Research Article
Volume 15:3, 2023

Journal of Bioanalysis & Biomedicine

ISSN: 1948-593X Open Access

Differentially Expressed Genes for Regulation of Fetal Hemoglobin Induction in Beta Thalassemia

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Abstract

Beta thalassemia is a disorder in which the body is unable to synthesise haemoglobin beta subunit due to deleterious mutations in the β-globin gene that results in underproduction of Adult Haemoglobin (HbA). Fetal Haemoglobin (HbF), which is composed of two α and two γ subunits, has been identified as a potential substitute for HbA with great clinical significance in β-thalassaemic patients. However, in the developmental stages, the expression of HbF is gradually minimized and overtaken by HbA. Our research found that the investigation of blood expression and its relationship to DEGs may aid in elucidating the role of these DEGs in beta thalassemia progression, and an RNA sequencing study indicated that the β globin gene is down regulated. There are 200 genes that are differently expressed in β thalassemia patients compared to healthy controls, as well as two key genes. *KLF1* and *MDM2* are two potential target genes for beta thalassemia patients that could be employed as diagnostic indicators. The differentially expressed genes include genes involved in heme biosynthesis, heme binding, erythrocyte homeostasis, iron ion binding, erythrocyte differentiation, gas transport and response to oxygen species metabolic processes, and other cellular processes. However, functional studies are needed to confirm their proposed relevance in beta thalassemia.

Keywords: Differential gene expression • RNA sequence • Beta-thalassemia • Fetal haemoglobin

Introduction

Hemoglobinopathies are endemic in numerous populations around the world, with hereditary carrier rates in Southeast Asia exceeding 40%. Mutations in β globin coding sequences that cause structural alterations in encoded proteins, as well as mutations that modify the expression of β globin genes, such as thalassemia, are examples of these illnesses. According to the World Health Organization (WHO), these disorders impact 7% of the population, with current research estimating that 300,000 to 400,000 infants are affected each year, with the prevalence predicted to climb in the future [1]. Hemoglobinopathies are Haemoglobin (Hb) abnormalities caused by mutations in the genes responsible for globin chain production. Over 1000 hemoglobinapathies have been found to date [2]. As a result of this positive selection, these disorders are quite common in the tropics and subtropics (Sub-Saharan Africa, the Mediterranean, and Southeast Asia). They have, however, become more common in non-endemic locations as a result of population migrations, making it a global health issue [3]. In the Var database, there are about 200 thalassemia alleles that have been described [4]. Unlike beta thalassemia, this is caused by one or a few nucleotide mutations in the globin gene (HBB). β alleles are HBB mutations that completely remove expression, whereas β^{+} or β^{++} alleles are HBB mutations that result in varying degrees of quantitative β globin expression reduction [5]. An imbalance between α and β chains causes the development of aberrant β4 tetramers in thalassemia. Both of these disorders cause anaemia owing to hemolysis, which is aggravated by a cellular stress response and insufficient erythropoiesis [6]. Hemolysis causes physiological iron excess, which is especially common in β thalassemia major. Increased inflammation and infection susceptibility may be linked to increased iron stores [7]. HbF increasing is a significant and important therapeutic tool to overcome the problem of hemolysis and anemia. During the first trimester, fetal haemoglobin has a high level in the foetus and then gradually lowers to an adult stage by maintaining the ratio of alpha to beta chain. The accumulation of alpha globin chains, a thyroid precursor, is reduced and, as a result, by boosting the oxygen supply to tissue and reducing clinical symptoms, the inefficient withdrawal process is inhibited. In recent research, the regulation of the fetal haemoglobin gene makes the developing process a complex process involving many different regulators. When connecting to the β globin locus, which lies between the foetal and adult genes, the BCL11A pathway, which generally

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Received: 10 November, 2022, Manuscript No. JBABM-23-85207; **Editor assigned:** 14 November, 2022, PreQC No. JBABM-23-85207 (PQ); **Reviewed:** 28 November, 2022, QC No. JBABM-23-85207; **Revised:** 06 February, 2023, Manuscript No. JBABM-23-85207 (R); **Published:** 13 February, 2023, DOI: 10.37421/1948-593X.2023.15.379

which generally cooperates with other repressors (e.g., Sox6), silences the β-globin genes [8]. We looked at the transcriptome profiles of two thalassemia patients with a new HBB gene mutation that may improve our understanding of the disease's heterogeneity and molecular mechanisms. RNA sequencing has been used to look at the gene expression of blood cells in patients with β thalassemia. The investigation of blood expression and its relationship to DEGs may aid in elucidating the role of these DEGs in beta thalassemia progression and formation.

