Acta Biomed 2025; Vol. 96, N. 6: 17281 DOI: 10.23750/abm.v96i6.17281

Original article

New generation sequencing in diagnosis of congenital anemias

Duran Canatan[1,2], Emel Altunsoy[2], Peter Bauer[3]

[1]Antalya Genetic Diseases Assessment Center, Antalya, Türkiye; [2]Antalya Bilim University, Antalya, Türkiye; [3]Centogene Rare Diseases Research Center, Rostock, Germany

Abstract. *Background and aim:* Congenital anemias are a wide spectrum of diseases including hypoproliferative anemia syndromes, dyserythropoietic anemias, sideroblastic anemias, red blood cell membrane and enzymatic defects, hemoglobinopathies, and thalassemia syndromes. Next-generation sequencing approach including targeted panel, whole-exome sequencing (WES) and Whole Genome Sequencing (WGS) have become more accessible as a diagnostic tool, and played an important role in more undiagnosed cases. In this study, we aimed to present NGS results of patients with congenital anemia who were admitted to our center and to discuss in literature studies. *Material and methods:* A total 11 patients (8 female, 3 male) the distribution of age was 1-25 years and mean±SD: 8.54±16.9 years. Bone marrow/anemia panel was applied to seven cases; WES was applied to three cases and WGS was applied to one case. *Results:* Eleven patients have been diagnosed with a definite diagnosis as followed up; autosomal dominant spherocytosis type-1in two patients, autosomal dominant spherocytosis type -2 in two patients, autosomal dominant elliptocytosis type 2 in one patient, autosomal recessive pyruvate kinase deficiency plus gamma-glutamyl cysteine synthetase deficiency in one patient, Hb Knosos plus δ0-thalassemia in one patient, diamond blackfan anemia type 1 in two patients, bone marrow insufficiency in one patient and MLASA Syndrome (myopathy lactic acidosis sideroblastic anemia) in one patient. As conclusion, in diagnosis success, patient selection, examination of clinical symptoms and medical records, and determination of their association with the possible genetic variations and mutations detected by WES/WGS, and close cooperation between clinician and genetic evaluation centre are crucial. (www.actabiomedica.it)

Key words: next generation sequencing, diagnosis, congenital, anemia

Introduction

Congenital anemias are a wide spectrum of diseases including hypoproliferative anemia syndromes, dyserythropoietic anemias, sideroblastic anemias, red blood cell membrane and enzymatic defects, hemoglobinopathies, and thalassemia syndromes. The various congenital anemia syndromes may have similar clinical and laboratory presentations. Conventional diagnosis methods include a complete blood count, blood smears, hemoglobin electrophoresis, red blood cell enzyme activity, ektacytometry, the osmotic fragility test, flow cytometry, and bone marrow studies are performed for the diagnosis (1). Most patients with anemia are diagnosed through clinical phenotype and basic laboratory testing. Nonetheless, in cases of rare congenital anemias, some patients remain undiagnosed despite undergoing an exhaustive workup. Therefore, genetic testing is crucial for accurate diagnosis of patients with congenital anemias. Genetic testing is complicated by the large number of genes that are involved in rare anemias, due to similarities in the clinical presentation. Thus, targeted next-generation sequencing (NGS) using custom-made gene panels

has been increasingly utilized, with a high success rate of diagnosis (2,3). NGS approach including targeted panel, whole-exome sequencing (WES) and Whole Genome Sequencing (WGS) have become more accessible as a diagnostic tool and played an important role in more undiagnosed cases. In this success, patient selection, examination of clinical symptoms and medical records, and determination of their association with the possible genetic variations and mutations detected by NGS and close cooperation between clinician and genetic evaluation centre are crucial. Targeted NGS assay is of major impact on congenital anemias. The assay should be used routinely in congenital anemias (4,5,6). In this study, we aimed to present NGS results of patients with congenital anemia who were admitted to our center without being diagnosed and to discuss in literature studies.

Patients and Methods

NGS analysis was performed in eleven patients (8 female, 3 male) who applied to our center due to anemia. The distribution of age was 1-25 years and mean±SD: 8.54±16.9 years. Detailed personal and familial history of the patients has been obtained, and physical examination has been performed. After obtaining the written informed consent, a detailed clinical report has been prepared, all clinical data of the case has been uploaded to the Centoportal system. Blood with 5 cc EDTA taken from the patient dropped on special cards, after drying it placed in an envelope and sent to Centogene. In Centogene, after DNA isolation from the cards, Bone marrow failure/anemia (BMFA) panel including 214 gene was applied to seven cases, WES was applied to three cases and WGS was applied to one case.

