RNA isolation and RT-qPCR

Total RNA was isolated from blood using EDTA as an anticoagulant and Trizol reagent (Sigma-Aldrich, Germany) following the manufacturer's instructions. One microgram of total RNA was used for cDNA synthesis with a QuantiTect reverse transcription kit (Qiagen). The concentration and quality of the cDNA from each sample were determined by absorbance at 260 and 280 nm, using a Nanodrop 2000c spectrophotometer (Thermo Fisher Scientific, Wilmington, DE). Relative quantification of β-globin cDNA was performed by real-time quantitative polymerase chain reaction (qPCR) using an iTaq™ Universal SYBR Green Supermix Kit (Bio-Rad) following the manufacturer's instructions. Briefly, qPCR was performed in a 10-µl volume, containing 5 µl SYBR Green Supermix, 2 µl cDNA (40 ng), 1 µl nuclease-free water, and 1 µl each of forward and reverse primers (3 µM). Primer pairs for β-globin cDNA (forward 5’-GAT
TAGHAVIFAR ET AL.

GAA GTT GGT GGT GAG GCC‐3' and reverse 5'-GCC CAT AAC AGC ATC AGG AGT G-3') as the target gene, and both ACTB (forward 5'-TGG CAC CAC ACC TTC TAC AAT G-3' and reverse 5'-GGT CTC AAA CAT GAT CTG GGT CA-3') and GAPDH (forward 5'-GGA AGG TGA AGG TCG GAG TC-3' and reverse 5'-ACA TGT AAA CCA TGT AGT GGT-3') as the endogenous reference genes were designed to obtain amplified sizes between 70 and 150 bp for the maximum qPCR efficiency, as recommended by Bio‐rad.

The qPCR was performed on a CFX96 Touch™ Real‐Time PCR Detection System (Bio‐Rad) following a thermal cycling profile of 95°C for 3 min, and 39 cycles of 95°C for 10 s and 57°C for 30 s. Samples were analyzed in triplicate, and the expression of HBB relative to reference genes was normalized to a control sample from a healthy individual with normal hematological parameters and hemoglobin profile. The relative fold change was calculated using the method of Pfaffl (Robinson et al., 2011). Bio‐Rad CFX Manager 3.0 software was used for amplification and melting curve analyses.

2.5 | Library construction and RNA sequencing

A quantity of 500 ng of the total RNA from each sample was used for RNA‐sequencing library preparation, and the quality of RNA was determined with a Bioanalyzer 2100. Libraries for RNA sequencing were prepared with a KAPA Stranded RNA‐sequencing Kit (Illumina), following the manufacturer's instructions. Sequencing was performed on an Illumina HiSeq 2500 system for pair‐ended reads with a length of 100 bp (2 × 100 bp). Preprocessing and data quality control were performed using Illumina proprietary software.

More than 39 million 100 bp pair‐ended reads were generated for each sample. Reads were aligned to the human reference genome with the STAR genome index (Quinlan & Hall, 2010), which included both the genome sequence (GRCh37.71) and the exon/intron structures of known reference sequence (RefSeq) transcripts. On average, 80% of the reads (using a similarity score of 66%) were aligned successfully with the genomic sequence.

Samtools (Trapnell et al., 2012) was used to sort and index the alignment files for visualization with the Integrative Genomics Viewer tool (Casero et al., 2015). The sorted binary sequence analysis file (BAM files) was also used to generate UCSC Browser tracks with a genome Coverage Bed from Bed Tools (Dennis et al., 2003). To this end, coverage files were normalized using the total signal for each sample.

The cufflinks (Senapathy, Shapiro, & Harris, 1990) suite of tools (version 2.2.1) was used for differential gene expression analysis, which provides and compares gene expression levels in units of Fragments Per Kilobase
of transcript per Million mapped reads (FPKM). The mapped reads were counted for RefSeq transcripts using cuffquant. The cuffquant outputs were passed to cuffdiff to test for significant differences in transcript abundance for each pair-wise comparison between the three samples (N [Normal control], M [Mother], and D [Daughter]). Genes were flagged as differentially expressed when the log2|fold change| was >1 and the adjusted p-value was <0.05. To allow cuffdiff analysis for a highly expressed gene, a default of a maximum of 1,000,000 fragments was used. This allows computation of the FPKM values for differentially expressed genes (DEGs), and allows comparison between expression levels of DEGs. The expression estimates were computed with HTSeq35 (unique hits and “intersection-nonempty” mode for version v0.6.1p2) to generate gene-level counts for each sample (Thein, 2013).

2.6 Analyzing effects of globin gene masking on gene expression analysis by RNA sequencing

One difficulty in analyzing gene expression in whole blood is that the majority of transcripts (over 70%) are from globin genes. The results of RNA-sequencing analysis from whole blood were masked using cufflinks (‐M/–mask‐file<mask. (gtf/gff)) to prevent interference from all the globin genes: HBA1, HBA2, HBB, HBD, HBG1, HBG2, HBE1, HBM, HBQ, and HBZ.

2.7 Gene ontology (GO) analysis using the DAVID annotation database

The Database for Annotation, Visualization and Integrated Discovery (DAVID) was used to determine the molecular functions, ontology terms, and the pathways significantly enriched in the DEGs among our samples (Li et al., 2015).

