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Prenatal and newborn screening for hemoglobinopathies

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SUMMARY

The hemoglobinopathies encompass a heterogeneous group of disorders associated with mutations in both the alpha-globin and beta-globin genes. Increased immigration of high-risk populations has prompted the implementation of prenatal and newborn screening programs for hemoglobinopathies across Europe and North America. In Canada, the UK, and other European countries, prenatal screening to identify hemoglobinopathy carriers and offer prenatal diagnostic testing to couples at risk is linked to newborn screening, while in the United States, it is still not universally performed. The structure of screening programs, whether prenatal or postnatal, universal or selective, varies greatly among these countries and within the United States. The laboratory methods used to identify hemoglobinopathies are based on the prevalence of hemoglobinopathies within the population and the type of screening performed.

Advances in molecular testing have facilitated the diagnosis of complex thalassemias and sickling disorders observed in ethnically diverse populations. This review summarizes the current approaches and methods used for carrier detection, prenatal diagnosis, and newborn screening.

INTRODUCTION

The hemoglobinopathies are a clinically heterogeneous group of inherited disorders associated with mutations in both the alpha (α)-globin and beta (β)-globin genes and are broadly classified as sickle cell disorders and thalassemias. Sickle cell disease is caused by sickle hemoglobin (Hb S), a structurally abnormal Hb variant due to a point mutation in the β-globin gene. Homozygous or compound heterozygous Hb S co-inherited with either another Hb variant (e.g., Hb C, D-Punjab, O-Arab) or a β-thalassemia mutation results in sickle cell disease (SCD).

The thalassemias are caused by decreased or absent production of otherwise normal α- or β-globin chains. Beta-thalassemia major occurs in individuals who are either homozygous or compound heterozygous for a β-thalassemia mutation. The phenotype of β-thalassemia major can also result from co-inheritance of a β-thalassemia mutation with Hb E (Hb E/β-thalassemia) or with a large deletion in the β-globin gene locus (δβ-thalassemia) or in conjunction with α-globin gene duplication.

Alpha-thalassemias are associated with variable clinical severity depending on the type and number of α-globin gene mutations involved. In the absence of sufficient α-globin chains, excess β- or γ-globin chains form tetramers, known as Hb H (β_4) or, in the fetus, Hb Bart's (γ_4). While individuals with a deletion or mutation in one or two of the four α-globin genes are asymptomatic, those with loss of three α-globin genes develop clinically significant Hb H disease (α -/- -). Co-inheritance of an α-globin gene point mutation and a deletion involving both α-globin genes on the same chromosome results in a more severe form of HbH disease (α^T/- -) [1]. Deletion of all four α-genes results in Hb Bart's hydrops fetalis (- -/- -), a condition usually incompatible with life.

EPIDEMIOLOGY

More than 270 million people worldwide are carriers of a clinically relevant hemoglobinopathy. One percent of pregnancies are at risk for disease, resulting in 330 000 affected births due to SCD (83%) or thalassemia (17%) each year [2]. Hemoglobin disorders are prevalent in sub-Saharan Africa, throughout Asia, the Middle East, and around the Mediterranean. Population migration from these regions has changed the demographic landscape in North America and Europe, where the carrier rate for these disorders is increasing. In the United States, the rise in previously uncommon thalassemias, such as Hb E/β-thalassemia and Hb H disease, reflects the steady growth in Chinese and South East Asian populations [3]. Formerly comprised of individuals of predominantly Italian and Mediterranean origin, the US β-thalassemia patient population is now 50% Asian. As a result of these demographic shifts, the hemoglobinopathies have become significantly more heterogeneous and complex, both genotypically and phenotypically, with emerging public health implications.

National surveillance data describing the prevalence and patient populations with Hb disorders in the United States are currently lacking. Data derived from newborn screening (NBS) programs estimate the sickle cell carrier frequency at 8-10% in African Americans and 0.6% in Hispanics, with a disease prevalence of 100 000 Americans [4]. Sources of epidemiologic data for thalassemia are limited to clinical networks (ClinicalTrials.gov Identifier: NCT00000623).