Results

All patients had anemia. The starting time of the complaints was between 30 days and 10 years (Table 1).

Eleven patients have been diagnosed by NGS method as followed up; autosomal dominant spherocytosis type-1in two patients, autosomal dominant spherocytosis type -2 in two patients, autosomal dominant elliptocytosis type 2 in one patient, autosomal recessive pyruvate kinase deficiency plus gamma-glutamyl cysteine synthetase deficiency in one patient, Hb Knosos plus δ0-thalassemia in one patient, diamond blackfan anemia type 1 in two patients, bone marrow insufficiency in one patient and MLASA Syndrome (myopathy lactic acidosis sideroblastic anemia) in one patient (Table 2).

Table 1. Demographic Features of Patients with Congenital Anemia

No	Sex	Age (Years)	Complaints	The duration of the complaints (Years)
1	F	14	anemia	14 years
2	F	1	anemia+neonatal icterus	30 days
3	F	2	anemia	1 year
4	F	25	anemia and icterus	10 years
5	F	3	anemia and icterus	2 years
6	M	9	anemia and growth retardation	2 years
7	M	9	anemia	4 months
8	F	4	anemia	2 years
9	F	13	anemia	2 months
10	M	10	anemia and thrombocythemia	4 years
11	F	4	anemia	2 months
	8 F 3 M	Mean±SD: 8.54±16.9		

Table 2. New generation sequencing analysis results of patients with congenital anemias

No	Pre liminary Diagnosis	Gene	NGS	Variant Coordinates Amino acide change	Definitive Diagnosis
1	Hereditary Membran Disorders	SPTB	PANEL	NM_001024858.3: c.3190C> T, p. (Gln1064*) Exon 15	Autosomal dominant spherocytosis type 2
2	Congenital Hemolytic Anemia	1. PKLR 2. GCLC	PANEL PANEL	NM_000298.5: c.1456C>T p. (Arg486Trp) Exon 10 NM_000298.5: c.1591C> p. (Arg531Cys) Exon 10 NM_001498.3: c.1332T>G p. (Phe444Leu) Exon 12 NM_001498.3: c.1486G>T p. (Ala496Ser) Exon 14	1. Autosomal recessive pyruvate kinase 2. Deficiency 3. Gamma-glutamylcysteine 4. synthetase deficiency
3	Congenital Hemolytic Anemia	SPTA1	PANEL	NM_003126.2: c.83G>A p. (Arg28His) SIFT: Exon 2	Autosomal dominant elliptocytosis type 2
4	Congenital Hemolytic Anemia	SPTB	PANEL	NM_001024858.2: c.1912C>T p. (Arg638*) Exon 15	Autosomal dominant spherocytosis type 2
5	Congenital Hemolytic Anemia	ANK1	PANEL	NM_001142446.1: c.2775T>G p. (Ty r925*)	Autosomal dominant Spherocytosis type 1
6	Growth, Motor and Mental retardation	HBB HBD	WES	NM_000518.4: c.82G>T p. (Ala28Ser) Exon 1 NM_000519.3: c.179del p. (Lys60Argfs*2) Exon 2	Hb Knossos δ0-thalassemia
7	Congenital Hemolytic Anemia	PUS1	PANEL	NM_025215.5: c.904del p. (Val302Trpfs*4)	Myopathy, lactic acidosis and sideroblastic anemia (MLASA)
8	Congenital Hemolytic Anemia	ANK 1	PANEL	NM_001142446.1: c.2775T>G p. (Ty r925*)	Autosomal dominant Spherocytosis type 1
9	Congenital Anemia	RPS19	WES	NM_001321485.1:c.416_417del p.(Arg139Profs*?)	Diamond-Blackfan anemia type 1
10	Pancytopenia	TERT	WGS	NM_198253.2: c.2093G>A p. (Arg698Gln) NM_198253.2:c.2419G>A p. (Asp807Asn)	Dysceratosis Congenita
11	Congenital anemia	RPS19	WES	NM_001321485.1: c.416_417del p. (Arg139Profs*?)	Diamond-Blackfan anemia type 1