3 RESULTS

3.1 Hematological indices and genomic DNA sequence analyses

Whole blood specimens were collected from a family in which the father (F) and daughter (D) were anemic, with hematological indices consistent with β-thalassemia minor and β-thalassemia intermedia, respectively. The mother (M) had a slight microcytic anemia with borderline indices (Table 1). Genomic DNA sequencing indicated that the father is heterozygous for a common β-thalassemia allele in which a thymidine nucleotide is deleted from codon 37 of the β-globin gene (HBB:c.112delT), resulting in a translational frameshift and early termination of translation (Figure 1). Sequencing of the mother’s DNA revealed that she is a carrier of a novel mutation in exon 1 of HBB, in which there is a C to T transition mutation (HBB:c.51C>T) in the third nucleotide of codon 16. The daughter (D) is a compound heterozygote for the maternal and paternal mutations, and has the most severe phenotype.

3.2 Novel mutation introduces a cryptic splice donor site, and is associated with downregulation of the β-globin gene

Analyses of the mRNA sequence and splicing patterns of HBB were conducted in our laboratory by RNA-sequencing analyses and RT/qPCR. The relative expression of β-globin in M, as estimated by the cDNA counts during RNA sequencing, was approximately 70% of that detected in N (the ratio

FIGURE 1 Sequencing of β-globin genomic DNA from the daughter (D), with the top panel highlighting the novel maternally derived mutation (HBB:c.51C>T) and the bottom panel highlighting the paternally derived mutation (HBB:c.112delT)
The downregulation of β-globin in M was also confirmed by RT/qPCR analyses, showing that the relative normalized expression of β-globin in M was 0.77 (±0.1) of that detected in N (Figure 2). This slight downregulation of the β-globin gene is consistent with the functional effect of the previously known cryptic splice site mutations at the HBB (Allan, Beattie, & Flint, 2008).

While the allele expression difference was minor in M and consistent with the mother’s slight microcytic anemia, the C>T transition represented 99% of the HBB transcripts in the compound heterozygote D (Figure 3). The strong prevalence of RNA transcribed from the maternal allele (HBB:c.51C>T) in the daughter was likely caused by preferential decay of the mRNA transcribed from the paternally derived allele (HBB:c.112delT) carrying a frame-shift mutation in codon 37.

RNA-sequencing reads with at least one gap in their genomic alignment were analyzed to establish the (putative) splicing patterns at the HBB locus. In both M and D, transcripts were detected that carried the HBB:c.51C>T mutation and spanned the novel joint of the putative splice site, confirming its use as a splice donor (Figure 4a–c). These aberrantly spliced products were found at a low frequency compared to the canonically spliced reads, but without information on the degradation of alternatively spliced mRNAs it is difficult to draw a conclusion about the exact extent of use of the cryptic splice site. It is noteworthy that a natural cryptic splice site spanning codon 17–19 is frequently used for splicing in all three samples (Figure 4b,c).

### 3.3 More than 300 genes are differentially expressed in β-thalassemic blood

RNA-sequencing analysis was used to characterize the expression of alpha and other β-like globin genes in D and M, to develop an understanding of compensatory gene expression in these individuals. Compared to a normal individual, the HBD gene encoding δ-globin was upregulated approximately 80-fold in D, and the HBG1 and HBG2 genes encoding γ-globin were upregulated 18- and 13-fold, respectively. It is possible that HbA2 and HBF were generated in substantially increased amounts to compensate for the defective production of HbA in D. The expression of HBA1 and HBA2 of normalized sequence reads was 285,022/410,599). The downregulation of β-globin in M was also confirmed by RT/qPCR analyses, showing that the relative normalized expression of β-globin in M was 0.77 (±0.1) of that detected in N (Figure 2). This slight downregulation of the β-globin gene is consistent with the functional effect of the previously known cryptic splice site mutations at the HBB (Allan, Beattie, & Flint, 2008).

While the allele expression difference was minor in M and consistent with the mother's slight microcytic anemia, the C>T transition represented 99% of the HBB transcripts in the compound heterozygote D (Figure 3). The strong prevalence of RNA transcribed from the maternal allele (HBB:c.51C>T) in the daughter was likely caused by preferential decay of the mRNA transcribed from the paternally derived allele (HBB:c.112delT) carrying a frame-shift mutation in codon 37.

RNA-sequencing reads with at least one gap in their genomic alignment were analyzed to establish the (putative) splicing patterns at the HBB locus. In both M and D, transcripts were detected that carried the HBB:c.51C>T mutation and spanned the novel joint of the putative splice site, confirming its use as a splice donor (Figure 4a–c). These aberrantly spliced products were found at a low frequency compared to the canonically spliced reads, but without information on the degradation of alternatively spliced mRNAs it is difficult to draw a conclusion about the exact extent of use of the cryptic splice site. It is noteworthy that a natural cryptic splice site spanning codon 17–19 is frequently used for splicing in all three samples (Figure 4b,c).

