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Review Article
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Current Status of Molecular Diagnosis of Hereditary Hemolytic Anemia in Korea

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ABSTRACT

Hereditary hemolytic anemia (HHA) is considered a group of rare hematological diseases in Korea, primarily because of its unique ethnic characteristics and diagnostic challenges. Recently, the prevalence of HHA has increased in Korea, reflecting the increasing number of international marriages and increased awareness of the disease. In particular, the diagnosis of red blood cell (RBC) enzymopathy experienced a resurgence, given the advances in diagnostic techniques. In 2007, the RBC Disorder Working Party of the Korean Society of Hematology developed the Korean Standard Operating Procedure for the Diagnosis of Hereditary Hemolytic Anemia, which has been continuously updated since then. The latest Korean clinical practice guidelines for diagnosing HHA recommends performing next-generation sequencing as a preliminary step before analyzing RBC membrane proteins and enzymes. Recent breakthroughs in molecular genetic testing methods, particularly next-generation sequencing, are proving critical in identifying and providing insight into cases of HHA with previously unknown diagnoses. These innovative molecular genetic testing methods have now become important tools for the management and care planning of patients with HHA. This review aims to provide a comprehensive overview of recent advances in molecular genetic testing for the diagnosis of HHA, with particular emphasis on the Korean context.

Keywords: Anemia; Hemolytic; Congenital; Diagnosis; Genetic Testing

INTRODUCTION

Hereditary hemolytic anemia (HHA) includes a group of heterogeneous congenital red blood cell (RBC) disorders resulting from genetic defects in proteins within the RBC membrane, globin chains, or RBC enzymes. Based on their etiology, these are further categorized into RBC membranopathy, hemoglobinopathy, and RBC enzymopathy.[1] Because of their rarity

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and lifelong chronic nature, accurate diagnosis, regular examinations, and appropriate treatment by hematologists are crucial to mitigate complications of these conditions.[2]

HHA represents rare hematological disorders caused by structural changes, transport disorders, metabolic irregularities, or defective production of RBCs.[3] The RBC Disorder Working Party (WP) of the Korean Society of Hematology (KSH) developed the Korean Standard Operating Procedure (SOP) for the diagnosed of HHA in 2007 and has continuously revised and updated it since then.[4-6] The updated Korean SOP for HHA is distributed to the members of the KSH, to standardize and improve the accuracy of HHA diagnoses in Korea.[2] In addition, several advanced laboratory technologies were integrated into the SOP, to improve HHA diagnosis in Korea. In the case of RBC membranopathy, since March 2013, the central laboratories of Korea have been using liquid chromatography-tandem mass spectrometry (LC-MS/MS) to assess RBC membrane proteins.[7] Similarly, the flow cytometric osmotic fragility test (OFT) and eosin-5′-maleimide (EMA) binding test were included in the New Health Technology Assessment of Korea in November 2014 and have been utilized since.[8,9] In the case of hemoglobinopathy, globin gene mutations are identified via direct sequencing or multiple ligation-dependent probe amplification (MLPA) of the β- and α-globin genes. Since March 2013, the central laboratories of Korea have diagnosed RBC enzymopathy via the multiplex RBC enzyme analysis using ultra-performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS).[7]

The latest Korean clinical practice guidelines for HHA diagnosis recommends performed next-generation sequencing (NGS) before analysis of RBC membrane proteins and enzymes. Recent advancements in molecular genetic testing methods, particularly NGS, are proving invaluable in uncovering diagnostic clues for patients with HHA whose diseases are unknown. This article aims to provide an overview of the current status of molecular genetic testing for the diagnosis of HHA and recent advances in this field, with a particular emphasis on the Korean context.

CURRENT STATUS AND EPIDEMIOLOGICAL TRENDS OF HHA

Large-scale epidemiological studies on various types of HHA have been reported worldwide. Hemoglobinopathy, which also includes thalassemia and sickle cell disease (SCD), has been traditionally known to be relatively common in certain races and regions, such as South Asia and Africa. The prevalence of hereditary spherocytosis (HS), thalassemia, and SCD in Europe, India, and Asia was studied and reported several decades ago. Based on these studies, guidelines for the diagnosis and treatment of these diseases have been established.[10-13]

RBC membranopathy includes HS, which is the most common HHA in the European and North American populations, affecting approximately 1 in 2,000 individuals,[14] and hereditary elliptocytosis (HE), which affects 3–5 in 10,000 individuals.[15] Less common types of membranopathy, such as hereditary pyropoikilocytosis or hereditary stomatocytosis also exist.[16] According to previous reports, HS is most common among the Northern European populations, whereas other types of membranopathy, including hereditary stomatocytosis and HE, are very rare.[17]

Hemoglobinopathies result from genetic defects in the α- or β-globin genes. Quantitative defects in globin production lead to thalassemia, whereas qualitative defects in globin production lead to abnormal hemoglobin (Hb) formation (as seen in SCD) or unstable Hb.

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α-Thalassemia is known to be the most common inherited Hb disorder, with an estimated 5% of the world's population being carriers and approximately 1,000,000 are affected.[18,19] α-Thalassemia is more common in Africa, Southeast Asia, and the Middle East, whereas β-thalassemia is common in Mediterranean countries, Southeastern Europe, Asia, and the Middle East.[20,21] The prevalence of β-thalassemia is reported to be approximately 1 in 100,000 people worldwide and 1 in 10,000 people in Europe.[22] β-Thalassemia includes various heterozygotes and carriers, such as β-thalassemia minor, intermedia, HbC/β, HbE/β, HbS/β, hereditary persistence of HbF and β-thalassemia, β-thalassemia-trichothiodystrophy, and X-linked thrombocytopenia with thalassemia, which are difficult to recognize by clinical manifestation alone and therefore require a molecular diagnosis.

