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A Hemoglobin Variant Associated with Neonatal Cyanosis and Anemia

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SUMMARY

Globin-gene mutations are a rare but important cause of cyanosis. We identified a missense mutation in the fetal G γ -globin gene (*HBG2*) in a father and daughter with transient neonatal cyanosis and anemia. This new mutation modifies the ligand-binding pocket of fetal hemoglobin by means of two mechanisms. First, the relatively large side chain of methionine decreases both the affinity of oxygen for binding to the mutant hemoglobin subunit and the rate at which it does so. Second, the mutant methionine is converted to aspartic acid post-translationally, probably through oxidative mechanisms. The presence of this polar amino acid in the heme pocket is predicted to enhance hemoglobin denaturation, causing anemia.

Hemoglobin abnormalities, including those resulting from globin-gene mutations, cause a variety of clinical symptoms, including cyanosis.¹ Hemoglobin is a tetramer of two α -like and two β -like globin protein chains, each of which binds one oxygen molecule through an associated heme prosthetic group. The α -like and β -like globin-gene loci are developmentally regulated² (the β -like cluster is shown in Fig. 1A). The predominant globins expressed in the fetus and neonate are α and γ , which combine to form hemoglobin F ($\alpha_2\gamma_2$). Postnatally, globin expression shifts from γ to β , producing hemoglobin A ($\alpha_2\beta_2$). Thus, physiologically significant mutations in γ -globin genes (*HBG1* or *HBG2*) cause symptoms in the fetus and neonate that gradually abate in the first months of life. Four known γ -globin mutants are associated with neonatal cyanosis: hemoglobins Fort Ripley, Osaka, Cincinnati, and Circleville (each named for the patient's city of origin).^{4–8} Mutations in the α -globin genes (*HBA1* or *HBA2*) can also cause neonatal cyanosis, although in such cases symptoms persist throughout life.⁹ In contrast, mutations in the β -globin gene (*HBB*) that cause cyanosis are manifested a few months after birth, as the switch from γ -globin to β -globin ensues. We characterized a new γ -globin (*HBG2*) mutation, hemoglobin Toms River, in a neonate with cyanosis and anemia.

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CASE REPORT

A full-term female infant weighing 2825 g was born by vaginal delivery to a 20-year-old woman who had been pregnant for the second time. The infant had Apgar scores of 8 at both 1 minute and 5 minutes and was described as a “happy blue baby” — that is, cyanotic but well appearing. Initial hemoglobin oxygen saturation, measured in ambient air with the use of pulse oximetry, was 30 to 50%, and the partial pressure of arterial oxygen (PaO₂) was 107 mm Hg. After intubation and delivery of 100% oxygen, hemoglobin saturation fluctuated around 85%, and the arterial PaO₂ reached 369 mm Hg. The physical examination revealed only cyanosis and moderate hepatomegaly. The infant was extubated, with a transition to oxygen delivery by means of nasal cannula. She was clinically well, although hemoglobin oxygen saturation remained low, at 80 to 90%, despite the absence of evidence of arterial hypoxia. Laboratory data (Table 1) were notable for moderate anemia with reticulocytosis. The methemoglobin level was normal, and the erythrocyte morphologic features were unremarkable. The results of chest radiography and echo-cardiography were normal. On the first day of life, electrophoresis performed with cellulose acetate and agarose gel showed that total hemoglobin consisted of approximately 90% hemoglobin F and 10% hemoglobin A, with no variant bands. The infant received erythrocyte transfusions, which raised her hemoglobin oxygen saturation from approximately 80% to more than 90%. She was discharged home at 6 days of age, with oxygen saturation in the range of 90 to 95%. Her clinical course was unremarkable, and by 2 months of age, her hemoglobin oxygen saturation was consistently higher than 95%.

The patient’s father had also had transient neonatal cyanosis, with hemoglobin oxygen saturation of approximately 80%, despite the use of supplemental oxygen and adequate arterial oxygenation (Table 1). Extensive testing for infections and metabolic abnormalities was unrevealing. His cyanosis resolved within 1 to 2 months, and he was subsequently healthy.