### 3.3 More than 300 genes are differentially expressed in β-thalassemic blood

RNA-sequencing analysis was used to characterize the expression of alpha and other β-like globin genes in D and M, to develop an understanding of compensatory gene expression in these individuals. Compared to a normal individual, the HBD gene encoding δ-globin was upregulated approximately 80-fold in D, and the HBG1 and HBG2 genes encoding γ-globin were upregulated 18- and 13-fold, respectively. It is possible that HbA2 and HBF were generated in substantially increased amounts to compensate for the defective production of HbA in D. The expression of HBA1 and HBA2 of normalized sequence reads was 285,022/410,599). The downregulation of β-globin in M was also confirmed by RT/qPCR analyses, showing that the relative normalized expression of β-globin in M was 0.77 (±0.1) of that detected in N (Figure 2). This slight downregulation of the β-globin gene is consistent with the functional effect of the previously known cryptic splice site mutations at the HBB (Allan, Beattie, & Flint, 2008).

While the allele expression difference was minor in M and consistent with the mother’s slight microcytic anemia, the C>T transition represented 99% of the HBB transcripts in the compound heterozygote D (Figure 3). The strong prevalence of RNA transcribed from the maternal allele (HBB:c.51C>T) in the daughter was likely caused by preferential decay of the mRNA transcribed from the paternally derived allele (HBB:c.112delT) carrying a frame-shift mutation in codon 37.

RNA-sequencing reads with at least one gap in their genomic alignment were analyzed to establish the (putative) splicing patterns at the HBB locus. In both M and D, transcripts were detected that carried the HBB:c.51C>T mutation and spanned the novel joint of the putative splice site, confirming its use as a splice donor (Figure 4a–c). These aberrantly spliced products were found at a low frequency compared to the canonically spliced reads, but without information on the degradation of alternatively spliced mRNAs it is difficult to draw a conclusion about the exact extent of use of the cryptic splice site. It is noteworthy that a natural cryptic splice site spanning codon 17–19 is frequently used for splicing in all three samples (Figure 4b,c).
encoding α-globin chains was represented at slightly higher FPKM levels in D, compared to N, perhaps as a consequence of heightened erythropoiesis rather than gene regulation at the cellular level.

The DEGs in samples M and D, compared to N, were determined using the cuffdiff analysis tool with the software settings adjusted to include highly expressed genes, as described in Materials and Methods. A separate analysis was performed to determine whether masking globin genes as targets in the assignment of reads using the cuffdiff software changes the FPKM value for non-globin genes. There was a high correlation of FPKM values \( (R > 0.997) \) between the two approaches (masking vs. not masking) for all but 10 genes, as shown in Figure 5. The genes below the diagonal are globin genes that appear to have null expression when their genomic targets are masked. The two genes above the diagonal are SMN2 and EIF3CL, which show an artificial increase in apparent expression when globin gene targets are masked. These may have adventitious similarity to globin reads and serve as an alternate target for cuffdiff. As the remaining genes lie closely along the diagonal, the masking (or not) of globin genes in the analysis does not appear to significantly bias the FPKM counts for non-globin genes.

In pairwise comparisons between the samples M, D, and N, there were a total of 398 significant DEGs, 312 of

---

**FIGURE 4** Gene expression at the HBB locus. (a) Normalized, whole-genome RNA-Seq coverage tracks are shown for D, M, and N. An asterisk represents the position of the novel mutation. Vertical black lines represent sequence reads of high abundance, and grey vertical lines represent reads of low abundance, showing their splicing locations. (b) Splice site usage in the exon 1 region of HBB, from samples D, M, and N. (c) A detailed view of the cDNA sequence reads spanning exon 1 (right) and exon 2 (left) of HBB, in samples D, M, and N. The horizontal lines indicate the sequence joints generated by splicing of these exons, and the canonical exon 1–exon 2 junction is shown at the bottom of the “N” examples. The cryptic splice donor site used in samples D and M are indicated by red asterisks. Dark and light bars indicate more or less frequently detected sequence reads, and reference information for this exon 1, intron 1, exon 2 region are indicated at the bottom.
which were differentially expressed in sample D compared to N. There were only 37 DEG by comparison of sample M with N, and 177 that were differentially expressed between D and M, in accordance with the severe hematological changes seen in the daughter compared to the slight anemia present in the mother. Of the 398 DEG in the overall list, 343 had expression levels with FPKM > 2 in all three comparisons (Table 2). The differential patterns of gene expression in samples D and M are shown in Figure 6, and depend on both changes in the cellular make-up of the blood and changes in gene expression within individual cell types. An increase in erythropoiesis, for example, in the daughter who is severely anemic, would result in an increase in erythroblast-associated transcripts in the total pool of buffy-coat RNA. The overall RNA-sequencing pattern is thus a snapshot of the heterogeneous blood compartment as a whole, and indicates the physiological state of that tissue.

### 3.4 Involvement of many DEGs in pathways relevant to the clinical management of β-thalassemia

The DAVID Bioinformatics Resources tools were used to organize the list of DEGs under ontologic terms, so that genes with common functions could be clustered. As shown in Table 2, there were highly significant clusterings of genes involved in erythrocyte development and function. The dysregulation of globin genes, the cause of β-thalassemia, is included under the ontologic term Iron Ion Binding. There are also multiple categories of the DEGs that are involved in hematopoiesis, response to oxidative stress, inflammation, immune response, protein modification, and apoptosis (Table 2). Although some DEGs belong to more than one ontologic category, almost all of the genes in erythrocyte homeostasis, hemopoiesis, iron ion binding, hemoglobin’s chaperone, blood group antigens, and response to oxidative stress categories are upregulated. Conversely, most of the DEGs belonging to the defense response, response to bacteria, chemotaxis, and immunoglobulin-like fold categories are downregulated.