Materials and Methods

Data acquisition and processing

The GSE96060 dataset, which contains expression profiles by array, was obtained from the NCBI GEO database. GEO collects data from a variety of investigations and makes gene expression profiles available [9]. We used the keywords "Beta thalassemia" and "Homo sapiens" to search GEO datasets for relevant gene expression profiles. GSE96060 comprises three control samples: Daughter (compound heterozygote for two HBB mutations), mother (heterozygote for a new HBB mutation), and normal (an individual with wild type HBB alleles and normal haematological indices).

Establishment of PPI networks and module analysis

To study the link between the DEGs in the datasets, we utilized the STRING web based tool (v11.5) to generate a PPI (protein-protein interaction) network [10]. We used a confident interaction value of 0.4 as the cutoff threshold to eliminate inconsistent PPI interactions from the dataset. As a result, we were able to put together a formidable PPI network. Using Cytoscape software (v3.9.0) and utilising the STRING tool results, we hypothesised the statistically significant DEGs' PPI interactions [11]. The group (cluster) determining extremities were charted using the Cytoscape plugin, Molecular Complex Detection plugin (MCODE); with Kappa score (K-core) set to 5, degree cutoff set to 2, max. depth set to 100, and Node score cutoff set to 0.2, to distinguish the intersected clusters from the acquired PPI network [12].

Gene Ontology (GO) analysis using the DAVID annotation database and ClueGO enrichment analysis

The Database for Annotation, Visualization, and Integrated Discovery (DAVID) is a useful tool for evaluating the functional characteristics of high throughput gene expression patterns. The DAVID database was used to identify biological activities, ontology concepts, and pathways that were significantly enriched in DEGs across all of our samples [13]. For GO functional annotation and KEGG pathway enrichment analysis, we employed the web based DAVID v6.8 programme. We employed the DAVID and ClueGO for integrative analysis programmes to look for DEGs that are linked to GO keywords and pathways. The DEG dataset was used to compile a list of Gene Ontological terms (GO) and disease-related pathways. The initial DEGs from the GEO2R tool were applied to ClueGO v2.5.8 and CluePedia v1.5.8. ClueGO combines KEGG or Reactome pathways with GO, resulting in a fundamentally ordered DEG dataset pathway network or GO [14]. DEGs were further investigated in terms of GO and pathway enrichment analyses for molecular/biological functions, with p-values less than 0.05 considered significant.

Results and Discussion

Data acquisition and identification of DEGs

The GSE96060 dataset included three control samples: Daughter (compound heterozygote for two HBB mutations), mother (a heterozygote for a new HBB mutation), and normal (an individual with wildtype HBB alleles and normal haematological indices) Table 1. *Homo sapiens* was used, and the RNA quality was assessed using a bioanalyzer 2100 [15].

Case	Age (year)	HGB (g/dl)	HBA%	HBA2%	HBF%
Mother (M) HBB	28	12.8, 13.4	96.2	3.4	0.4
Father (F) HBB	35	10.5, 11.2	94.4	5	0.6
Daughter (D) HBB	5	7.1, 7.6	93.5	4.8	1.7

Note: HBA: Haemoglobin A; HBA2: Haemoglobin A2; HBF: Fetal Haemoglobin; HGB: Haemoglobin; HBB: Haemoglobin B

Table 1. GSE96060 hemological parameter of family.

Establishing PPI networks and analyzing modules

The STRING programme was used to investigate the physical and functional interactions between proteins of beta thalassemia DEGs. The PPI network is depicted, which has 185 nodes and 475 edges. The nodes represent the quantity of proteins, while the edges represent the interactions between them. The MCODE v1.5.1 Cytoscape plugin interpreted the protein network's tightly interconnected sections as clusters. The PPI network's top three clusters that are significant and have MCODE scores of above 5 were chosen. Figure 1 illustrates these clusters graphically. Cluster 1 was created from the KLF1 node. Cluster 2 was created using node *MDM2*.