SCD is reported to affect 1 in 500 African-Americans and 1 in 1,200 Hispanic-Americans.[10,23] The overall global prevalence of SCD is not clear but is estimated to be approximately 300,000 births annually, according to a World Health Organization report in 2011.[24] SCD is an autosomal recessive disorder; therefore, patients with homozygous HbS mutations with symptoms are considered sickle cell anemia patients. Heterozygous compound forms, such as S/β-thalassemia or sickle cell anemia are also referred to as SCD.[10]

The prevalence of enzymopathy is currently underresearched. Currently, the most common enzymopathy is glucose-6-phosphate dehydrogenase (G6PD) deficiency. The global prevalence is estimated at 4.9%, affecting at least 400 million people worldwide,[25] especially in Africa, southern Europe, the Middle East, Southeast Asia, and the Central and Southern Pacific islands. These regions are also endemic for malaria.[26] The second most common enzyme deficiency is pyruvate kinase deficiency (PKD), with an estimated global prevalence ranging from 3 in 1,000,000 to 1 in 20,000.[27,28]

Recent advances in molecular genetic approaches for screening and diagnosis have influenced the estimated prevalence and medical burden of these diseases. Therefore, many epidemiological studies have recently focused on the differences between phenotypic manifestations and genetic diagnosis.[29-31] Advances in molecular genetic testing have also changed the strategies for prenatal and neonatal screenings.[32-35]

CURRENT LANDSCAPE OF HHA IN KOREA: EPIDEMIOLOGY, CLINICAL FEATURES, AND MOLECULAR DIAGNOSIS

Epidemiology of HHA in Korea

In 1991 a nationwide multi-center epidemiological study of HHA in Korea covered the period from 1981 to 1990. This study covered 315 patients, including 162 pediatric patients aged 0–15 years.[36] Since 1997, epidemiological studies on HHA have been conducted in 10-year cycles by the RBC Disorder WP of the KSH. The first 10-year national epidemiological study of HHA, covering the period from 1997 to 2006, was published in 2007.[4] This study covered 431 subjects, including 333 pediatric patients from 35 hospitals across Korea. The second 10-year nationwide Korean epidemiological study, covering the period from 2007 to 2016, was reported in 2020.[6] This study included 369 pediatric patients with HHA diagnosed using the Korean SOP for HHA.[4,5] These epidemiological studies between 1997 and 2016, HS is the most common type of HHA, accounting for over 70% of all nonimmune hemolytic anemia. From 2007 to 2016, 253 pediatric patients were diagnosed with HS, whereas 10 of the 369 patients with nonimmune hemolytic anemia were diagnosed with HE.

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Thalassemia major and other hemoglobinopathies were previously uncommon. However, recent increases have occurred[4,6] primarily because of increased thalassemia minor (carriers of thalassemia) attributed to an increase in international marriage contacts.[37] Additionally, clinicians may have increased their knowledge of hemoglobinopathy in Korean children with anemia.[2]

RBC enzymopathies are rare in Korea, with G6PD deficiency and PKD the most common.[38,39] From 2007 to 2016, 23 pediatric patients with RBC enzymopathy were recorded, including 12 with PKD and nine patients with G6PD deficiency. More recently, the incidence of PKD has increased markedly compared with the period 2007 to 2016.[6] One factor may be external immigration, especially from South-East Asia, because of international marriages.[40-43] The increase in the diagnostic confirmation of RBC enzymopathy may be attributed to the increased availability of genetic diagnostic techniques, including NGS.[16,44,45] **Fig. 1** illustrates the shifts in the epidemiology of HHA in Korea. Furthermore, **Table 1** provides a summary of HAA diagnosis in Korea, encompassing all HAA patients recruited in the three preceding nationwide studies.[4,6,36]

Clinical and laboratory features of HHA in Korea

HHA is suspected with symptoms such as anemia, jaundice, and splenomegaly.[2] The most common symptom/sign of HHA is neonatal jaundice, which may require medical intervention.[46]

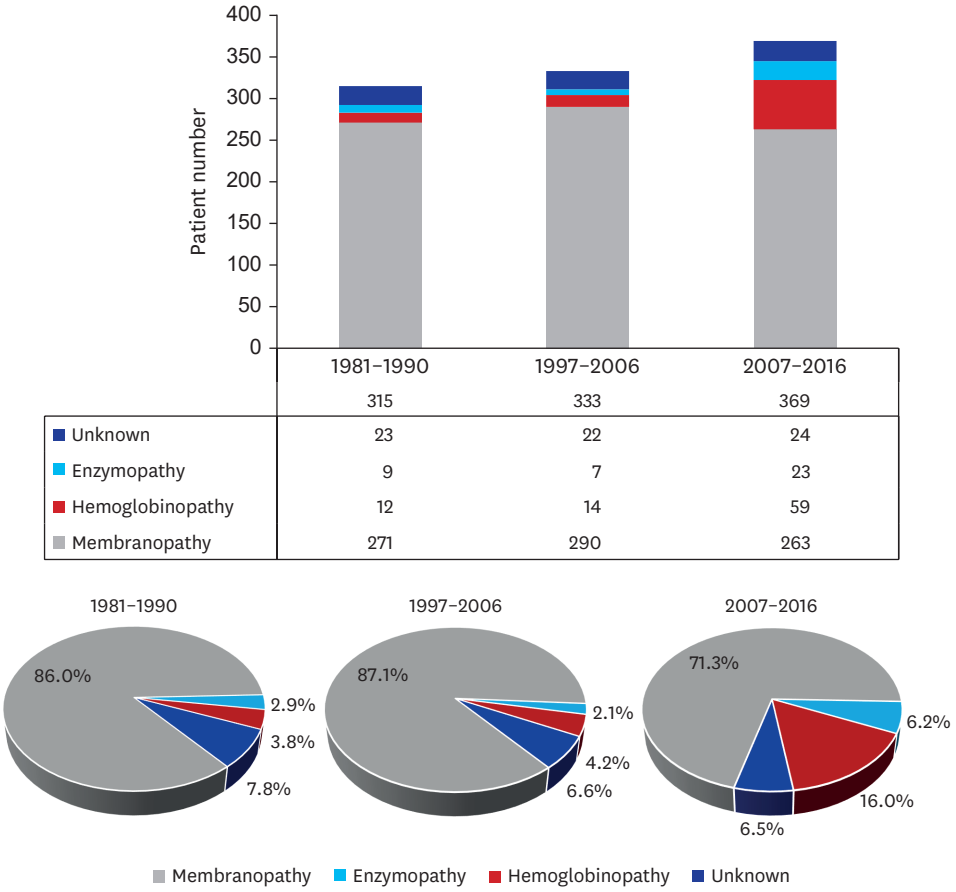

Fig. 1. Changes in the epidemiology of hereditary hemolytic anemia in Korea over three decades. Although membranopathy is predominant, there has been a marked increase in the detection rates of hemoglobinopathy and red blood cell enzymopathy as well.