The most significantly upregulated gene in D (>1,100-fold, compared to N) was IFI27, an INFα inducible gene that encodes an intracellular product used as a marker of epithelial cell senescence and fibroblast overgrowth (Cai et al., 2015). The IGFBP5 gene is expressed at low levels in cell lines of hematopoietic lineages, and the dramatic increase in its RNA levels in the blood compartment of D may reflect a derangement of the bone marrow or increased population of fibrocytes associated with inflammation elsewhere in the body (Mukaiyama et al., 2015).

The microenvironment of the bone marrow may be profoundly affected by the disease β-thalassemia and some of the changes may be mediated by hyaluronic acid (HA), a major component of the extracellular matrix in bone marrow. For example, the receptor EGFR (epidermal growth factor receptor) is 35-fold upregulated in D and is involved in fibroblast to myofibroblast differentiation, mediated by HA and in response to TGF-β1 signaling (Lee et al., 2009). The extracellular matrix proteoglycans BCAN (breviscan, 66-fold upregulated in D) and NCAN (neurocan core protein, 47-fold upregulated in D) have roles in fibrosis, inflammation, and wound recovery, and bind to HA (Gileles-Hillel, Kheirandish-Gozal, & Gozal, 2015). The upregulation of RAP1GAP (Rap1 GTPase-activating protein) by 140-fold in D may help reduce inflammation mediated by Rap1 and NFκB (Xi, He, Zhang, Xue, & Zhou, 2014), by returning Rap1 to an inactive (GDP bound) state.
Osteoporosis is one of the leading causes of morbidity in individuals with β-thalassemia, and the ALPL (alkaline phosphatase) gene required for proper osteoblast function was 33-fold downregulated in D compared to N. This reduction in expression may be a regulatory consequence of iron overload (Kramer et al., 2001). Dual specificity tyrosine-phosphorylation-regulated kinase 3 (DYRK3, 140-fold upregulated in D) may also be important in bone homeostasis as it acts as a negative regulator of osteoclastogenesis in mice (Raghavachari et al., 2012). Solute carrier family 11A1 (SLC11A1, encoding a divalent metal ion transporter), was eightfold downregulated in D compared to N, which may also be a response to iron overload from hemolysis (Biggs et al., 2001).

We observed differential expression of genes involved in the regulation of circadian rhythms, for example the circadian sleep–wake cycle process. Sleep disturbances, and particularly sleep-disorders of breathing have been frequently observed in hemoglobinopathies such as SCD and β-thalassemia, and also in both iron overload and iron deficiency anaemia (Kihm et al., 2002). In particular, PROK2, encoding prokineticin 2 protein, is 24-fold downregulated in D compared to N, is expressed in the suprachiasmatic nucleus and