Between 2001 and 2011, the California NBS program identified 1,594 newborns, representing 1 : 3300 births, affected with a clinically significant hemoglobin disorder (Table 1). Similar to previous reports, the birth prevalence of SCD has remained stable at 17.4 per 100 000 screened, affecting 1 : 350 Black births and 1 : 2560 Hispanic births [5, 6]. These figures parallel those recently reported in New York state, citing annual incidence rates for SCD of 1 : 230 non-Hispanic Black births and 1 : 2320 Hispanic births [7].

Thalassemic disorders are also prevalent in California, occurring in 12.1 per 100 000 newborns screened during the 10-year period. These disorders affect predominantly Asian populations with an annual incidence of 1 : 1000 births. While births due to β-thalassemia are ethnically diverse, those due to Hb E/β-thalassemia are virtually exclusive to Laotian, Vietnamese and Cambodian populations, with a

Table 1. Birth prevalence rates for hemoglobin disorders identified by the California newborn screening program, 1/2001–12/2010 (n = 5 419 093)

Diagnosis	No. identified	Birth prevalence, per 100 000
Sickle cell disease		
Hb SS	483	8.9
Hb SC	259	4.8
Hb S/beta thalassemia	151	2.8
Hb SE	19	0.4
Hb S/HPFH	14	0.3
Hb S/variant*	12	0.2
	938	17.4
Alpha thalassemia syndromes		
Hb H disease	476	8.8
Hb H Constant Spring	51	0.9
Hb Bart's (hydrops fetalis)	6	0.1
	527	9.8
Beta thalassemia syndromes		
Beta thalassemia	28	0.5
Hb E/beta thalassemia	51	0.9
Hb C/beta thalassemia	29	0.5
Hb D/beta thalassemia	20	0.4
	129	2.3
Clinically significant hemoglobin disorders	1594	29.5

*Includes Hb D, O-Arab, Lepore and other rare hemoglobin variants.

prevalence of 1 : 2600 births among South East Asians. The genotypic heterogeneity of these disorders is illustrated by the broad range of mutations observed in these newborns and parallels the racial and ethnic origins reported by parents (Figure 1, Table 2).

In California, where Asians make up 13% of the population, Hb H disease is the second most common hemoglobinopathy detected by NBS [5]. The majority of cases are South East Asian, with a Laotian, Vietnamese or Cambodian ancestry (40%), and the remaining are Filipino (15%), Chinese (13%), or other Asian (9%). The distribution of Hb H genotypes among California newborns has been previously reported [6, 8]. The majority of cases are caused by deletions in the α-gene locus, with the - -SEA/-α3.7 genotype constituting 61% of cases, followed by the - -FIL/-α3.7 and the - -SEA/-α4.2 constituting another 23% cases. Ten percent of Hb H cases have the more severe Hb H/Constant Spring (Hb H/CS) caused by a point mutation $\alpha^{\text{[Codon 142 TAACAA]}}$ in combination with the - -SEA, - -FIL, or - -Thai deletion. Hemoglobin Constant Spring (Hb CS) is the most prevalent nondeletional α-thalassemia variant worldwide, with gene frequencies reaching 6% in Thailand and parts of South East Asia [9]. Other nondeletional Hb H genotypes, including the α-globin point mutations, $\alpha^{\text{Quong Sze Codon 125 CTGCCG}}$, $\alpha^{\text{Codon 35 TCCCCC}}$, α^{PolyA}, and $\alpha^{\text{Initiation codon ATGAG}}$, were found in 5% of newborns in this subgroup. The relatively high prevalence and more severe clinical phenotype associated with Hb H/CS provided the justification for adding Hb H disorders to California's NBS panel [5, 10].