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Table 1. Trend of studies on the epidemiology of hereditary hemolytic anemia in Korea

Variables	Ahn et al.[36]	Cho et al.[4]	Shim et al.[6]
Study period	1980–1991	1997–2006	1997–2016
No. of institution enrolled	38	35	38
No. of patients enrolled	934	431	369
Department enrolled	Adults (531)/ Children (403)	Adults (98)/ Children (431)	Children
Diagnosis			
Membranopathy	292 (92.7%)	382 (88.6%)	263 (71.3%)
Hemoglobinopathy	10 (3.1%)	20 (4.6%)	59 (16.0%)
Enzymopathy	13 (4.1%)	7 (1.6%)	23 (6.2%)
Other (unknown)	514[a]	22 (5.1%)	24 (6.5%)
Method of diagnosis	History & physical examination		
	· Osmotic fragility test	· Osmotic fragility test	· Osmotic fragility test (flow cytometric analysis, EMA-binding test added)
	· Enzyme activity: G6PD	· SDS-PAGE	· Enzyme activity: UPLC-MS/MS method enzyme assay added
	· Hemoglobin EP for thalassemia	· Enzyme activity: G6PD, PK, Enolase	· Hemoglobin EP
		· Hemoglobin EP, MLPA for thalassemia	· MLPA for Thalassemia
		· Case series of Sanger sequencing	· Molecular diagnosis: Sanger sequencing, NGS, WGS

G6PD = glucose-6-phosphate dehydrogenase, EP = electrophoresis, SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis, PK = pyruvate kinase, MLPA = multiple ligation-dependent probe amplification, EMA = eosin-5′-maleimide, UPLC-MS/MS = ultra-performance liquid chromatography-tandem mass spectrometry, NGS = next-generation sequencing, WGS = whole-genome sequencing, HA = hemolytic anemia, PNH = paroxysmal nocturnal hemoglobinuria, CDA = congenital dyserythropoietic anemia.
[a]Acquired hemolytic anemia (including immune-mediated HA), PNH, CDA were included in this study.

With RBC membranopathy, most patients with autosomal dominant-type HS have a mild phenotype. whereas those with autosomal recessive type HS may have severe anemia requiring transfusions.[46] Most patients with HS may also show splenomegaly.[12] Those with well-compensated anemia in HS may receive a diagnosis later in life because of cholelithiasis.[47] For example, in one study on HHA, jaundice and splenomegaly were the most common symptoms among patients with RBC membranopathy.[6]

Patients with hemoglobinopathy may show facial changes, characterized by enlarged maxillary sinuses resulting from blood cell production. These facial changes can lead to ear, nose, and throat infections in patients, especially those with thalassemia major, to infections of the ears, nose, and throat. Adults with thalassemia major with chronic transfusion-dependent anemia and iron overload may develop complications such as cardiomyopathy, diabetes mellitus, hypopituitarism, hypoparathyroidism, hypothyroidism, or testicular/ovarian failure.[20] The most common presentation in Korea is thalassemia minor, which is usually asymptomatic. In tests conducted on Koreans with HHA, the median value of Hb was the highest, whereas those of the RBC profiles were the lowest. The median value of corrected reticulocytes was also low, as was that of lactate dehydrogenase. In contrast, the median value of haptoglobin was high in Korean individuals with hemoglobinopathy, suggesting that hemolysis is not severe among patients with hemoglobinopathy in Korea.[6]

For RBC enzymopathy, patients with G6PD deficiency typically develop jaundice a few days after birth.[46] However, beyond infancy, most G6PD-deficient patients have no symptoms under normal conditions. Hemolysis in these individuals is triggered by oxidative stressors such as infections or some medications.[26,46] When exposed to oxidative stressors, G6PD-deficient patients can experience abrupt hemolytic anemia, with pallor, jaundice, and the presence of red-brown urine. Patients with PKD present a wide spectrum of clinical manifestations.[48] The severity of hemolysis in PKD can be exaggerated during infections or pregnancy.[49] Some patients with PKD have lifelong, fully compensated, or mild anemia, whereas others experience severe anemia requiring repetitive RBC transfusions.[50] In severe cases of PKD, patients may develop transfusion-dependent iron overload

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or cholelithiasis.[48,49] In a Korean epidemiological study for HHA, patients with RBC enzymopathy exhibited the most severe degree of anemia among individuals with HHA. Additionally, the diagnostic age of individuals with RBC enzymopathy was the lowest among the different HHA groups.

Genetic characteristics of HHA in Korea

Studies utilizing confirmation tests for patients with HHA in Korea are scarce because of the limited availability of certain confirmation test facilities. Lee et al.[51] conducted a study on 27 patients with HS and identified various types of HS, using sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE): spectrin deficiency in 7.4% of cases (2/27), ankyrin deficiency in 29.6% (8/27), combined spectrin and ankyrin deficiency in 3.7% (1/27), band 3 deficiency in 11.1% (3/27), and protein 4.2 deficiency in 14.8% (4/27). These findings indicated that ankyrin deficiency alone and its combination with spectrum deficiency accounted for 33.3% of cases (9/27), as the most common biochemical defects. Notably, protein 4.2 deficiency was more prevalent among Koreans than Caucasians. Protein defects were not observed in nine out of the 27 patients (33.3%).[51]

Park et al.[52] reported 42 suspected patients with HS that were screened via OFT and confirmed using the molecular tic test of Sanger sequencing. Of these, 25 patients carried one heterozygous mutation of *ANK1* (n = 13) or *SPTB* (n = 12).[52] Deleterious mutations, including frameshift, nonsense, and splice site mutations were found in 91% (21/23) of cases, with non-hotspot mutations dispersed across multiple exons. This was the first large-scale single-center study to examine *ANK1* or *SPTB* mutational characteristics and genotype-phenotype correlation. Anemia was most severe in patients with HS who carried mutations on the *ANK1* spectrin-binding domain, whereas *SPTB* mutations in HS patients spared the tetramerization domain, where HE and pyropoikilocytosis mutations are typically located. The study also found a high frequency of splenectomy in *ANK1*-mutant HS (32%), as compared to that in *SPTB*-mutant HS (10%) ($P = 0.028$).