Discussion

Genetic diagnosis of congenital haematological disorders is complicated by the overlap of the clinical and laboratory presentation across different diseases and the large number of genes involved in each syndrome. In recent years, a novel method of targeted next generation sequencing using gene panels was developed (7). WES covers approximately 20,000 genes, and all protein coding regions in the genome are analysed at the same time and provides 85% information about the mutations in the genome. WGS covers InDels, structural variants (SVs), large copy number variants (CNV)AS almost 99% of a gene. (8). Congenital anemia that caused by hereditary erythrocyte membrane disorders may result from mutations in genes encoding structural membrane proteins or membrane transport proteins. Approximately thirty genes have been identified for the hereditary erythrocyte membrane disorders. Mutations have been frequently detected in SPTA1, SPTB, ANK1, SLC4A1 and EPB42 which encode the structural membrane proteins (9,10). Agarwal et.al reported NGS panel includes 28 genes encoding red blood cell (RBC) cytoskeletal

proteins, membrane transporter, RBC enzymes, and certain bilirubin metabolism genes. They identified pathogenic/likely pathogenic variants in 111/456 (24%) patients that were responsible for the disease phenotype. The most common mutated genes were membrane cytoskeleton genes SPTA1, and SPTB, followed by PKLR as enzyme gene (11). Nieto et.al. developed a panel including 48 gene developed to diagnose hemolytic membrane anemia by NGS in a large cohort of 165 patients from 160 unrelated families. They were divided patients two groups, as A group and B group. Diagnostic performance was 83.5% in group A who had a suspicion of a specific type of HA (n = 109), and 35.7% of patients B group who had a suspicion of HA but with no clear type (n = 56). achieved a genetic diagnosis. They recommended that the use of NGS is a sensitive technique to diagnose hemolytic anemias and it shows better performance when patients are better characterized (12). Fermo et.al. reported a 43 genes targeted NGS panel in diagnosis of congenital hemolytic anemia in 122 patients and the overall sensitivity of NGS was 74% (13). Mansour -Hendili et.al reported probable genetic cause of disease was identified in 82.5% of the patients with hemolytic anemias (14). Kedar et.al. has performed 76 genes panel known to cause anemia syndromes by gene capture followed by NGS in 17 (80.9%) of 21 transfusion-dependent patients and undiagnosed by conventional workup (2). Li et.al. developed 217 known genes of congenital anemias by NGS in a total of 46 patients were enrolled in this study. They defined 28 (60.9%) of 46 patients became confirmed cases after targeted NGS (4). Averbuch et.al achieved in 13 out of 21 patients (62%) with congenital anemias by NGS (5). Jamwal et.al. enrolled 43 patients with clinical and laboratory evidence of unexplained hemolytic anemia. Initially, 13 patients were tested using a commercial (TruSight One) panel, and remaining cases underwent targeted sequencing using a customized 55-gene panel. Overall, 63% cases received a definite diagnosis (15). Sun et.al confirmed that a novel mutation in ANK1 may be causative of HS, which plays an important role in expanding the mutational spectrum of ANK1 mutation (16). Lin et al. reported five causative variants, including two ANK1, two SPTA and one SPTB variants, were detected in four patients. WES analysis is an efficient tool for determining genetic etiologies of RBC membrane disorders and can facilitate accurate diagnosis and genetic counseling (6).

We applied a bone marrow panel/anemia including 214 gene and well characterized all patients so that we diagnosed autosomal dominant spherocytosis type -1 in two patients with a variant in ANK1gene, autosomal dominant spherocytosis type-2 in two patients with a variant in SPTB gene and , autosomal dominant elliptocytosis type 2 in one patient with a variant in SPTA1 gene. Enzyme deficiencies are as important as membrane disorders in congenital anemia. Pyruvate kinase deficiency (PKD) is an autosomal recessive condition, caused due to homozygous or compound heterozygous mutation in the PKLR gene resulting in non-spherocytic hereditary hemolytic anemia. Dongerdive et al. reported the mutational landscape of 45 unrelated PKD cases from India. They evaluated the phenotypic and molecular spectrum of PKLR gene disorders and also emphasizes the importance of combining both targeted next-generation sequencing with bioinformatics analysis and detailed clinical evaluation to elaborate a more accurate diagnosis and correct diagnosis (17). Del Orbe Barreto et.al. designed a custom panel for sequencing coding regions from 40 genes known to be involved in the pathogenesis of congenital anemias.They identified pathogenic mutations in membrane defects (SPTB, ANK1, SLC4A1 and EPB41), and enzyme deficiencies (GPI, TPI1 and GSS), one had a mutation in the HBB gene (18). We diagnosed autosomal recessive pyruvate kinase deficiency plus gamma-glutamyl cysteine synthetase deficiency in one patient with BMFA panel. Inherited bone marrow failure syndrome (IBMFS) is a group of clinically heterogeneous disorders characterized by significant hematological cytopenias of one or more hematopoietic cell lineages. The genetic etiology of IBMFS includes germline mutations impacting several key biological processes, such as DNA repair, telomere biology, and ribosome biogenesis. These may cause four major syndromes: Fanconi anemia, dyskeratosis congenita, Diamond-Blackfan anemia, and Shwachman-Diamond syndrome (19). Diamond-Blackfan anemia (DBA) is a rare congenital disorder presenting remarkable phenotypic overlap with other inherited bone marrow failure syndromes, making