PRENATAL SCREENING FOR HEMOGLOBINOPATHIES

Increased immigration of high-risk populations has prompted the implementation of prenatal and NBS programs for hemoglobinopathies across Europe and North America. Universal screening programs aimed at detecting carriers and offering prenatal diagnosis in pregnancies at risk for thalassemia have been adopted in Canada, the UK, and other European countries [11–13]. These programs also selectively screen for Hb S and other structural Hb variants in all pregnant women from racial and ethnic groups known to be at increased risk for hemoglobinopathies (Asian, African, and Mediterranean). In the

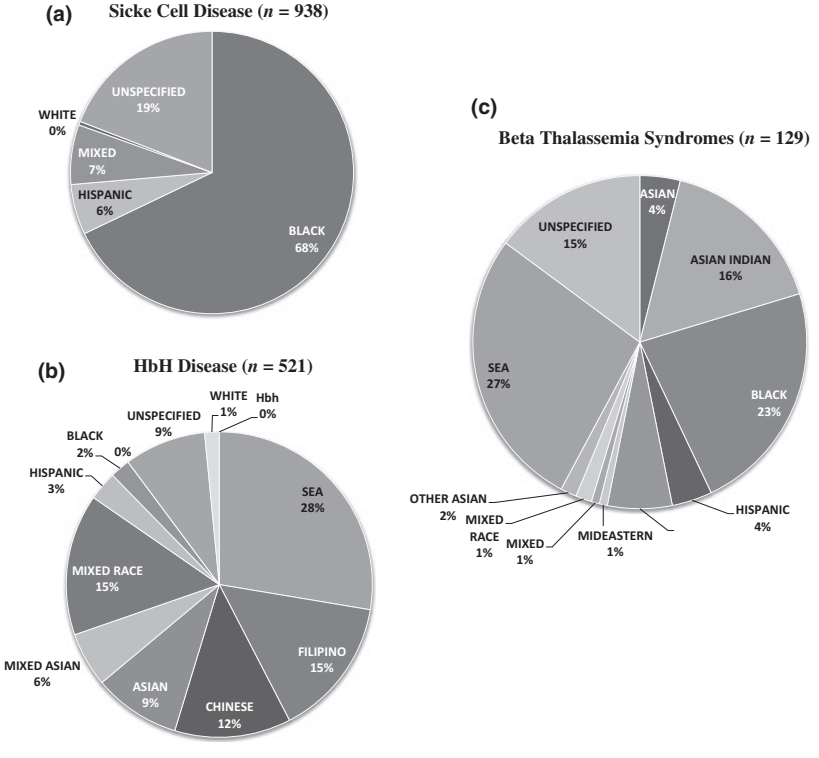

Figure 1. Racial and ethnic breakdown of hemoglobin disorders identified in California newborn population, 2001-2010 (n = 5 419 093). Racial and ethnic breakdown are given for: (a) Sickle cell disease; (b) Hemoglobin H disease; (c) Beta thalassemia syndromes, including beta thalassemia major, Hemoglobin E beta thalassemia and compound heterozygous thalasemias.

Table 2. Distribution of beta thalassemia mutations in CA newborns with a clinically significant beta thalassemia syndrome, 01/2001-09/2011