Figure 1. From the PPI network, two clusters with a cluster score of more than 5 were discovered. Cluster 1 was created from the *KLF1* node. Cluster 2 was created using nodes *MDM2* and *MDM3*.

DAVID enrichment analysis

The DAVID v6.8 web server was utilized to functionally annotate DEGs. Biological processes, molecular functions, and signaling pathways related to KEGG pathways were used to determine the KEGG pathway-enriched genes and the probable GO (Gene Ontology) category. We discovered that DEGs from the complex PPI network were enriched in transcription from RNA polymerase II promoter (GO: 0006366), heme biosynthetic process (GO: 0006783), chemotaxis (GO: 0006935), transcription DNA template (GO: 0006351), positive regulation of cell death (GO: 0010942), and oxygen transport by analyzing biological processes (GO: 0015671).

DEGs were found to be involved in RNA polymerase II regulatory region sequence specific DNA binding (GO: 0000977), DNA binding (GO: 0003677), oxygen transport activity (GO: 0005344), iron ion binding (GO: 0005506), and transcription binding factor (GO: 0005506), according to the Gene Ontology Molecular Functions analysis (GO: 0008134). The DEGs were found to be associated with the FOXO signalling pathway (hsa04068) and the PI3K-Akt signalling pathway in the KEGG pathway enrichment analysis (hsa04151). The following terms' annotated results were tabulated (Table 2).

Category	Term	Count	%	P-value	Fold enrichment	FDR	Gene
(GO: 0006366)	Transcription from RNA polymerase II promoter	7	4.04	3.36E	1.46	0.99	*EGR1, JUN, MYB, FOXO3, NFIX, GATA1, PBX1,*
(GO: 0006783)	Heme biosynthetic process	3	1.73	1.58E	21	0.99	*FECH,HMBS, CPOX*
(GO: 0006935)	Chemotaxis	5	2.89	2.65E	4.41	0.67	*C5, CCRL2, PROK2, FPR2, CCR3*
(GO: 0006351)	Transcription DNA, template	17	9.82	7.32E	9.36	0.99	*KANK2, DNMT1, NFE4, NFIX,RNF14, BCL11A, TCEANC, ZNF23, KLF1, TFDP1, BCL6,TAL1, BCL3 SOX6, ZBTB7A, BIRC2, LTF*
(GO: 0010942)	Positive regulation of cell death	3	1.73	2.29E	11.13	0.67	*HBB, HBA2, HBA1*
(GO: 0015671)	Oxygen transport	10	5.78	1.87E	71.7	1.97	*HBZ, HBM, HBG2, HBG1, HBE1, HBB, HBA1, HBA2, HBD, HBQ1*
(GO: 0000977)	RNA polymerase II regulatory region sequence specific DNA binding	5	2.78	1.27E	2.58	0.98	*SP1, EGR1, BCL6, TAL1, GATA1*
(GO: 0003677)	DNA binding	19	10.98	2.83E	1.22	0.98	*EGR1, JUN, DNMT1, NFIX, TCEANC, ZNF23, FOXO3, GATA1, PBX1, TFDP1, SP1, BCL3, MYB, E2F2, TLR8, SOX6, ZBTB7A, HIST1H2BD, LTF*
(GO: 0005344)	Oxygen transport activity	10	5.78	7.46E	76.8	2.47	*HBZ, HBM, HBG2, HBG1, HBB, HBE1, HBA2, HBD, HBQ1, HBA1*
(GO: 0005506)	Iron ion binding	13	7.51	1.82E	9.31	1.51	*HBZ, HBG2, HBG1, HBE1, CYP4F3, HBB, HBA2, HBD, HBA1, HBM, CYP1B1, HBQ1, LTF*

(GO: 0008134)	Transcription binding factor	6	3.468	1.234E	2.27	0.98	*JUN, TFDP1, SP1, BCL3, E2F2, PBX1*
(hsa04068)	$FOXO_3$ signalling pathway	4	2.312	1.64E	2.81	1	*BCL6, MDM2, FOXO3, EGFR*
(hsa04151)	PI3K-Akt signalling pathway	4	2.312	7.07E	1.09	1	*MYB, FOXO3, EGFR, MDM2*

Table 2. Gene Ontology (GO) terms for DEGs related with beta thalassemia from DAVID, including biological process, molecular activities, and KEGG pathways.