| TABLE 2 | Selected gene ontology terms that were significantly enriched among the differentially expressed genes in the daughter (D) |
| --- | --- |
| Ontology terms | Genes | p-value | Gene examples |
| Erythrocyte homeostasis | 8 | 3.2 × 10⁻⁵ | BPGM, BCL6, KLF1, SOX6, TAL1, DYRK3, EPB42, TRIM10 |
| Hemopoiesis; immune system development | 15 | 1.4 × 10⁻⁴ | BPGM, BCL3, BCL6, KLF1, RASGRP4, SOX6, TAL1, DYRK3, EGR1, EPB42, AHSP, MMP9, PBX1, SPTA1, TRIM10 |
| Iron ion binding | 18 | 8.1 × 10⁻⁵ | CISD2, RESFD, STEAP4, C5orf4, CYP1B1, CYP4F3, FECH, HBA2, HBA1, HBB, HBE1, HBG1, HBM, HBQ1, ISCA1, LTF, MPO, RSAD2, SLC11A1 |
| Hemoglobin chaperone | 6 | 6.0 × 10⁻⁶ | GATA1, CPOX, AHSP, FECH, HBB, HMBS |
| Inflammatory response | 14 | 7.8 × 10⁻³ | CCR3, C5, FPR2, IL8, KRT1, LY96, MEFV, MMP25, ROK2, SLC11A1, TLR6, TLR8, TFRC, VNN1 |
| Defense response | 23 | 2.6 × 10⁻³ | BCL3, MEFV, BPI, CCR3, C5, DEFAS, FPR2, IL8, KRT1, LTF, LILRA2, LILRB3, LY96, MPP25, MPO, PROK2, RSAD2, SLC11A1, TLR6, TLR8, TFRC, VNN1 |
| Response to oxidative stress | 8 | 3.4 × 10⁻² | CRYAB, EGFR, GCLC, JUN, KRT1, MPO, SELK, VNN1 |
| Blood group antigen | 8 | 6.0 × 10⁻⁷ | ART4, KEL, XK, AQP1, BCAM, ERMAP, GYPA, SLC14A1 |
| Response to bacterium | 11 | 3.3 × 10⁻³ | BCL3, ADM, BP1, DEFA3, IRAK3, JUN, LTF, LY96, SLC11A1, THBD, TLR6 |
| Regulation of apoptosis | 23 | 4.6 × 10⁻² | BCL3, BCL6, CITED2, NRLP12, ARHGEF12, BIRC2, COL18A1, CRYAB, DAPK2, DYNL1L1, EGFR, FEM1B, GCLC, HSPB1, HSPA1B, HSPA1A, ITSN1, JUN, MMP9, MPO, NRG1, NET1, PROK2, VNN1 |
| Gas transport | 8 | 1.9 × 10⁻⁸ | AQP1, CA2, HBA1, HBA2, HBB, HBE1, HBG1, HBM, BQ1 |
| Iron ion homeostasis | 5 | 4.3 × 10⁻³ | ABCB6, EPB42, LTF, SLC11A1, TFRC |
| Chemotaxis (chemokine activity) | 10 | 3.1 × 10⁻³ | CMTM2, CCR3, CCR2, CXCL5, CKLF, C5, FPR2, IL8, PLAUR, PROK2 |
| Regulation of cell shape | 5 | 1.8 × 10⁻² | ARAP3, CDC42EP2, EPB42, LST1, SPTA1 |
| Immunoglobulin-like fold | 24 | 2.9 × 10⁻⁴ | FCGR1T, FCRL3, FCRL5, HEPACAM2, BCAM, BTN1L8, CEACAM3, CEACAM4, ERMAP, EPB42, IL1R2, LRFN1, LILRA2, LILRA3, HLA-DQA2, NRG1, NFATC2, OSCAR, PILRA, SIGLEC14, SIGLEC5, SIRPB1, SIRPB2, TREM1 |
| Protein kinase cascade | 13 | 4.5 × 10⁻² | BCL3, NRLP12, RASGRP3, C5, CRYAB, DAPK2, EGFR, LY96, LPAR2, MAP4K5, PROK2, TLR6, TLR8 |
| Disulfide bond | 70 | 1.5 × 10⁻³ | KIAA1324, CTS1L1, CCRL2, CXCL5, CLIC2, ST3GaL4, ST6GaLNAC2, ALDH5A1, DIL3, DPEP2, EMR3, EMR3, HRR2, KREMen1, LGRI1, MGAM, MME, NTNG2, PLAUR, PTN, PRGR4, PTGDS, PTP4A3, SCARF1, SOSTDC1, SPRF2, TNFRSF10C |
| Carboxylic acid transport | 8 | 2.0 × 10⁻² | XK, AQP9, CPT1B, SLC11A1, SLC16A1, SLC16A3, SLC19A1, SLC7A5 |
| Membrane fraction | 24 | 2.9 × 10⁻² | ABCC4, RASGRP4, ACSL6, NCEH1, CEACAM4, CPT1B, CSPG5, CSPG5, CYP4F3, DGAT2, DYNL1L1, GYPA, HSPA13, ITSN1, KREMen1, LNPEP, MME, SLC16A1, SLC16A3, SLC19A1, SLC2A1, STX3, YES1 |
may act as an output component to transmit behavioral circadian rhythm and regulate other circadian rhythms such as sleep–wake cycles, feeding, endocrine rhythms, and a suggestion of signaling circadian day (Gallagher, Liem, Wong, Weiss, & Bodine, 2005). In addition, EGFR (epidermal growth factor receptor) indirectly controls inhibited locomotor activity and disrupted circadian sleep–wake cycles via the alteration of TGF-α (Nirupam et al., 2012), and it is 35-fold upregulated in D.

Scatter plots (log–log) were used to compare the relative expression of individual genes in samples D, M, and N over a wide range of expression (Figure 7a–c). An equivalence between FPKM values in the pairwise comparisons is shown as a diagonal line, and its offset from the data is likely to indicate a robust process of hematopoiesis in D and M compared to N. The comparison also indicates substantially greater numbers of genes that are up- or downregulated in D compared to M.

**FIGURE 6** Significantly differentially expressed genes demonstrated by pairwise comparisons of samples N, M, and D, using the software package cuffdiff. The heat-map is divided into five parts, and in each part, the first and second columns represent the data for, respectively, D versus N and M versus N pairwise comparisons. A red color indicates upregulation, and a blue color indicates downregulation. The numbers in the cells show the log base 2 of the fold change (numbers are rounded). The heat map is generated using selected genes with differential expression greater than ~6× (log2 > 2.5) or less than ~1/6 (log2 < −2.5). Also, the genes with log2 (fold change) equal to inf or –inf have been omitted. The red and blue colors indicate upregulation and downregulation respectively.
3.5  β-Thalassemia shows important similarities and differences with SCD at the transcriptome level

The list of DEGs in β-thalassemia was compared to the previously published RNA-sequencing results for SCD (Raghavachari et al., 2012). In the case of SCD, there were 331 DEGs in whole blood from individuals with SCD compared to normal samples, and approximately one third of them (110 genes) were in common with the 343 DEG in β-thalassemia, as shown in Table 3. Many genes involved in hematopoiesis, erythrocyte hemostasis, and iron-binding proteins were differentially expressed in both β-thalassemia and SCD, which might be expected since both disorders are hemolytic anemias. Improving the ability of erythrocytes to deliver oxygen to tissues would also be a predictable response in both β-thalassemia and SCD, and upregulation of BPGM (bisphosphoglycerate mutase) is indeed seen in both cases. One of the genes upregulated in both β-thalassemia and SCD was KLF1 (Kruppel-like factor 1), a master regulator of red blood cell gene expression. Twenty-five DEGs in both β-thalassemia and SCD were also found in the published list of the DEGs in circulating erythroblasts from a homozygous KLF1-null neonate with hydrops fetalis (Magor et al., 2015).