Choi et al.[53] conducted the first multi-center genetic study of Korean patients with HS. Genetic variations in RBC membrane protein-encoding genes were examined using multi-gene targeted NGS (t-NGS) in patients with HS diagnosed by Korean HHA guidelines. These findings were compared with clinical features. A total of 43 genes were included, comprising 17 RBC membrane protein and 20 RBC enzyme-encoding genes, along with six additional candidate genes, for differential diagnoses (thalassemia, congenital dyserythropoietic anemia [CDA], paroxysmal nocturnal hemoglobinuria, and Gilbert syndrome). Of the 59 patients with HS, 50 (84.7%) had one or more critical variants in the RBC membrane protein-encoding genes. A total of 54 critical variants, including 46 novel mutations, were detected in six RBC membrane protein-encoding genes. The highest number of variants were found in *SPTB* (n = 28), followed by those in *ANK1* (n = 19), *SLC4A1* (n = 3), *SPTA1* (n = 2), *EPB41* (n = 1), and *EPB42* (n = 1). Concurrent mutations in genes encoding RBC enzymes (*ALDOB*, *GAPDH*, and *GSR*) were also detected in three patients. *UGT1A1* mutations were present in 24 patients (40.7%). The positive rate of OFT was 86.8% among patients harboring HS-related gene mutations. As a result, many single-gene confirmatory tests that utilized Sanger sequencing have transitioned to NGS, particularly t-NGS for HHA.[45,53-55] Following these studies, cases with novel mutations have continued to be reported using Sanger sequencing, t-NGS, or whole-exome sequencing (WES).[56-60]

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DIAGNOSTIC HURDLES AND NEW TECHNIQUES

Traditional laboratory diagnosis

RBC membrane stability test

Flow cytometric OFT is recommended because it is well standardized and offers higher sensitivity and specificity than the traditional method.[61,62] Flow cytometric analysis of EMA-labeled intact RBCs demonstrates high sensitivity and specificity. EMA binds to membrane proteins on RBCs (such as band 3, CD47, Rh protein, and Rh glycoprotein), which are indirectly associated with the spectrin-based RBC cytoskeleton.[63] A reduction in the fluorescence intensity of the EMA-labeled RBCs upon flow cytometry analysis indicates disruption of the membrane protein.

RBC deformability is measured to identify the different deformability profiles of RBC membrane disorders.[64] For RBC membranopathy, studies using RheoScan, osmotic gradient ektacytometry, conducted using laser-assisted optical rotation cell analyzers, have shown modifications in the shape of RBCs as they flow through capillaries.[65-67] Key parameters evaluated for differential diagnosis include the omin-value (the osmolality at which deformability reaches its minimum, similar to the 50% hemolysis point in a conventional OFT) and elongation index max (corresponding to the maximum deformability or elongation obtained near the isotonic osmolality). Although RBC ektacytometry is included in many diagnostic algorithms for RBC membranopathy,[55,64] it is not widely available for clinical use in Korea.

RBC membrane protein analysis

Traditionally, the quantification of RBC membrane proteins was performed using SDS-PAGE.[17] However, more recently, mass-spectrometry-based techniques, including matrix-assisted laser desorption/ionization-time of flight mass spectrometry and LC-MS/MS, have become available.[68] These mass spectrometry-based methods provide sensitive approaches to identify the profile of RBC membrane proteins.

RBC enzyme analysis

Classical spectrophotometric methods for measuring RBC enzyme activity are generally labor-intensive and time-consuming because they require multiple reactions, each one measuring a specific enzyme activity separately. To overcome these disadvantages, LC-MS/MS-based multiplex RBC enzyme assays were developed to measure the activities of enzymes involved in RBC enzymopathy. Since 2013, the central laboratories of Korea have used ion-pairing UPLC-MS/MS for multiplex RBC enzyme analysis,[69] which measures the activities of six important RBC enzymes, including G6PD, pyruvate kinase (PK), pyrimidine 5'-nucleotidase, hexokinase (HK), triosephosphate isomerase (TPI), and adenosine deaminase (AD). The UPLC-MS/MS assay enables reproducible multiplex assessment of the activity of enzymes associated with RBC enzymopathy.

Molecular diagnosis of HHA

The molecular testing strategies for HHA have developed with the adoption of NGS in many clinical laboratories. Many causative genes are associated with a single HHA entity, and these may overlap between diseases with different phenotypes.[64] In addition, the clinical presentation of HHA can overlap and lack specificity, requiring complex analyses and consequent complicate the diagnosis.[47,70] In one study, multi-gene analysis modified the original clinical diagnosis in 45.8% of patients with congenital anemia, including HHA. For example, 45.5% of the probands initially classified as CDA were diagnosed with chronic

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anemia because of enzymatic defects, primarily caused by mutations in the *PKLR* gene.[54] In another study, t-NGS enabled molecular diagnosis in 38.6% of patients, which accounted for only 13.6% of the known cases, leading to altered treatment approaches.[71] Co-occurrence of multiple disorders can also complicate phenotypic assessment, necessitating molecular testing. Recent studies report co-inheritance of G6PD in 15% of patients with HS,[72] as well as that of G6PD and dehydrated stomatocytosis,[54] and combined metabolic deficiencies with RBC membrane defects.[73] Accurate diagnosis is crucial to avoid complications or unnecessary interventions for HHA. In cases of hereditary stomatocytosis, splenectomy is contraindicated, whereas in PKD, a new oral activator of PK may offer therapeutic potential.[50,74]

The British Society of Hematology and the European Hematology Association recently published a practice paper on the use of NGS for inherited anemias, including HHA, and recommended NGS after characterization of the phenotype, including gene panels for Diamond-Blackfan anemia, CDA, congenital sideroblastic anemia, RBC enzymopathy, and RBC membranopathy.[55] Patients suspected of a specific type of HHA achieved higher diagnostic yields than those with an unspecified HHA subtype.[73] However, in Korea, because of the widespread availability of t-NGS testing and limited access to tests characterizing the phenotype, such as RBC membrane analysis, ektacytometry, or RBC enzyme testing, molecular diagnosis is recommended as the initial step in the diagnostic workup, with phenotypic correlation performed later in the process.[2] Several reports of t-NGS results for HHA in Korea have been published, especially for HS, followed by genetic profiling to confirm the HHA diagnoses.[53,57,58,75]