METHODS

DNA SEQUENCE ANALYSIS

Experimental details of the DNA sequence analysis are provided in the Supplementary Appendix, available with the full text of this article at NEJM.org.

PRODUCTION AND ANALYSIS OF RECOMBINANT HEMOGLOBIN PROTEINS

Recombinant hemoglobin F was produced with the use of the plasmid pHE9 provided by Shen and coworkers.¹⁰ Experimental details are provided in the Supplementary Appendix and have been described previously.¹¹ The hemoglobin proteins were then analyzed with the use of mass spectroscopy.

FERRICYANIDE-MEDIATED HEME OXIDATION

Erythrocyte lysates were diluted to a final heme concentration of 25 μ M in 10 mM HEPES buffer (pH 7.4). After the addition of an equimolar mixture of potassium ferricyanide, spectral changes between wavelengths of 400 to 700 nm were recorded at room temperature at 1-second intervals with the use of a spectrophotometer with photodiode array (PiStar-180, Applied Photophysics). Heme concentrations were calculated by means of spectral deconvolution to known standards for deoxyhemoglobin, oxyhemoglobin, and methemoglobin.

RESULTS

The clinical history suggested an inherited fetal hemoglobinopathy, which we confirmed through DNA sequencing (Fig. 1A). The patient and her affected father were found to be heterozygous for a novel γ -globin (*HBG2*) missense mutation (Fig. 1B). A substitution of guanine for adenine at nucleotide position 202 (202G→A) replaces valine with methionine at codon 68, or amino acid 67, in the post-translationally processed γ -globin chain product and is referred to here as γ -globin V67M. This represents the 11th amino acid of γ -globin helix E (i.e., E11) with the thiol ether side chain in the oxygen-binding pocket (Fig. 1C). We named this mutant hemoglobin Toms River, after the infant's birthplace. The larger E11 methionine is expected to fill the back portion of the ligand-binding pocket and sterically impair oxygen uptake and binding to the heme iron (Fig. 1C). An analogous E11 valine-to-methionine missense mutation in the β -globin gene (*HBB*) results in an unstable hemoglobin molecule (hemoglobin Bristol-Alesha), with consequent congenital hemolytic anemia.^{12–17} In this mutant hemoglobin A, the β -E11 methionine is converted post-translationally to aspartic acid, probably through oxidative mechanisms.¹⁴ The results of mass spectrometry were consistent with these features, showing mutant γ -globin chains with methionine and aspartic acid at position E11 in hemolysate from the infant with the hemoglobin Toms River mutation but not in the hemolysate from a neonatal control, in which valine was at the E11 position (data not shown).

To study the biochemical consequences of the hemoglobin Toms River mutation, we used an *Escherichia coli* expression system to synthesize recombinant hemoglobin F ($\alpha_2\gamma_2$) in which both γ -chains contain the E11 V67M substitution.¹⁸ Oxidation of the side chain was prevented by storage of the protein in complex with carbon monoxide. The mutant hemoglobin F was produced at yields similar to those for wild-type hemoglobin F. Thus, the V67M substitution does not appear to affect γ -globin synthesis, stability, or assembly with α -globin in *E. coli*. Initial studies indicated that the oxygenated ($\alpha_2\gamma^{V67M}_2$) hemoglobin tetramer was not excessively prone to oxidation, heme loss, or denaturation, as compared with wild-type hemoglobin F.