The genes differentially expressed in β-thalassemia but not in SCD included AHSP (alpha hemoglobin-stabilizing protein), and the transcription factor GATA-1 that induces

FIGURE 7  Diagonal (log–log) plots of expression levels of individual genes (FPKM units) among the three samples. (a) D versus N comparison, (b) M versus N comparison, and (c) D versus M comparison. The diagonal red lines indicate equivalence of expression. FPKMs, fragments per kilobase of transcript per million mapped reads.
AHSP expression (Gallagher et al., 2005; Kihm et al., 2002). AHSP prevents aggregation of α-globin during erythroid cell development and is predicted to moderate pathological states of α-globin excess such as β-thalassemia. SCD is not associated with α/β chain imbalance, and so it is understandable that upregulated expression of AHSP is unnecessary. A surprising finding is that most genes differentially expressed in β-thalassemia but not in SCD are involved in inflammation and immunity. Among these were genes that are activated in response to bacterial pathogens such as ADM, BCL3, BP1, DEFA3, IRAK3, JUN, LTF, LY96, SLC11A1, THBD, and TLR6 (see Data S1).

4 | DISCUSSION

We described the downregulation of the β-globin gene in β-thalassemia by RNA-sequencing analysis using a novel mutation in HBB in a family with an unexpectedly severe case of β-thalassemia. The RNA-sequencing analysis allows comparison of the effects of β-thalassemia on β-globin and other genes between β-thalassemia and other hemoglobinopathies. We present results of sequencing analysis that show that the mother has a novel β-globin mutation that introducing a cryptic splice site that explains why her phenotype is very mild compared to the expected phenotype of a carrier parent for β-thalassemia. The novel codon 16 mutation introduces a cryptic splice donor site in exon 1 and is associated with downregulation of β-globin expression as confirmed by RNA-sequencing analysis and qPCR. The newly described mutation contributes to a severe β-thalassemia intermedia in a compound heterozygous daughter (D), but her mother (M) carrying the novel mutation in combination with the wild-type allele is phenotypically asymptomatic and has borderline normal hematologic parameters. The moderate downregulation of HBB expression in M is consistent with the functional effect of the previously known cryptic splice site mutations at the HBB gene that exhibit a recessive pattern of inheritance (Allan et al., 2008). The expression of HBB in D is more complicated, since the paternal allele bears a frameshift mutation and the maternal allele introduces a cryptic splice site leading to a truncated protein product. While the steady-state HBB transcript levels in D are approximately 93% of N, most arising from the maternal allele, a smaller subset of transcripts will encode a full-length β-globin protein.
Blood transcriptome analysis has not been previously reported for a human case of β-thalassemia, and this study allows a better understanding of the regulatory changes and disease processes taking place in affected individuals. Over 300 genes were identified as being differentially expressed (DEGs) between the samples from D and M, in comparison with a normal sample (N). The DEGs in β-thalassemia were interpreted through GO enrichment analysis. This revealed that the genes most affected in β-thalassemia are involved in the hematopoietic response to oxidative stress, inflammation, immune response, protein modification, and apoptosis, as shown in Table 2.

A comparison of DEGs in β-thalassemia versus SCD and KLF1-null anemia shows that while many DEGs involved in response to hemolysis, iron homeostasis, and anemia were common to these disorders, over 200 DEGs were unique to β-thalassemia. These results indicate fundamental differences between the anemias.

While this study only provides information on two individuals, a carrier mother and her severely affected daughter, it provides a wealth of data on how the disease is manifested in that family. For example, the expression of the gene IFI27 is upregulated more than 1,100-fold in D and this may be indicative of a process of myelofibrosis or fibrotic scarring of the bone marrow. This has only been recently reported in a single case of β-thalassemia (Nirupam et al., 2012), and it may be an overlooked element of the pathology of the disease. Exhaustion of the bone marrow epithelial microenvironment and subsequent fibroblast overgrowth would be predicted to interfere with the robust rate of erythropoiesis needed to replace cells lost to hemolysis, and therapeutic approaches that minimize or prevent myelofibrotic scarring might be helpful. Osteoporosis is a significant cause of morbidity in β-thalassemia, and downregulation of ALPL expression by more than 30-fold in D provides an important clue as to how that may develop. There are also indications that dysregulation of several regulatory genes (PROK2, EGFR) may be at the root of the sleep disturbances and apneas that are among the problems associated with β-thalassemia. This study, the first genome-wide RNA-seq analysis of the blood transcriptome in β-thalassemia, reveals some new molecular biomarkers responding to the disease, which can be examined in future studies as therapeutic targets to improve the quality of life for those affected with β-thalassemia.

ACKNOWLEDGMENTS

We acknowledge substantial contributions to the initial analysis of the RNA seq. data by Dr. David Casero at UCLA.