Several steps are required before implementing t-NGS for HHA diagnosis.[76] Target genes should be carefully selected to include potential causative genes for HHA and diseases that mimic or overlap with HHA. The clinical t-NGS panels reported in the Genetic Testing Registry for HHA typically include 28–40 genes, and a recent Korean clinical practice guideline for HHA diagnosis recommends specific genes for testing.[2]

Regarding the type of genetic variants, all possible pathogenic variants, including single nucleotide variants, indels, or copy number variations, should be considered and evaluated. If necessary, valid ancillary assays should be performed to complement the analysis.[55] As copy number variations cannot be fully analyzed in some t-NGS panels, additional molecular testing is required in conjunction with t-NGS, especially for RBC hemoglobinopathy. Additionally, common variants detected in the population can be considered for the screening test.[77,78] In HS, common pathogenic modifiers (e.g., alpha-LeLY/LePRA) are present in over 1% of the general population and should be checked in patients suspected of HS or HE, along with other *SPTA1* variants. Although alpha-LeLY is usually included in most t-NGS panels, alpha-LePRA (c.4339-99C>T) may not be covered within ± 10 base pairs of the exons in many panels, or might be lost during data processing; thus, it should be considered. However, alpha-LePRA is often co-inherited with the α-Bughill allele (missense variant p.[Ala970Asp]), which is covered by standard t-NGS panels, allowing confirmation of α-LEPRA using alternate methods.[79,80] For thalassemia, common globin gene deletions are analyzed using gap-PCR or MLPA,[81] and t-NGS simultaneously tests for *HBA1*, *HBA2*, and *HBB*. Depending on the chosen method, the laboratory should be aware of any limitations and either reduce them or clearly state what has not been tested in the report. In cases where no causative variants are identified, WES or whole-genome sequencing (WGS) may be considered, although this remains under research.[16,71,82] Although t-NGS may offer testing for a limited number of genes and variable phenotypes, comprehensive genetic analyses using WES have allowed the diagnosis of G6PD deficiency in patients initially suspected of

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CDA and the identification of genetic variants in non-HHA genes that explained part of the phenotypes. Some laboratories have opted for WES over t-NGS (because of its increasing availability) and have explored the identification of causative genes via trio-analysis of WES.[83-85] Various methods for diagnosing HHA have been succinctly summarized in **Table 2**, including their respective strengths, weaknesses, sensitivities, and specificities.

However, despite advances in sequencing techniques, genetic causes may remain unexplained in some patients with HHA.[86] In addition, the clinical importance of some detected mutations may be unclear, often classifying them as a 'variant of uncertain importance.' Even if a genetic abnormality is found, the genotype may not align with the patient's phenotype. Therefore, characterizing a patient's phenotype using traditional laboratory tests remains essential for individual patient diagnosis, severity assessment, and medical management planning. Several traditional diagnostic laboratory methods for HHA have undergone improvements in Korea.

CURRENT GUIDELINES ON HHA DIAGNOSIS

General guidelines on diagnosis of HHA in other countries

Diagnosis of HHA is often challenging because of the potential overlap of clinical symptoms and laboratory test results. In some cases, identifying the causative gene may be necessary to establish a clear diagnosis, and to avoid unnecessary investigations and ensure timely

Table 2. Comparison of different methods to differentiate and diagnose HHAs

Assay	Purpose	Methods	Advantages	Disadvantages
Osmotic fragility test	To screen for hereditary spherocytosis, to assess resistance of RBC to hemolysis in hypotonic solutions	Spectrophotometry, flow cytometry	Simple, rapid turnaround time, low cost, widely available	· Low sensitivity and specificity for HS. · Positive in other immune hemolytic anemias, RBC enzyme deficiencies
Eosin-5-maleimide binding test	To screen for hereditary spherocytosis, detect decrease of band 3 and Rh-related proteins using fluorescent dye	Flow cytometry	High sensitivity, rapid turnaround time	· Fresh sample required. · Decreased in other hemolytic anemias such as hereditary pyropoikilocytosis, Southeast Asian Ovalocytosis, other autoimmune hemolytic anemias, false negative in mild cases of HS.
RBC Membrane protein assay	Identify composition and integrity of RBC membrane proteins	Mass spectrometry, SDS-PAGE	Identify specific protein deficiencies	· Complex and expensive. · Not standardized. · Require expertise for testing and interpretation
RBC Enzyme assay	Measure activity of RBC enzymes	Spectrophotometry, mass spectrometry	Identify specific enzyme deficiencies	· Some enzyme deficiencies may show normal activity, requires specific conditions for accurate measurement, require expertise for testing and interpretation.
Hemoglobin analysis	Qualitative and quantitative Hb analysis	HPLC, capillary zone electrophoresis, gel electrophoresis	Rapid, high throughput	· Not for definitive diagnosis, poor separation of HbS depending on the methods.
Targeted next-generation sequencing panel	Identify variants for HHA	Molecular methods	Relatively low cost compared to that of WES and WGS. Less time for interpretation than WES and WGS.	· Can only identify variants in the target regions. · Copy number variants may be hard to assess.
Whole-exome sequencing	Identify variants for HHA	Molecular methods	Covers all exons, cost lower than WGS	· Require expertise for interpretation. · Copy number variants may be hard to assess. · Ethical issues for incidental findings should be considered prior to testing.
Whole-genome sequencing	Identify variants for HHA	Molecular methods	Assess all genomes	· High cost, highly skilled personnel for interpretation, and long turnaround time. · Ethical issues for incidental findings should be considered prior to testing.

HHA = hereditary hemolytic anemia, RBC = red blood cell, HS = hereditary spherocytosis, SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Hb = hemoglobin, HPLC = high-performance liquid chromatography, WES = whole-exome sequencing, WGS = whole-genome sequencing.

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and appropriate treatment. Therefore, many countries have developed their own practice guidelines and recommendations tailored to their unique epidemiological and medical environments. However, the core approach to diagnosing HHA remains similar across these guidelines. When suspecting HHA in a patient, a comprehensive diagnostic process is essential, which includes a thorough physical examination, as well as a detailed review of the patient's medical and family history. The laboratory evaluation process may vary depending on the specific type of HHA suspected or the patient's epidemiological/ethnic background.