ClueGO/CluePedia enrichment analysis

The functional enrichment of the DEGs from the GSE96060 dataset was investigated using the Cytoscape plugin ClueGO/CluePedia. ClueGo assisted in visualising the found PPI complex network's GO keywords. Figure 2 depicts the complex PPI network's biological process, molecular functions, and functional enrichment analyses. A two-sided hypergeometric test with p 0.05, Benjamini-Hochberg correction, and a primary criterion of kappa score 0.4 were used to establish the statistical alternatives for ClueGO enrichment analysis. Myeloid cell differentiations (GO: 0030099), erythrocyte differentiation (GO: 0030218), reactive oxygen species metabolic process (GO: 0072593), response to toxic substances (GO: 0009636), homeostasis of cells number (GO: 0048872), gas transport (GO: 0015669), cellular detoxification (GO: 1990748), heme binding (GO: 0020037) and haemoglobin binding (GO: 0030492). Figure 3 were predominantly enriched in the biological process, molecular functions of DEGs from the complex PPI network. The KEGG and REACTOME pathway analysis from ClueGO showed that many DEGs were significantly enriched in erythrocytes take up oxygen and release carbon dioxide (R-HSA: 1237044), erythrocytes take up carbon dioxide and release oxygen (R-HSA: 1247673), Bladder cancer (KEGG:05219), pertussis (KEGG: 05133), oxygen/carbon dioxide exchange in erythrocytes (R-HSA: 1480926), heme biosynthesis (R-HSA: 189451), scavenging of heme from plasma (R-HSA: 2168880), misregulation of transcription in cancer (KEGG: 05202) (Table 3).

The results of the David and ClueGO enrichment clearly reveal that DEGs modify the metabolic behaviour of signalling pathways and are intimately associated to beta thalassemia, with genes involved in erythrocyte production and function clustered in extremely important ways. The ontologic term Iron Ion binding refers to the β thalassemia-causing dysregulation of globin genes. The genes in the erythrocyte homeostasis, heme biosynthesise, iron ion binding; erythrocyte differenation, gas transport and response to oxygen species metabolic process categories are elevated. The bulk of DEGs in chemotaxis are up-regulated, while bladder cancer is down regulated. Furthermore, the dysregulated pathways discovered by our bioinformatics enrichment study could be essential in the aetiology of beta thalassemia (Table 4).

Figure 2. The ClueGO/CluePedia plugin from Cytoscape was used to illustrate enrichment by Gene Ontology (GO) terms. With the precise gene connections, vital Molecular Functions (MF) and Biological Processes (BP) involved in the DEGs are shown. The 200 DEGs PPI network was used to infer the MF and BP enrichment studies. With a kappa score of 0.4, the connection of the GO keywords network is represented by functional nodes and edges that are shared between the DEGs. Only significant GO keywords (p-value 0.05) are shown in the enrichment. The node size is shown by p 0.05 values. The colour coding of a node denotes the functional class in which it is involved. The various molecular functions and biological processes involved in the enrichment analysis are represented by the various colours.

Figure 3. The ClueGo/CluePedia plugin from Cytoscape is used to visualise pathway term enrichment. The DEG enrichment analysis plugin includes KEGG and REACTOME pathways, as well as a full enrichment analysis for DEGs. With a kappa score of 0.4, the connection of the pathways in the network is represented by functional nodes and edges that are shared between the DEGs. Only relevant paths (p-value 0.05) are highlighted in the enrichment. The node size is shown by p 0.05 values. The colour coding of a node denotes the functional class in which it is involved. The different colours reflect different molecular pathways that were used in the enrichment analysis of the DEGs that were found.