We used partial laser photolysis and rapid mixing methods to measure the association (k'_{O_2}) and dissociation (k_{O_2}) rate constants for the last step of oxygen binding to individual globin subunits in wild-type and V67M γ -hemoglobin F tetramers^{11,19} (Table 2). These parameters reflect how quickly oxygen is released (k_{O_2}) and taken up (k'_{O_2}) by each subunit at high levels of oxygen saturation, and the ratio provides an estimate of the dissociation equilibrium constant for oxygen binding to the high-affinity allosteric form of hemoglobin (i.e., $P_{50} = k_{O_2}/k'_{O_2}$). The γ -V67M subunits take about 25 times as long as the wild-type subunits to bind oxygen and carbon monoxide (for the values of k'_{O_2} and k_{O_2} , see Table 2). Similar decreases in k'_{O_2} caused by the replacement of valine (E11) with large apolar amino acids have also been observed in mammalian myoglobin and β subunits of hemoglobin A^{20–22} as a result of the filling of the back portion of the ligand-binding pocket, which inhibits oxygen capture. In contrast, the γ -globin V67M mutation has little effect on the rate of oxygen release (k_{O_2}) by hemoglobin F. Consequently, the increase in P_{50} (approximately 12 μ M, or 7 mm Hg) in the mutated γ subunit is 30 times as high as that in wild-type subunits in partially saturated hemoglobin, which exists in a high oxygen-affinity conformation (often termed the R quaternary state).^{20–22} On complete deoxygenation of hemoglobin F, the switch to the low oxygen-affinity conformation (the T quaternary state) is expected to increase the P_{50} of each hemoglobin F subunit by a factor of about 200.²³ Thus, the fully deoxygenated γ -V67M subunit could have a P_{50} as high as 2400 μ M, or 1400 mm Hg, which would markedly inhibit oxygen uptake by hemoglobin F Toms River in the lungs or placenta, accounting for the presence of cyanosis in newborns with this mutation.

Analysis of the infant's intact erythrocytes and soluble hemoglobin at 6 days of age revealed a normal P_{50} for oxygen binding (Table 1), presumably because the percentage of mutant γ -globin was low (16.3%) after erythrocyte transfusions and increased endogenous erythropoiesis, which accelerates the switch from γ -globin to β -globin. However, the blood sample obtained when the patient was 6 days old was characterized by an abnormally increased initial rate of methemoglobin production after incubation with ferricyanide (Fig. 2), indicating the presence of a small fraction of hemoglobin molecules with high rates of oxygen dissociation and auto-oxidation, a finding that would be expected (as indicated by mass spectroscopy) if some γ -V67D subunits were present. This biochemical abnormality resolved by the time the infant was 6 months of age, when the switch from γ -globin to β -globin was complete.

DISCUSSION

Mutations in the γ -globin gene are an uncommon but important cause of neonatal cyanosis. Clinical clues include a positive family history and reduced hemoglobin oxygen saturation without arterial hypoxemia^{1,7} in an infant who otherwise appears to be healthy and has no evidence of heart or lung disease. The reason for establishing this diagnosis is to offer accurate genetic and prognostic counseling that will "provide adequate reassurance and prevent iatrogenic misadventures that might arise under the mistaken impression that the patient has a cardiac or pulmonary disorder."⁹

Four previously described γ -globin mutations cause neonatal cyanosis, either by inhibiting the binding of oxygen to ferrous (Fe^{2+}) hemoglobin or by promoting its spontaneous oxidation (auto-oxidation) to ferric (Fe^{3+}) methemoglobin, which cannot bind oxygen.^{4,5,8} These variants are referred to as M hemoglobins. The infant's level of methemoglobin was normal, and recombinant hemoglobin F containing γ -V67M exhibited only slightly increased rates of auto-oxidation and heme loss, as compared with wild-type hemoglobin F (data not shown). These data suggest that when methionine is present at the E11 position, hemoglobin Toms River is relatively stable and remains primarily in the reduced state. Only oxygen uptake is considerably impaired (Table 2). The observation that substitution of the E11 valine for methionine has little effect on the stability of the related protein myoglobin²⁴ is consistent with these findings.