CONFLICT OF INTEREST

The authors have no conflict of financial interest.

AUTHOR’S CONTRIBUTION

FT carried out the molecular genetic studies, including qPCR, participated in the sequence alignment, and participated in drafting the manuscript. MH collected samples, conducted clinical studies, performed nucleotide sequence determination of the β-globin genes, and multiplex ligation-dependent probe amplification of the α–globin genes. GS conducted physical examination and initial clinical studies.

ORCID

Mohammad Hamid https://orcid.org/0000-0002-4625-0713

REFERENCES

Allan, G. J., Beattie, J., & Flint, D. J. (2008). Epithelial injury induces an innate repair mechanism linked to cellular senescence and fibrosis involving IGF-binding protein-5. Journal of Endocrinology, 199(2), 155–164. https://doi.org/10.1677/JOE-08-0269

Biggs, T. E., Baker, S. T., Botham, M. S., Dhital, A., Barton, C. H., & Perry, V. H. (2001). Nramp1 modulates iron homoeostasis in vivo and in vitro: Evidence for a role in cellular iron release involving de-acidification of intracellular vesicles. European Journal of Immunology, 31(7), 2060–2070. https://doi.org/10.1002/1521-4446(200107)31:7<2060::AID-IMMU2060>3.0.CO;2-L

Cai, Y., Sukhova, G. K., Wong, H. K., Xu, A., Terzankar, V., Vanhouette, P. M., & Tang, E. H. (2015). Rap1 induces cytokine production in pro-inflammatory macrophages through NfκB signaling and is highly expressed in human atherosclerotic lesions. Cell Cycle, 14(22), 3580–3592. https://doi.org/10.1080/15384101.2015.1100771

Casero, D., Sandoval, S., Seet, C. S., Scholes, J., Zhu, Y., Ha, V. L., … Crooks, G. M. (2015). Long non-coding RNA profiling of human lymphoid progenitor cells reveals transcriptional divergence of B cell and T cell lineages. Nature Immunology, 16(12), 1282. https://doi.org/10.1038/nili.3299

Chong, S. S., Boehm, C. D., Higgs, D. R., & Cutting, G. R. (2000). Single-tube multiplex-PCR screen for common deletional determinants of α-thalassemia. Blood, 95(1), 360–362.

Dennis, G., Sherman, B. T., Hosack, D. A., Yang, J., Gao, W., Lane, H. C., & Lempicki, R. A. (2003). DAVID: Database for annotation, visualization, and integrated discovery. Genome Biology, 4(9), R60.

Gallagher, P. G., Liem, R. I., Wong, E., Weiss, M. J., & Bodine, D. M. (2005). GATA-1 and Oct-1 are required for expression of the human α-globin-stabilizing protein gene. Journal of Biological Chemistry, 280(47), 39016–39023. https://doi.org/10.1074/jbc.M506062200

Gileles-Hillel, A., Khierandish-Gozal, L., & Gozal, D. (2015). Hemoglobinopathies and sleep – The road less traveled. Sleep Medicine Reviews, 24, 57–70. https://doi.org/10.1016/j.smrv.2015.01.002

Gorden, A., Yang, R., Yerges-Armstrong, L. M., Ryan, K. A., Speliotes, E., Borecki, I. B., … Gerhard, G. S. (2013). Genetic variation at the NCAN locus is associated with inflammation and fibrosis in non-alcoholic fatty liver disease in morbid obesity. Human Heredity, 75(1), 34–43. https://doi.org/10.1159/000346195

Khandros, E., Thom, C. S., D’Souza, I., & Weiss, M. J. (2012). Integrated protein quality control pathways regulate free α-globin in murine...
β-thalassaemia. *Blood*, 119, 5265-5275. https://doi.org/10.1182/blood-2011-12-397729

Kihm, A. J., Kong, V. I., Hong, W., Russell, J. E., Rouda, S., Adachi, K., … Weiss, M. J. (2002). An abundant erythroid protein that stabilizes free α-haemoglobin. *Nature*, 417(6890), 758. https://doi.org/10.1038/nature00830

Kramer, A., Yang, F.-C., Snodgrass, P., Li, X., Scammell, T. E., Davis, F. C., & Weitz, C. J. (2001). Regulation of daily locomotor activity and sleep by hypothalamic EGF receptor signaling. *Science*, 294(5551), 2511–2515.

Lee, Y., Ha, J., Kim, H. I., Kim, Y.-S., Chang, E.-J., Song, W.-J., & Kim, K.-H. (2009). Negative feedback inhibition of NFATc1 by DYRK1A regulates bone homeostasis. *Journal of Biological Chemistry*, 284(48), 33343–33351. https://doi.org/10.1074/jbc.M109.042234

Li, S., Xie, Y., Zhang, W., Gao, J., Wang, M., Zheng, G., … Tao, X. (2015). Interferon alpha-inducible protein 27 promotes epithelial–mesenchymal transition and induces ovarian tumorigenicity and stemness. *Journal of Surgical Research*, 193(1), 255–264. https://doi.org/10.1016/j.jss.2014.06.055

Mager, G. W., Tallack, M. R., Gillinder, K. R., Bell, C. C., McCallum, N., Williams, B., & Perkins, A. C. (2015). KLF1-null neonates display hydrops fetalis and a deranged erythroid transcriptome. *Blood*, 125(15), 2405–2417. https://doi.org/10.1182/blood-2014-08-590968.