differential diagnosis challenging and its confirmation often reached with great delay. Errichiello et.al. unravelled the presence of pathogenic variants affecting genes already known to be involved in DBA pathogenesis (RPL5 and RPS19) in three patients with otherwise uncertain clinical diagnosis with WES analysis (20). Russo et.al. obtained an overall diagnostic yield of 64.9% in hereditary anaemias such as hyporegenerative anemias, as congenital dyserythropoietic anemia (CDA) and Diamond-Blackfan anemia (21). We applied WES analysis to two cases with suspected DBA and identified the RPS19 variant in both cases. We applied the WGS analysis for a patient with pancytopenia because he was an undiagnosed patient for a long time. We detected a variant in the TERT gene. This variant was related to telomeres and caused bone marrow failure.

Myopathy, lactic acidosis, and sideroblastic anemia (MLASA) is a rare mitochondrial disorder characterized by MLASA. Variable features of this condition include failure to thrive, and developmental delay or intellectual disability. MLASA has previously been associated with mutations in pseudouridylate synthase 1 (PUS1) and YARS2. Oncul et al. reported on 2 Turkish sisters 4 and 11 of years with an MLASA with PUS1 gene revealed a novel homozygous p.Glu311* mutation (22).

We diagnosed MLASA in one patient with BMFA panel. The mutation causing MLASA syndrome was c.904del p. (Val302Trpfs*4) in the PZO gene. Congenital anemias due to the variation in hemoglobin synthesis, are generally classified into two groups; deletion of alpha/beta/gamma/delta globin genes leads to thalassemia, and structural abnormalities of these globin chains generate variants of hemoglobin. In the thalassemia group, 220 alpha, 344 beta, 34 delta, 42 delta/beta and 28 εδβeta thalassemia have been published, and in the variants of hemoglobin group, 460 alpha, 601 beta, 99 gamma, 74 delta chain (23,24). Hb Knossos (beta 27 (B9) Ala----Ser) is discovered hemoglobin variant first time in a case from Algeria. The propositus also has homozygous delta (0)-thalassemia (25). Olds et.al. defined the molecular basis of normal HbA2 beta-thalassaemia associated with Hb Knossos in Egytp family. DNA sequence analysis of the delta globin gene in cis with beta Knossos showed deletion of a single A in codon 59 leading to a premature termination at codon 60 (26). We diagnosed Hb Knosos plus δ0-thalassemia with mutation c.82G>T p. (Ala28Ser) Exon 1. NM_000519.3: c.179del p. (Lys60Argfs*2) Exon 2 in HBB gene with WES analysis in one patient. A total eleven patients with congenital anemia admitted to the genetic assessment center, and underlying genetic abnormalities were determined using NGS methods. Although between sixty and ninety-four percent successes have been achieved in the literature in NGS methods, all patients were diagnosed in our genetic centre. In conclusion, in recent years, NGS technologies including targeted panel tests, WES and WGS have become more accessible as a diagnostic tool in diagnosis of congenital anemias. In diagnosis success, patient selection, examination of clinical symptoms and medical records, and determination of their association with the possible genetic variations and mutations detected by WES/WGS, and close cooperation between clinician and genetic evaluation centre are crucial.

Conflict of Interest: The authors of this paper have no conflicts of interest, including specific financial interests, relationships, and/or affiliations relevant to the subject matter or materials included.

Authors contribution: DC, EA: Concept; DC, EA, PB: Design; EA, PB: Data collection or processing; PB, DC: Analysis or interpretation; DC, EA, PB: Writing.

Declaration on the Use of AI: We didn't use chatbot any AI.

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Correspondence:
Received: 21 June 2025
Accepted: 19 August 2025
Prof. Dr. Duran Canatan, M.D.,
Antalya Bilim University,
Çıplaklı, Akdeniz Blv. No:290/A, 07190
Döşemealtı/Antalya- Turkey
E-mail: durancanatan@gmail.com
ORCID: 0000-0001-8128-82694