Guidelines for diagnosing HS are abundant because of its high incidence, whereas those for diagnosing other types of HHA are relatively limited. In most cases of RBC membranopathy and hemoglobinopathy, the key lies in interpreting the peripheral blood smear findings, which then guide the subsequent evaluation. When HS is suspected, many countries recommend the OFT as a screening test, whether the classical method or the EMA-binding test. However, it is important to note that OFT can yield both false positives and false negatives, especially in neonates or younger patients with conditions such as CDA type II (CDA-II) or other membranopathies. To address these challenges, guidelines in the UK and from the International Council for Standardization in Haematology recommended membrane protein analysis as a screening or diagnosis test.[12,17] More recent guidelines advocate for molecular genetic testing such as PCR or NGS, especially in neonates or individuals who do not have documented family histories.[87] A recent Chinese protocol also suggests NGS as the next step after screening tests such as the OFT, assessment of G6PD levels, and acidified glycerol lysis test, instead of the SDS-PAGE test.[88]

However, a recent meta-analysis on the diagnostic methods for RBC disorders in Asia revealed varying proportions of using genetic analysis to identify mutations, with Sanger sequencing, NGS, and WES being used in 33.3%, 22.2%, and 13.8% of cases, respectively.[35] Surprisingly, proteomics was employed in only 5.5% of the total studies, which is a relatively low proportion given the existing recommendations and guidelines. In contrast, guidelines for diagnosing enzymopathy are relatively scarce, with the most recent (2019) recommendations for PKD provided by EuroBloodNet, the European Reference Network in Rare Hematological Diseases representing one of the latest guidelines for enzymopathy.[89]

Current SOP of HHA in Korea

The RBC Disorder WP of KSH has introduced an SOP for the diagnosis of HHA, as illustrated in **Fig. 2**.[5,15] When dealing with patients suspected of having HHA, it is crucial to conduct a thorough review of their medical history, including familial and ethnic backgrounds, in addition to appropriate laboratory tests for HHA. The primary focus should initially be on complete blood count, with RBC indices and peripheral blood smear findings. If RBC membranopathy is suspected, especially HS, OFT, flow cytometric OFT, or EMA-binding test are recommended. Although the EMA-binding test is highly predictive for HS, the fluorescence intensity of RBCs in Southeast Asian ovalocytosis, cryohydrocytosis, and some cases of CDA-II can fall within the same range as that expected for HS. Additionally, patients with HS who have isolated ankyrin deficiency may exhibit normal fluorescence results. Clinicians should, therefore, correlate these findings with family studies and other test results, for comprehensive diagnosis and accurate assessment.[90]

For diagnosis of RBC membranopathy, the International Council for Standardization in Haematology guidelines recommends traditional methods for quantifying RBC membrane proteins, using SDS-PAGE. Although more advanced techniques have emerged, including

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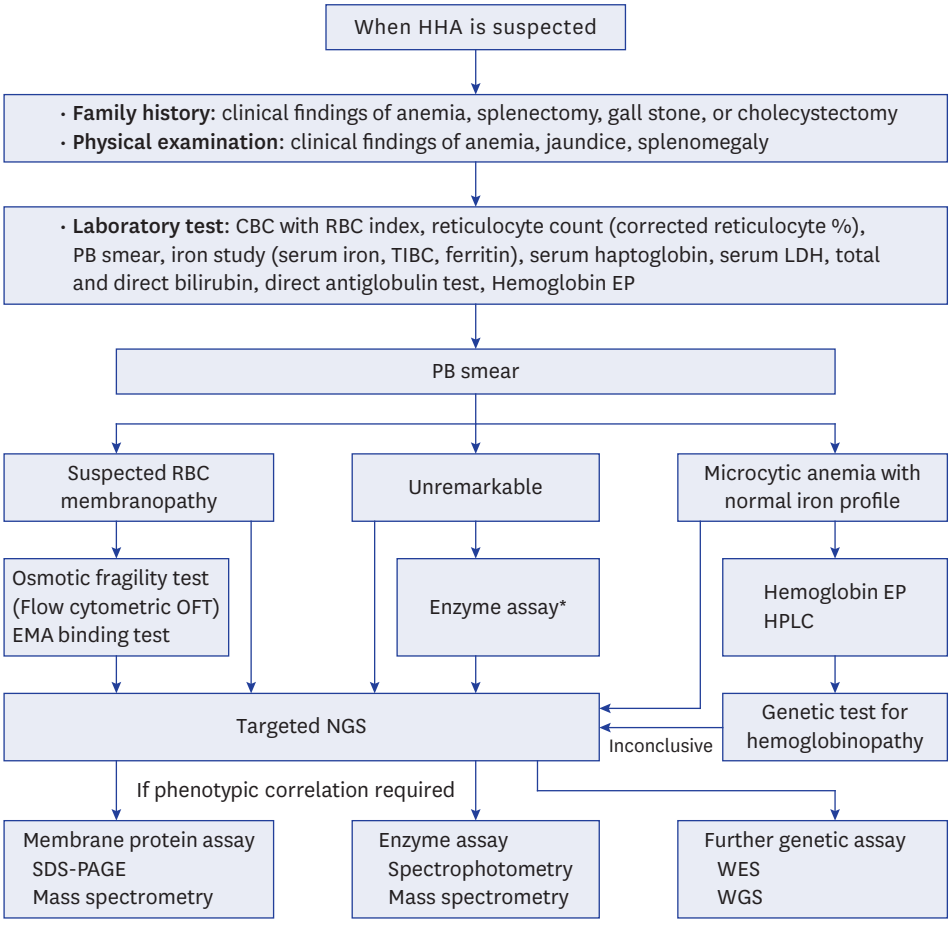

Fig. 2. SOP for the diagnosis of HHA by the RBC Disorder Working Party of the KSH.[5,15]
HHA = hereditary hemolytic anemi, CBC = complete blood count, RBC = red blood cell, TIBC = total iron binding capacity, LDH = lactate dehydrogenase, EP = electrophoresis, PB = peripheral blood, OFT = osmotic fragility test, EMA = eosin 5-maleimide, HPLC = high-performance liquid chromatography, NGS = next-generation sequencing, SDS-PAGE = sodium dodecyl sulfate-polyacrylamide gel electrophoresis, WES = whole-exome sequencing, WGS = whole-genome sequencing, SOP = standard operating procedure, KSH = Korean Society of Hematology.
*Prioritize enzyme analysis before transfusion in suspected patients; if unclear, perform NGS testing afterwards.

matrix-assisted laser desorption/ionization-time of flight mass spectrometry or LC-MS/MS, these are currently not available in Korea.[54,91,92] Therefore, in cases of RBC membranopathy in Korea, the recommendation is to confirm the diagnosis using molecular genetic methods, which can provide accurate results.