Mutation	HGVS nomenclature	Type	Population ethnic distribution	No. observed
Transcriptional : promoter regulatory elements				
−101 C>T	HBB:c.−151C>T	β+ (silent)	Mediterranean	2
−90 C>T	HBB:c.−140C>T	β+	Portugese	2
−88 C>T	HBB:c.−138C>T	β+	US Black, Asian Indian	15
−87 C>G	HBB:c.−137C>G	β+	Mediterranean	2
−32 C>T	HBB:c.−82C>T	β0 or β+	Hispanic	2
−29 A>G	HBB:c.−79A>G	β+	US Black, Chinese	44
−28 A>G	HBB:c.−78A>G	β+	US Black, SEA	4
*619 bp del	NG_000007.3:g. 71609_72227del619	β0	Asian Indian	2
RNA processing: splice junction				
IVS I:1 G>A	HBB:c.92+1G>A	β0	Med, Middle East	7
IVS I:1 G>T	HBB:c.92+1G>T	β0	Asian Ind, SEA, Chinese	2
IVS I:2 T>A	HBB:c.92+2T>A	β0	Algerian, Italian	2
IVS I:2 T>C	HBB:c.92+2T>C	β0	US Black, Algerian, Russian	3
IVS II:1 G>A	HBB:c.315+1G>A	β0	Med, US Black	2
RNA processing: consensus splice sites				
IVS I:5 G>C	HBB:c.92+5G>C	β+ Isevere)	Asian Ind, SEA, Melanesian	9
IVS I:5 G>T	HBB:c.92+5G>T	β+ (severe)	Med, N.European	3
IVS I:5 G>A	HBB:c.92+5G>A	β+ (severe)	Med, Algerian	6
IVS I:6 T>C	HBB:c.92+6T>C	β+	Med	2
RNA processing: cryptic splice sites in introns				
IVS I:110 G>A	HBB:c.93-21G>A	β+	Med	13
IVS I:130 G>C	HBB:c.93-1G>C	β0	Korean, Russian, Med	1
IVS II:654 C>T	HBB:c.316-197C>T	β+ (severe)	Chinese, SEA	11
IVS II:745 C>G	HBB:c.316-106C>G	β+	Med	4
IVS II:837 T>G	HBB:c.316-14T>G	β0 or β+	Asian Indian	1
RNA processing: cryptic splice sites in exons				
CD24 T>A	HBB:c.75T>A	β+	US Black	1
CD26 GAG>AAG (HbE)	HBB:c.79G>A	β+	SEA	52
CD27 GCC>TCC (Hb Knossos)	HBB:c.82G>T	β+	Med	1
Poly A (T>C) AATAAA>AACAAA	HBB:c.*+110T>C	β+	US Black	3
RNA translation: nonsense codons				
CD15 G>A	HBB:c.47G>A	β0	Asian Indian	1
CD17 A>T	HBB:c.52A>T	β0	Chinese, Malaysian, Thai, Korean	9
CD35 C>A	HBB:c.108C>A	β0	Thai	1
CD39 C>T	HBB:c.118C>T	β0	Med	4
RNA translation: frameshift				
CD5 –CT	HBB:c.17_18delCT	β0	Med, Asian Indian, Middle East	2

Table 2. (Continued)

Mutation	HGVS nomenclature	Type	Population ethnic distribution	No. observed
CD8/9 +G	HBB:c.27_28insG	β0	Asian Indian, Med, Middle East	8
CD36/37 –T	HBB:c.112delT	β0	Kurdish, Middle East	2
CD41/42 –TTCT	HBB:c.126_129delCTTT	β0	Chinese, SEA, Asian Indian	12
CD47/48, +ATCT	HBB:c.146_147insATCT	β0	Asian Indian	1
CD71/72 +A	HBB:c.216_217insA	β0	Chinese, SEA	3
CD106/107 +G	HBB:c.321_322insG	β0	US Black, Egyptian	4
				243

*Clinically significant disorders include homozygous or compound heterozygous β-thalassemia syndromes.

UK, for example, an initial screen for thalassemia carriers is performed in all pregnant women, and additional testing for sickle cell carriers is targeted to areas with a high prevalence of SCD. In low-prevalence areas, further testing is offered based on reported ancestry (China, South East Asia, Greece, Turkey, Cyprus, or unknown origin) [14]. If the mother is found to be a carrier of a hemoglobinopathy, follow-up genetic counseling and screening of the father are provided. Prenatal diagnostic testing is offered to couples found to be at risk for having an affected child, with the option of termination or continuation of the pregnancy.

Although many European countries advocate for prenatal screening as an effective means to prevent births with clinically significant hemoglobinopathies, there is no such political or social mandate in the United States. Historically, acceptance of prenatal diagnosis among at-risk pregnant women is low and lower yet in pregnancies that are at risk for SCD vis-à-vis thalassemia [15]. Although cultural and social factors play a role, the decision to terminate a pregnancy affected with SCD is influenced by the lack of measurable markers of disease severity and inability to predict prognosis in affected individuals [16].

Prenatal screening thus continues to be offered on a targeted, ad hoc basis in the US Guidelines published by the American College of Obstetrics and Gynecology (ACOG), which recommend that couples at high risk, based on self-reported ancestry, be identified and offered voluntary screening, but provide no consistent screening protocol or policy for practitioners, other than performing a complete blood count (CBC) early in pregnancy [17]. In California, prenatal screening is offered retrospectively, following the birth of an affected child identified through NBS. Counseling and assessment of fetal risk must take into account the ethnic and likely geographic origin of the parents, especially for thalassemias. A questionnaire detailing the social, medical, and family history, modeled after programs such as those implemented in the UK, should be included at the initial prenatal visit to help identify pregnancies at risk.