Unlike the four other hemoglobin variants that cause neonatal cyanosis, hemoglobin Toms River affects amino acid E11 of γ -globin. Because this position localizes to the distal ligand-binding pocket, nonconservative substitutions are likely to have functional consequences. Rees et al.¹⁴ summarized five hemoglobin variants with amino acid substitutions at the E11 position in β -globin that cause cyanosis or hemolytic anemia. Although the presenting feature of hemoglobin Toms River was neonatal cyanosis, both affected patients (father and daughter at birth) also had moderate anemia and reticulocytosis, suggesting that the mutant hemoglobin is also unstable. The equivalent mutation in β -globin (E11 V67M, hemoglobin Bristol-Alesha) causes congenital hemolytic anemia, with no reports of associated cyanosis. In both hemoglobin Bristol-Alesha and hemoglobin Toms River, the mutant E11 methionine is gradually converted to aspartic acid post-translationally, probably through oxidative mechanisms (as noted above and also reported by Rees et al.¹⁴). Generation of a highly polar side chain of aspartic acid at the E11 position of γ subunits is expected to markedly enhance both auto-oxidation and globin denaturation, on the basis of studies of myoglobin in which valine E11 is replaced with a highly polar asparagine²⁴⁻²⁷ and studies in patients with hemoglobin Bristol-Alesha.¹⁴

Considering these findings, we speculate that the substitution of methionine for valine at E11 in patients with the hemoglobin Toms River mutation mainly causes cyanosis.

Subsequent conversion of the mutant methionine to aspartic acid destabilizes the hemoglobin tetramer, resulting in hemolysis and anemia without the accumulation of soluble methemoglobin. Differences in phenotypes between patients with hemoglobin Bristol-Alesha (which is mainly hemolytic) and hemoglobin Toms River (mainly cyanotic) may depend on the relative rates at which the mutant E11 methionine is converted to aspartic acid. These rates are probably controlled by ambient oxygen concentrations, oxidant exposure, and intrinsic differences between the γ -globin and β -globin chains carrying the analogous mutation at position E11. Hemoglobin Bristol-Alesha probably produces a more severe phenotype because the mutation is present in one of two β -globin genes, whereas in our patient with the hemoglobin Toms River the mutation was present in one of four γ -globin genes (Fig. 1A). It will be informative to determine the conditions that promote the conversion of the E11 amino acid to aspartic acid and the effects this change has on the properties of recombinant hemoglobin F Toms River. The identification of this mutation and subsequent laboratory studies illustrate how unusual disorders caused by “experiments of nature” offer unique opportunities to gain a better understanding of both medicine and biology.²⁸

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. The Hemoglobin Toms River Mutation

Panel A depicts the human β -globin gene cluster on chromosome 11. Affected persons are heterozygous for a transition from guanine to adenine (G→A) at nucleotide position 202 of the G γ -globin gene. This switch substitutes methionine (Met) for valine (Val) at codon 68, or amino acid 67, after the post-translational cleavage of methionine at position 1. The mutant methionine residue is converted to aspartic acid (Asp) post-translationally. Valine is at position 11 in α -helix E of γ -globin. Panel B depicts the hemoglobin Toms River pedigree, with squares representing male members and circles female members and shading indicating members with neonatal cyanosis and anemia. The individual G γ -globin genotypes for each person are indicated. Panel C shows the structure of the oxygen-binding pockets of the γ -globin subunits in persons with wild-type (left image) and mutant (right image) V67M deoxygenated hemoglobin F. The wild-type subunit shows valine at position 67; the other subunit shows the predicted structure of the Toms River mutation, in which methionine has been inserted into the wild-type structure at position 67. In both images, the γ subunits are depicted as yellow ribbons. The planar heme group and its associated proximal histidine are depicted with red and yellow stick models, respectively. The iron atom is shown as an orange sphere. Oxygen enters the heme pocket in the wild-type subunit (indicated by the blue arrow) and binds iron on the upper face of the heme ring next to the valine 67 side chain. In the mutant subunit, the residues at position 67 are depicted as spheres, with blue indicating the additional methionine atoms that prevent oxygen from having access to the iron atom in the center of the heme ring. The insets show wild-type valine and mutant methionine in stick and dot format to indicate the difference in size between the amino acids.