Category	Term	Percentage associated gene	Count	Gene
R-HSA:189451	Heme biosynthesis	28.5	4	*ABCC2, CPOX,FEC4,HMBS*
KEGG:05133	Pertussis	7.89	6	*C5, CALM1, CXCL8, JUN, LY96, NOS2*
KEGG:05202	Transcriptional misregulation in cancer	5.73	11	*BCL6, BIRC2, BMP2K, CXCL8, DEFA3, MDM2, MMP9, MPO, PBX1, SP1, TSPAN7*
KEGG:05219	Bladder cancer	14.63	6	*CXCL8, DAPK2, E2F2, EGFR, MDM2, MMP9*
R-HSA:1237044	Erythrocytes take up carbon dioxide and release oxygen	46.15	6	*AQP1, CA1, CA2, HBA1, HBA2, HBB*
R-HSA:1247673	Erythrocytes take up oxygen and release carbon dioxide	66.67	6	*AQP1, CA1, CA2, HBA1, HBA2, HBB*
R-HSA:1480926	O_2/CO_2 exchange in erythrocytes	46.15	6	*AQP1, CA1, CA2, HBA1, HBA2, HBB*
R-HSA:2168880	Scavenging of heme from plasma	23.08	3	*HBA1, HBA2, HBB*

Table 3. Shows some of the Gene Ontology (GO) concepts linked with beta thalassemia, such as biological process and molecular activities of DEGs.

Caterory	Term	Percentage associated gene	Count	Gene
(GO: 0030099)	Myeloid cell differentiations	4.04	18	*ACVR1B, AHSP, BCL6, BPGM, CA2, DYRK3, EPB42, GATA1, JUN, KLF1, LILRB3, LTF, MMP9, RASGRP4, TAL1, TFRC, TRIM10, ZBTB7A*
(GO: 0030218)	Erythrocyte differentiation	8.66	11	*ACVR1B, AHSP, BCL6, BPGM, DYRK3, EPB42, GATA1, KLF1, TAL1, TRIM10, ZBTB7A*
(GO: 0072593)	Reactive oxygen species metabolic process	7.06	19	*BIRC2, CLCN3, CRYAB, CYP1B1, EGFR, FOXO3,*

				FPR2, HBA1, HBB, HBD, HBE1, HBG1, HBG2, HBM, HBQ1, HBZ, MPO, NOS2, TLR6
(GO: 0009636)	Response to toxic substances	5.76	16	*ABCB6, ABCG2, CLIC2, CYP1B1, FECH, HBA1, HBB, HBD, HBE1, HBG1, HBG2, HBM, HBQ1, HBZ, MDM2, MPO*
(GO: 0048872)	Homeostasisof number of cells	4.01	12	*ACVR1B, AHSP, BCL6, BPGM, DYRK3, EPB42, GATA1, KLF1, SPTA1, TAL1, TRIM10, ZBTB7A*
(GO: 0015669)	Gas transport	46.15	12	*AQP1, CA2, HBA1, HBA2, HBB, HBD, HBE1, HBG1, HBG2, HBM, HBQ1, HBZ*
(GO: 1990748)	Cellular detoxification	9.85	13	*ABCB6, ABCG2, CLIC2, HBA1, HBB, HBD, HBE1, HBG1, HBG2, HBM, HBQ1, HBZ, MPO*
(GO: 0020037)	Heme binding	9.76	16	*ABCB6, CYP1B1, CYP4F3, HBA1, HBA2, HBB, HBD, HBE1, HBG1, HBG2, HBM, HBQ1, HBZ, MPO, NOS2, STEAP4*
(GO: 0030492)	Haemoglobin binding	46.15	6	*AHSP, HBB, HBD, HBE1, HBG1, HBG2*

Table 4. Cluego's gene Ontology (GO) terminology for DEGs related with beta thalassemia, including KEGG pathways and REACTOME pathways.

In this study, the DEGs were studied between the gene expression profiles that were extracted from the GSE96060 dataset, which included three control samples: Daughter (compound heterozygote for two HBB mutations), Mother (heterozygote for a new HBB mutation), and Normal (an individual with wildtype HBB alleles and normal haematological indices) (Homo sapiens), and the RNA quality was assessed using a Bioanalyzer 2100 [16].