Carrier testing

Screening for hemoglobinopathies usually begins with a complete blood cell count (CBC) in conjunction with a serum ferritin or free erythrocyte protoporphyrin (FEP) to exclude iron-deficiency anemia. Microcytosis with a mean cellular volume (MCV) <80 fL and/or hypochromia with a mean cellular hemoglobin (MCH) <27 pg and an elevated RBC count suggest a thalassemia mutation or structural variant with thalassemic effects, such as Hb E. Abnormal findings on the CBC should prompt additional testing.

Techniques such as high-performance liquid chromatography (HPLC) or capillary electrophoresis (CE) are performed to quantitate Hb A2 and Hb F in screening for thalassemia and to resolve uncommon hemoglobin variants, such as Hb C-Harlem and Hb D-Punjab. An elevated Hb A2 level (>3.5%) indicates β-thalassemia trait, but levels higher than 10% should raise suspicion for the presence of Hb E (25–30%) or a δβ-thalassemia deletion (15%). Decreased MCV (<80 fL) and/or MCH (<27 pg) with a normal electrophoresis pattern or HPLC result, particularly in individuals of South East Asian or Mediterranean

ancestry, suggests α thalassemia trait. As the MCV and MCH may be normal in individuals who are doubly heterozygous for an α- and β-thalassemia, inclusion of HPLC or CE with the initial CBC should be considered in ethnically diverse populations. In rare cases, with a normal MCV/MCH, normal or reduced Hb A2 levels, and elevated Hb F levels, further testing for δ β-thalassemia and HPFH should be performed.

Electrophoretic techniques, such as isoelectric focusing (IEF) and citrate agar electrophoresis, are used to distinguish the most common Hb variants responsible for SCD. Some uncommon Hb variants (e.g., Hb G, D, and Lepore) will migrate with the same mobility as Hb S on electrophoresis at alkaline pH. As these variants do not cause sickling, a solubility test may be helpful in making this distinction. A sickle solubility test may also be helpful in identifying other less common Hb S variants, such as Hb S-Antilles, that result from a second mutation in addition to the sickle mutation (double heterozygous) in the β-globin gene.

Prenatal diagnostic testing

Prenatal diagnostic testing is offered to couples when screening results indicate a high-risk pregnancy. Although genetic prediction of risk for an affected child with β-thalassemia is relatively straightforward, predicting the risk of having a child with α-thalassemia is more complicated. Alpha-thalassemia trait carriers with a mutation that deletes both α-genes on the same chromosome, that is, in *cis* (- -/αα) are at risk for a pregnancy affected with Hb H disease or Hb Bart's hydrops fetalis. If both parents carry an α-thalassemia deletion in cis, as either heterozygous (- -/α α) or hemoglobin H disease (- -/-α), prenatal diagnosis should be performed. Serial fetal ultrasound assessing the fetal cardiothoracic ratio or Doppler measurement of middle cerebral artery blood flow velocities may also be used to identify an affected fetus. If an abnormality is detected, confirmatory molecular testing of fetal cells should be performed followed by referral to a specialty center for further counseling and management.

Although Hb Barts hydrops fetalis is usually fatal *in utero*, in cases with deletions that spare the embryonic ζ-globin genes (e.g., - -SEA and - -MED), the fetus may survive through ongoing production of embryonic hemoglobins, Hb Gower 1 (ζ2ε2) and Portland 1 (ζ2γ2). Prenatal diagnosis and successful treatment with intrauterine red cell transfusions have led to a reevaluation of the approach to this disorder [18].