For RBC enzymopathy, the classical spectrophotometric methods used to measure RBC enzyme activity are typically labor-intensive and time-consuming. To address the limitations, a recommended approach is to use the ion-pairing UPLC-MS/MS method. It is important to note that the current UPLC-MS/MS RBC enzyme activity assay covers only six enzymes. Thus, clinicians should be aware of which specific enzymes are tested and avoid ruling out untested diagnoses. Additionally, when interpreting enzyme assay results, factors such as previous RBC transfusions, which can affect accuracy, must also be considered.

Hemoglobinopathies in Korea are typically screened using either high-performance liquid chromatography or hemoglobin electrophoresis. Abnormal results obtained from these methods are usually confirmed via genetic testing, primarily employing Sanger sequencing and/or MLPA.[93]

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Molecular diagnosis of HHA in Korea

RBC membrane disorders are usually caused by membrane structural defects or altered membrane transport function. As further evaluations for membrane proteins are not available in Korea, molecular genetic tests can be considered. Genetic variations reported in Korea include those related to cytoskeleton and membrane protein quantitative deficiencies including ankyrin, erythrocytic α- and β-spectrin chains, band 3 and protein 4.2.[53,80] Although genetic testing is a powerful tool in the diagnosis of these disorders, approximately 50% of patients remain undiagnosed despite NGS testing.[80]

The diagnosis of enzymopathy in Korea is currently dependent on UPLC-MS/MS RBC enzyme activity assay, which has a limitation of covering six enzymes. Therefore, molecular genetic tests are recommended as the next step for patients who are suspected of enzymopathy.

For every patient suspected of having HHA, a genetic test is strongly recommended as a confirmation test. t-NGS is now routinely used in undiagnosed patients and family members with HHA. t-NGS is preferred over WES or WGS because of its lower cost, quicker turnaround time, simpler data analysis, better coverage of relevant regions of interest, and reduced risk of incidental findings. The RBC Disorder WP of the KSH has created a t-NGS panel that encompasses genes associated with RBC membranopathy, enzymopathy, and other diseases that share overlapping features with HHA (**Table 3**), including *ADA*, *AK1*, *ALDOA*, *ANK1*, *CYB5R3*, *EPB41*, *EPB42*, *G6PD*, *GCLC*, *GPI*, *GSR*, *GSS*, *HBA1*, *HBA2*, *HBB*, *HK1*, *NT5C3A*, *PFKM*, *PGK1*, *PIEZO1*, *PKLR*, *SLC4A1*, *SPTA1*, *SPTB*, *TPI1*, and *UGT1A1*.[2] It is worth noting that in Korea, t-NGS is often considered before RBC enzyme or membrane protein analysis because of its greater availability. Of note, specific considerations may be required for the detection of large deletions/duplications, variants in introns, and copy number variations related to hemoglobinopathy.

In the case of hemoglobinopathy, especially thalassemia, it is recommended to perform Sanger sequencing and MLPA as the initial approach, rather than t-NGS. A retrospective epidemiological study of HHA conducted in Korea revealed that most of the hemoglobinopathies were β-thalassemia minor (83.1%) or α-thalassemia minor (15.3%). In the case of β-thalassemia, the primary mutations involve single nucleotide variants in the *HBB* gene, which can be detected using sequence analysis. However, this may also encompass variants in non-coding regions, and rarely, large deletions. If *HBB* sequencing reveals only one pathogenic variant or none at all, deletion/duplication analysis is typically conducted. In contrast, α-thalassemia cases in Korea are primarily caused by a deletion in the *HBA1* or *HBA2* genes, accounting for up to 85% of all causative mutations. As mentioned above, such large deletions can be missed in sequencing or t-NGS. It is worth noting that, despite the importance of MLPA in diagnosing α-thalassemia, it is not routinely performed in Korea because of it not being covered by the health insurance, potentially leading to an underestimation of α-thalassemia in the country.

CLINICAL IMPLICATIONS OF MOLECULAR GENETIC TEST RESULTS IN HHA TREATMENT

Molecular genetic tests are crucial for the development of accurate HHA diagnosis and treatment guidelines, to prevent misdiagnoses.[94] Comprehensive genetic testing has resulted in corrected diagnoses, with some cases of two concurrent types of HHA identified at

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Table 3. Genes recommended for hereditary hemolytic anemia gene panel testing

Gene symbol	Gene name	OMIM	Phenotype	Inheritance
ADA	Adenosine deaminase	608958	ADA deficiency	AR
AK1	Adenylate kinase 1	103000	AK1 deficiency	AR
ALDOA	Aldolase A	103850	ALDOA deficiency	AR
ANK1	Ankyrin 1	607008	Spherocytosis	AD/AR
CYB5R3	Cytochrome b5 reductase 3	613213	Methemoglobinemia	AR
EPB41	Erythrocyte membrane protein band 4.1	130500	Elliptocytosis	AR
EPB42	Erythrocyte membrane protein band 4.2	177070	Spherocytosis	AR
G6PD	Glucose-6-phosphate dehydrogenase	305900	G6PD deficiency	XL
GCLC	Glutamate-cysteine ligase, catalytic subunit	606857	GCLC deficiency, hyperbilirubinemia, hemolytic anemia	AR
GPI	Glucose phosphate isomerase	172400	Acute/chronic hemolytic anemia	AR
GSR	Glutathione reductase	138300	GSR deficiency	AR
GSS	Glutathione synthetase	601002	GSS deficiency	AR
HBA1	Hemoglobin alpha locus 1	141800	alpha-thalassemias	AR
HBA2	Hemoglobin alpha locus 2	141850	alpha-thalassemias	AR
HBB	Hemoglobin beta locus	141900	beta-thalassemia	AR
HK1	Hexokinase 1	142600	Hemolytic anemia	AR
NT5C3A	5′-nucleotidase, cytosolic IIIA	606224	NT5C3A deficiency, hemolytic anemia	AR
PFKM	Phosphofructokinase, muscle	610681	PFKM deficiency, glycogen storage disease 7	AR
PGK1	Phosphoglycerate kinase 1	311800	Phosphoglycerate kinase 1 deficiency	XLR
PIEZO1	Piezo-type mechanosensitive ion channel component 1	611184	Xerocytosis	AR
PKLR	Pyruvate kinase (liver and RBC)	609712	PKLR deficiency, hemolytic anemia	AR
SLC4A1	Solute carrier family 4, anion exchanger, member 1, band 3	109270	Spherocytosis, blood group variation, anemia, stomatocytosis, acanthocytosis kernicterus, ovalocytosis	AD/AR
SPTA1	Spectrin alpha	182860	Elliptocytosis, spherocytosis, pyropoikilocytosis, elliptopoikilocytosis	AD/AR
SPTB	Spectrin beta	182870	Elliptocytosis, spherocytosis	AD/AR
TPI1	Triosephosphate isomerase 1	190450	TPI1 deficiency	AR
UGT1A1	UDP glycosyltransferase 1 family, polypeptide A1	191740	Crigler-Najjar syndrome, hyperbilirubinemia (unconjugated), Gilbert syndrome	AR