Miller, S. A., Dykes, D. D., & Polesky, H. F. R. N. (1988). A simple salting out procedure for extracting DNA from human nucleated cells. *Nucleic Acids Research*, 16(3), 1215. https://doi.org/10.1093/nar/16.3.1215

Modell, B., & Darlington, M. (2008). Global epidemiology of haemoglobin variants and thalassaemia mutations for population and sequence variation studies. *Nucleic Acids Research*, 36(Database issue), D537–541. https://doi.org/10.1093/nar/gknh006

Mukaiyama, K., Kamimura, M., Uchiyama, S., Ikegami, S., Nakamura, Y., & Kato, H. (2015). Elevation of serum alkaline phosphatase (ALP) level in postmenopausal women is caused by high bone turnover. *Aging Clinical and Experimental Research*, 27(4), 413–418. https://doi.org/10.1007/s40520-014-0296-x

Nirupam, N., Maheshwari, A., Rath, B., Chandra, J., Kumar, P., Basu, S., & Nangia, A. (2012). Myelofibrosis: A cause of increased transfusion requirement in a child with β-thalassemia intermedia. *Journal of Pediatric Hematology/Oncology*, 34(2), 143–145. https://doi.org/10.1097/MPH.0b013e3182278266

Patrinos, G. P., Giardine, B., Riemer, C., Miller, W., Chui, D. H., Anagnostou, N. P., … Hardison, R. C. (2004). Improvements in the HbVar database of human hemoglobin variants and thalassaemia mutations for population and sequence variation studies. *Nucleic Acids Research*, 32(Database issue), D537–541. https://doi.org/10.1093/nar/gkh006

Quinlan, A. R., & Hall, I. M. (2010). BEDTools: A flexible suite of utilities for comparing genomic features. *Bioinformatics*, 26(6), 841–842. https://doi.org/10.1093/bioinformatics/btq033

Raghavachari, N., Barb, J., Yang, Y., Liu, P., Woodhouse, K., Levy, D., … Kato, G. J. (2012). A systematic comparison and evaluation of high density exon arrays and RNA-seq technology used to unravel the peripheral blood transcriptome of sickle cell disease. *BMC Medical Genomics*, 5(1), 28. https://doi.org/10.1186/1755-8794-5-28

Ribeil, J. A., Arlet, J. B., Dussiot, M., Cruz Moura, I., Courtois, G., & Hermine, O. (2013). Ineffective erythropoiesis in β-thalassemia. *The Scientific World Journal*, 2013, 394295. https://doi.org/10.1155/2013/394295

Robinson, J. T., Thorvalsdottir, H., Winckler, W., Guttman, M., Lander, E. S., Getz, G., & Mesirov, J. P. (2011). Integrative genomics viewer. *Nature Biotechnology*, 29(1), 24. https://doi.org/10.1038/nbt.1754

Senapathy, P., Shapiro, M. B., & Harris, N. L. (1990). Splice junctions, branch point sites, and exons: Sequence statistics, identification, and applications to genome project. *Methods in Enzymology*, 183, 252–278. https://doi.org/10.1016/0070-6679(90)83018-5

Thein, S. L. (2005). Pathophysiology of β thalassemia—A guide to molecular therapies. *ASH Education Program Book*, 2005(1), 31–37.

Thein, S. L. (2013). The molecular basis of β-thalassemia. *Cold Spring Harbor Perspectives in Medicine*, 3(5), a011700. https://doi.org/10.1101/cshperspect.a011700

Trappell, C., Roberts, A., Goff, L., Pertea, G., Kim, D., Kelley, D. R., … Pachter, L. (2012). Differential gene and transcript expression analysis of RNA-seq experiments with TopHat and Cufflinks. *Nature Protocols*, 7(3), 562. https://doi.org/10.1038/nprot.2012.016

Wanachwanawin, W. (2000). Infections in E-β thalassemia. *Journal of Pediatric Hematology/Oncology*, 22(6), 581–587. https://doi.org/10.1097/00043426-200011000-00027

Xi, B. O., He, D., Zhang, M., Xue, J., & Zhou, D. (2014). Short sleep duration predicts risk of metabolic syndrome: A systematic review and meta-analysis. *Sleep Medicine Reviews*, 18(4), 293–297.

Yu, J., Cao, J., Li, H., Liu, P., Xu, S., Zhou, R., … Guo, X. (2016). Bone marrow fibrosis with fibrocytic and immunoregulatory responses induced by β-catenin activation in osteoprogenitors. *Bone*, 84, 38–46. https://doi.org/10.1016/j.bone.2015.12.003

**SUPPORTING INFORMATION**

Additional supporting information may be found online in the Supporting Information section at the end of the article.

**How to cite this article:** Taghavifar F, Hamid M, Shariati G. Gene expression in blood from an individual with β-thalassemia: An RNA sequence analysis. *Mol Genet Genomic Med*. 2019;7:e740. [https://doi.org/10.1002/mgg3.740](https://doi.org/10.1002/mgg3.740)