Figure 2. Rates of Ferricyanide-Induced Methemoglobin Formation in the Patient and a Control Blood samples from the patient with the hemoglobin (Hb) Toms River mutation were analyzed when she was 6 days old and again when she was 6 months old. Erythrocyte lysates were diluted to a final heme concentration of 25 μM in 10 mM HEPES buffer (pH 7.4). The change in heme spectra was monitored after the addition of 25 μM of potassium ferricyanide, which oxidizes ferrous (Fe^{2+}) Hb to ferric (Fe^{3+}) methemoglobin. The initial rate of oxidation was more rapid when the patient was 6 days old. Similar results were obtained at a range of ferricyanide concentrations. HbA indicates data derived from purified hemoglobin A, which was used as an additional control.

Table 1

Laboratory Data for the Patient and Her Father as Neonates.*

Variable	Normal Range	Patient		Patient's Father
		1 Day of Age	6 Days of Age	
Hemoglobin (g/dl)	13.5–19.0	11.9	11.3	11
Reticulocytes (%)	2–6	33.4	9.3	20
Hemoglobin quantification (%) [†]				Not measured
Hemoglobin F		≈90	16.3	
Hemoglobin A		≈10	81.2	
Hemoglobin A2			2.5	
Oxygen saturation in ambient air (%)	>95	80–90	≈90	85
Lactate dehydrogenase (U/liter)	Not measured	Not measured	Not measured	6258
Total bilirubin (mg/dl)	0–11.9	2.5	1.7	22 (maximum) [‡]
Methemoglobin (%)	0–2.8	2.3	Not measured	Not measured
Antiglobulin				
Direct	Negative	Negative	Not measured	Negative
Indirect	Negative	Negative	Not measured	Negative
Chest radiograph	Normal	Normal	Not obtained	Normal
Echocardiogram	Normal	Normal	Not obtained	Normal
Blood and CSF cultures	Negative	Negative	Not performed	Negative
Hemoglobin–oxygen affinity (P ₅₀) [§]	Not measured	Not measured	Normal*	Not measured

*CSF denotes cerebrospinal fluid.

[†]Hemoglobin quantification was performed with the use of cellulose acetate and agarose electrophoresis on the patient's 1st day of life and of high-pressure liquid chromatography on the 6th day of life.

[‡]"Maximum" refers to the highest value for bilirubin reached during the father's neonatal period.

[§]Oxygen hemoglobin dissociation curves were performed on intact erythrocytes with the use of a HEMOX Analyzer (TCS Scientific). The P₅₀ value represents the concentration of oxygen for half saturation (Kd) for the high-affinity state of the hemoglobins. The patient's average P₅₀ value was 29.1±0.3 mm Hg, which is similar to that in controls. However, this analysis was performed when the patient was 6 days old, after she had received multiple transfusions. At this time, the patient's hemoglobin F fraction was only 16.3%.

Rate and Equilibrium Constants for Oxygen Binding to High-Affinity Forms of the α , β , and γ Subunits of Hemoglobin F (Fetal) and Hemoglobin A (Postnatal) at pH 7.0, 20°C.*

Table 2

Hemoglobin Subunit	Variant	$k'_{O_2} \mu M^{-1} s^{-1}$	$k_{O_2} s^{-1}$	$P_{50} (k_{O_2}/k'_{O_2}) \mu M$	$k'_{CO} \mu M^{-1} s^{-1}$
Hemoglobin F ($\alpha_2\gamma_2$)					
α	Wild type	40	12	0.30	4.0
γ	Wild type	88	37	0.42	7.0
γ	V67M	3.6	43	12	0.22
Hemoglobin A ($\alpha_2\beta_2$) [†]					
α	Wild type	29±11	14±8	0.48±0.11	4.0±1.1
β	Wild type	60±12	31±13	0.53±0.15	7.0±2.0

* Plus-minus values are means ±SD. P50 is the concentration of oxygen for half saturation (Kd) for the high-affinity state of the hemoglobins. The constant k' O2 represents the bimolecular association-rate constants for oxygen binding, kO2 is the unimolecular-rate constant for oxygen dissociation, and k' CO is the bimolecular-rate constant for carbon monoxide binding.

[†] Parameters for wild-type recombinant hemoglobin A tetramers are from Birukou et al. 11