Prenatal diagnosis by DNA analysis can be performed using fetal cells obtained by chorionic villus sampling (CVS) or amniocentesis [19]. These techniques are invasive and not without associated risks to the fetus. The recent development of noninvasive prenatal diagnostic testing using cell-free fetal DNA from maternal plasma is currently an area of active investigation as it offers the potential to avoid invasive testing [20, 21]. Another advantage of this approach is that fetal DNA can be isolated from maternal blood as early as 6 weeks of gestation, while CVS is typically performed at 10–12 weeks and amniocentesis at 15–20 weeks of gestation. Various techniques have been applied to isolate and amplify fetal DNA from maternal blood, but obtaining highly purified fetal DNA has been a challenge because of the high background of maternal DNA contamination. A promising method, incorporating pyrophosphorolysis-activated polymerization (PAP), has recently been developed for noninvasive prenatal diagnosis (NIPD) of β-thalassemia major and sickle cell disease (SCD) [22]. The coupling of pyrophosphorolysis and polymerization permits detection of a single allele with a high degree of specificity. Using this technique in 13 test couples screened for informative paternal-specific alleles by melting curve analysis, followed by amplification of fetal DNA with PAP primers targeting these alleles, the paternal allele was successfully identified in all cases.

NEWBORN SCREENING

In the United States, the purpose of NBS for hemoglobin disorders has historically been to identify infants with SCD to prevent life-threatening infection through early initiation of penicillin prophylaxis [23]. While NBS programs outside of the United States intend to identify infants with β-thalassemia as well as SCD, nonsickling hemoglobin disorders are secondary outcomes of NBS in most states [24].

As in other countries, testing for hemoglobinopathies in the United States is performed as part of the existing NBS program for metabolic and other inherited disorders using the initial filter paper blood spot obtained by capillary heel stick. Laboratory services for hemoglobinopathy diagnosis vary significantly

between states in their organizational models, diagnostic testing methods, and referral rates of specimens for molecular characterization of thalassemia and uncommon hemoglobin mutations. The California NBS program uses a two-tiered approach, incorporating both biochemical and molecular methods, to identify both SCD and thalassemic disorders. The general testing and reporting protocol used by the California NBS program is schematically shown in (Figure 2).

Newborn screening methods

Most NBS programs employ high-performance liquid chromatography (HPLC) as the primary screening method to make a presumptive diagnosis of a clinically significant hemoglobinopathy, followed by a complementary method such as IEF or citrate and cellulose acetate electrophoresis to confirm SCD. HPLC is preferred for population screening because it is fully automated and high throughput, accommodating 1500 samples per day with a 2 min per sample run time [25, 26]. In addition to identifying common Hb variants, HPLC permits quantitation of Hb F and Hb Bart's, making it possible to screen for Hb H disease in the newborn period. A threshold of >25% Hb Bart's ensures that all newborns with a suspected diagnosis of Hb H disease are detected and referred for further testing [9]. Several ratio computations of specific HPLC retention times permit the presumptive identification of a thalassemic disorder. For example, a Hb S/Hb A ratio >2.0 is highly suggestive of Hb S/β+ thalassemia rather than Hb AS trait. Primary screening with HPLC can thus help to streamline the follow-up tests needed for the identification of a hemoglobin disorder in the majority of cases. Limitations of HPLC include its inability to distinguish β-thalassemias from benign conditions (e.g., both Hb E/β-thalassemia and Hb EE show an FE pattern by HPLC) or detection of unusual Hb variants.

Confirmatory diagnostic testing

Demographic (age, racial/ethnic background) information and a blood sample from one or both parents are requested to accompany a liquid blood sample from the newborn to help guide the sequence of confirmatory diagnostic tests for specific hemoglobinopathies. Complementary methods such as IEF and electrophoresis are usually sufficient to confirm the diagnosis of SCD, but molecular methods are required to diagnose