AD = autosomal dominant, AR = autosomal recessive, XL = X-linked, XLR = X-linked recessive, RBC = red blood cell.

times.[48,95-98] Transfusions, splenectomy, and iron chelation are the conventional therapies applied in the treatment of thalassemia. The management approach of this condition is related to the transfusion status of the patients as transfusion-dependency is a crucial factor in treatment decisions. However, advancements in genetic knowledge and ongoing clinical trials have led to the emergence of genetic information-based therapies, expanding treatment options.[99] The new therapies employed in clinical trials aim to address ineffective erythropoiesis and target the Janus kinase pathways. In addition, molecular genetic testing plays an important role in the treatment of thalassemia, by distinguishing the disease entities that rarely require transfusions, such as thalassemia trait and HbE. Nevertheless, both can be passed on to descendants, thus underscoring the importance of genetic counseling for family members. In the case of G6PD deficiency, long-term care guidelines are relatively well-established. Some drugs and substances should be avoided, and this list is continually updated. Thus, obtaining a precise diagnosis using genetic testing is of utmost importance for the lifelong care of patients with G6PD deficiency.[100]

PKD can exhibit a wide range of clinical manifestations, from mild asymptomatic anemia to severe fetal hydrops.[48] The patient's symptoms may not align with the severity of anemia. Instead, issues such as immune deficiencies, gallstones, and complications arising from iron overload are more common in symptomatic patients. With over 300 reported mutations in the *PKLR* gene and the ongoing identification of new mutation types,[89] the complexity of PKD is evident. Patients frequently have compound heterozygous mutations, resulting in diverse mutation profiles. The future of PKD management lies in personalized care guided by genetic information.[48,101] Metapivat (AK-348), a recently developed PK activator, has recently

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gained FDA approval for the treatment of adult PKD.[102,103] In phase 2 trials, it effectively improved Hb levels and reduced transfusion needs. However, it showed limited efficacy in patients with two non-missense mutations, leading to their exclusion from the trials. Given these examples, future treatments for HHA are expected to consider the genetic information of patients as well.[104,105]

When considering splenectomy or hematopoietic stem cell transplantation for patients with HHA, an exact diagnosis and accurate information regarding genetic mutations are needed. Patients with CDA-II, hexokinase deficiency, G6PD type 1 deficiency, and PKD, who require transfusions, may be candidates for splenectomy.[106] Grace et al.[48] showed that individuals with two non-missense mutations in PKD responded poorly to splenectomy, as observed in Amish patients. Therefore, these factors should be considered when planning treatment. As for splenectomy, clinical outcomes may vary between total and partial splenectomy for different diseases. According to a large registry report, certain patients with HS who have complete α-spectrin deficiency because of biallelic null *SPTA1* mutations show no improvement with splenectomy. They should instead be treated with chronic transfusions and chelation therapy or undergo hematopoietic stem cell transplantation.[107,108] Patients with SCD are at a higher risk of thrombotic events after splenectomy than patients with other anemias.[96] Registry data also suggest that some patients with rare CDA-II and PKD may not benefit from splenectomy and are better suited for repeated transfusions or stem cell transplantation or both.[109,110] Therefore, the collection of data from large registries in combination with clinical and molecular genetic information, is critical for the development of tailored treatment plans for these patients.

DISCUSSION

HHA is a rare but increasing disease in Korea, with occurrences linked to advancements in diagnostic methods and the influx of immigrants. Continuous epidemiological investigations have increased interest and awareness of HHA in Korea. The changing disease epidemiology underscores the need to expand healthcare capacity and develop and support health policies to address this evolving healthcare challenge.

Molecular diagnostics play a crucial role in identifying genetic mutations responsible for HHA. Diagnostic approaches for HHA in Korea have shifted from the traditional clinical and laboratory-based methods to molecular diagnostic techniques. The introduction of NGS tests using t-NGS has made HHA diagnosis more accessible. However, factors such as the relatively high associated costs (although it is now becoming more affordable), limited insurance coverage, and a lack of public understanding can still pose barriers to genetic testing. To improve the accuracy of genetic diagnosis of HHA, efforts should focus on discovering additional genes and new variants that can be examined. In cases where t-NGS does not provide a diagnosis, WES or WGS may be considered as alternative methods to identify possible genetic causes of HHA. It is important to note that confirming a patient's phenotype using traditional laboratory tests remains essential because genetic causes of HHA may remain undetected in some patients, despite advances in sequencing techniques. Furthermore, genetic abnormalities may not always correlate directly with a patient's phenotype, and mutations detected in some patients may have uncertain clinical importance, leading to their classification as 'variants of uncertain significance.' Therefore, the scope of health insurance coverage for diagnostic tests related to HHA must be expanded.

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In addition to epidemiological studies and the development of diagnostic guidelines for HHA, the RBC Disorder WP of the KSH is also involved in a new initiative – the establishment of a nationwide HHA registry. This registry will enroll patients both retrospectively and prospectively, to comprehensively understand the clinical characteristics of patients with HHA in Korea. This registry is expected to markedly improve patient management and prognosis via accurate diagnosis of HHA, provision of new treatment opportunities, and better management of complications associated with the condition.

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