This study involved two participants, a carrier mother and her affected daughter. There were 19030 DEGs in all, but only the top 200 DEGs were chosen for further study. From the PPI networks, we discovered two important clusters that are differentiated by high scores and strongly related regions. The MCODE clusters were frequently displayed in the PPI network processes, and cluster representation is critical for the functional and full comprehension of network features [17]. The PPI network's strongly interconnected nodes and less connected vertices were weighed using the MCODE plugin's core clustering coefficient. The gene cluster might be examined to see which genes are relevant, how they interact, and how they relate to beta thalassemia pathogenesis and progression. Following computation, an algorithm evaluated the weighted graph to extract highly connected parts known as clusters, which reflect DEG produced molecular complexes [18]. The *MDM2* and *KLF1* genes, which were discovered in Table 2, may have a role in the pathway's differential regulation. To present a thorough picture of the DEGs involved in beta thalassemia, we used the ClueGO plugin to identify the differentially regulated molecular pathways and their significant gene interactions based on p-values and kappa statistics. The functional annotation of the 200 DEGs discovered in biological processes and molecular functions of beta thalassemia, which we identified among the enriched identified among the enriched biological processes and molecular pathways, was determined using the constructed GO and KEGG enrichment. These DEGs were discovered to be particularly abundant in myeloid cell differentiation, erythrocyte differentiation, reactive oxygen species metabolic processes, toxic chemical response, cell number homeostasis, gas transport, and cellular detoxification. The DEGs were considerably enriched in heme binding and haemoglobin binding, according to a GO analysis of biological processes and molecular activities. Similarly, the DEGs are involved in bladder cancer, pertussis, transcription misregulation in cancer, and the DEGs are involved in erythrocytes taking up oxygen and releasing carbon dioxide, erythrocytes taking up carbon dioxide and releasing oxygen, oxygen/carbon dioxide exchange in erythrocytes, heme biosynthesis, and scavenging of heme from plasma, according to KEGG pathway enrichment analysis. Impaired heme production causes haematological disorders, tissue degeneration, and ageing, whereas heme binding protects cells by activating the Heme Oxygenase-1 (HO-1) gene. As a result, heme can be employed as a therapeutic agent in a number of heme-related disorders, either alone or in conjunction with other treatments [19].

This study showed that the genes *KLF1* and *MDM2* play a role in beta thalassemia progression and are implicated in dysregulated molecular pathways. Bianchi E, 2010 hypothesised that low MYB levels result in fewer cell-cycle events early in erythropoiesis and that early maturation of erythroblasts results in red cells with higher levels of HbF. Overall, MYB plays an important role in erythropoiesis, and recent research suggests that it does so in part by transactivating the expression of *KLF1*.

Our research has shown a link between core DEGs and dysregulated pathways in beta thalassemia patients. Hematologists have long been interested in the fetal to adult haemoglobin flip and silencing of Foetal Haemoglobin (HbF) because clinical stimulation of

HbF production offers great promise in alleviating the clinical symptoms of β-thalassemia. Further studies based on molecular investigations that have discovered regulators such as *MYB*, and *KLF1* for generating more focused and effective HbF induction approaches are needed [20].

Conclusion

New pharmacological and genetic targets for HbF activation have emerged as a result of recent findings and understanding of the transition from fetal to adult hemoglobin. The results of identifying thalassemia markers in beta thalassemia patients and healthy controls are quite limited. With the use of DEGs, the research of blood expression and its relationship to the role of DEGs in beta thalassemia progression may be explained. Our study found 200 genes that have varied expression patterns and are associated with beta thalassemia patients. DEGs are enriched in β–thalassemia related pathways, such as genes involved in heme biosynthesis, heme binding, erythrocyte homeostasis, iron ion binding, erythrocyte differentiation, gas transport and response to oxygen species metabolic process, and other cellular processes. We searched for two potential target genes, *KLF1* and *MDM2*, that could be useful in the development of β thalassemia therapies. *KLF1* promotes HbF repression by activating both beta globin and HbF. γ-globin gene expression is influenced by altered MYB expression. These transcription factors (*KLF1*, and *MYB*) could be important molecular targets for therapeutic induction of foetal haemoglobin. Some factors that play equally important roles in the switch, such as *KLF1* and *MYB*, have many other roles in red cell development and function, making it less clear that they could be manipulated in the context of increasing HbF production in adult cells without having negative effects on other aspects of erythropoiesis. However, functional studies are needed to confirm their proposed relevance in beta thalassemia.

Competing Interest

The authors state that they have no known competing financial interests or personal ties that may have influenced the work presented in this study.

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How to cite this article: Soumya, Khare, Chatterjee Tanushree, Gupta Shailendra and Patel Ashish. "Differentially Expressed Genes for Regulation of Fetal Hemoglobin Induction in Beta Thalassemia." *J Bioanal Biomed* 15 (2023): 379.