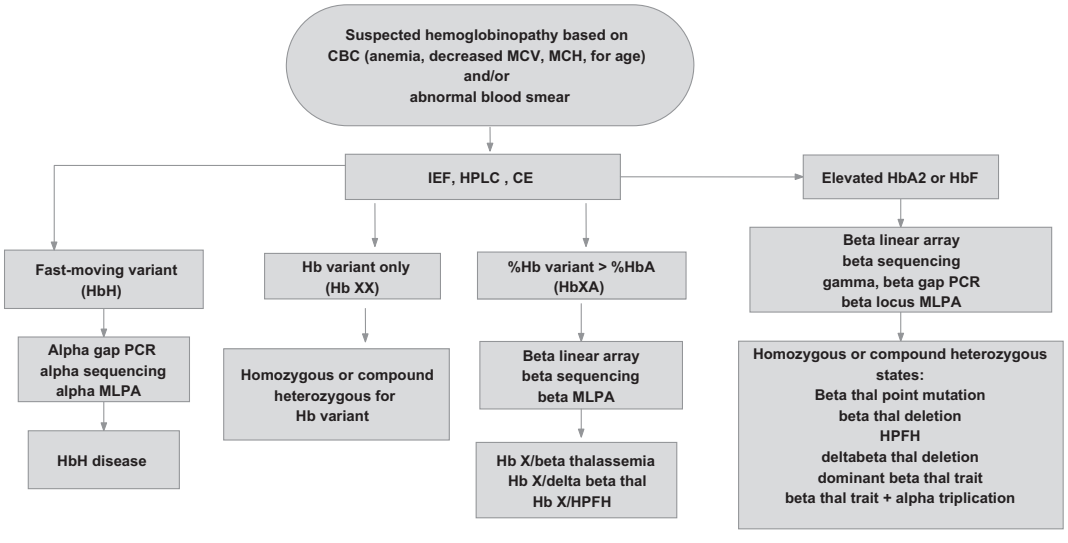

Figure 2. Laboratory investigation for hemoglobinopathies. Simplified flow chart illustrating how hemoglobin variants and mutations can be detected using biochemical and molecular methods. Hb X indicates hemoglobin variant. CE, capillary electrophoresis; HPFH, hereditary persistence of fetal haemoglobin; HPLC, high-performance liquid chromatography; IEF, isoelectric focusing; MCH, mean cell hemoglobin; MCV, mean cell volume; MLPA, multiplex ligation-dependent probe amplification.

a thalassemic disorder. Genotyping for β-thalassemia mutations includes various PCR-based assays, such as primer-specific amplification and reverse oligonucleotide probe hybridization. Gap-PCR is used to test for common α-thalassemia deletions or duplications, as well as HPFH-1, HPFH-2, HPFH-3, HPFH-7, and Hb Lepore deletions. Direct DNA sequencing will identify point mutations in the γ-, α-, and β-globin genes, but may miss mutations that are within introns or distal to the gene locus, as well as large deletions. Large β-globin locus deletions account for only a very small number of β-thalassemia mutations, but are the most difficult to detect because gap-PCR relies on knowledge of the deletion breakpoints. In such cases, multiplex ligation-dependent amplification (MLPA) may be used to determine the presence of an unidentified α- or β-globin gene deletion, by assessing DNA copy number changes [27]. This is followed by long-range sequencing to define the deletion breakpoints. Comparative genomic hybridization (CGH), in which long oligonucleotide (60 bp) probes are spotted or 'tiled' on an array, is a relatively new method to identify deletion breakpoints and DNA copy number with high resolution [28].

Reporting and follow-up

California's NBS program for hemoglobinopathies includes a follow-up strategy that incorporates a web-based screening information system and regional coordinating centers to track positive results and ensure timely enrollment of infants into a comprehensive treatment program [29]. Clinical interpretation and patient referral recommendations are sent together with the confirmatory results to submitting providers and clinical care centers. Consultation and published guidelines for the comprehensive care of infants, children, and adolescents are provided to genetic counselors, nurse practitioners, and physicians [30].

Although NBS has improved the prognosis for individuals with Hb disorders in the United States, there are ongoing problems with timely follow-up and implementation of comprehensive care. Wide variation in reporting of screening results by states has led to delays in early intervention [31]. Other states have reported gaps in compliance with early medical intervention, parental education, and provision of comprehensive health services [32]. These problems are compounded by the increasing number of patients who are underinsured or uninsured due to cutbacks in public healthcare spending. The decline in reimbursement for services from public insurance programs has become an obstacle to providing adequate clinical care and education to affected patients. New strategies are needed to minimize existing practice variations across states and ensure appropriate delivery of health care, despite limited resources, to this growing population of patients.

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