Hemoglobin Hirose: $\alpha_2\beta_2$37(C3) Tryptophan Yielding Serine

By KOTARO YAMAOKA

During an electrophoretic screening survey for hemoglobinopathies in western Japan, a slow-moving variant of hemoglobin A, to be designated hemoglobin Hirose, was found in a family of Japanese origin. Chemical characterization of hemoglobin Hirose revealed that tryptophan at the 37th position of the β-chain was replaced by serine, the third residue of C-helix of the β-chain involving contacts between α_1 and β_2 subunits. Even though the oxygen equilibrium of this hemoglobin was abnormal, none of the family members showed any clinically significant symptoms.

IN AN EXTENSIVE SURVEY of hemoglobinopathies in western Japan, conducted during the period 1957–1968, 20 structural and synthetic variants of hemoglobin were discovered.[1] The present paper is concerned with hematologic findings and the chemical characterization of a structural variant of the β-chain, to be designated hemoglobin Hirose, that was found in a family residing in an urban area of Yamaguchi Prefecture in 1965.

MATERIALS AND METHODS

Hemoglobin solutions were prepared by the method of Drabkin.[2] Thin-layer starch gel electrophoresis of the hemolysate containing hemoglobin at a concentration of 10% was carried out at 20 V/cm for 2 hr by the method of Baur[3] with a pH 8.6 Tris-EDTA-borate buffer,[4] and the gels were stained with Amido Black 10B (Merck). For identification of the affected chain, hybridization experiments were performed by the technique of Gammack et al.[5] with some modifications. Alkali-resistant hemoglobin was determined by the method of Singer et al.[6] Heinz body formation test was done by the method of Huehns et al.[7] The heat denaturation test was performed by the methods of Dacie et al.[8] or Huehns et al.[7] These heat denatured hemolysates were checked with 540/280-mμ ratio[9] by spectrophotometer (Hitachi 101). Structural characterization of the hemoglobin was first carried out by isolating the abnormal component on column chromatography using carboxymethyl cellulose according to the procedures described by Huisman and Meyering.[10] The abnormal component was eluted with a buffer of pH 7.37 containing 0.01 *M* Na_2HPO_4, 0.01 *M* $NaHPO_4$, and 0.01 *M* potassium cyanide. The eluted fractions were concentrated by ultrafiltration in vacuo using cellophane tubes (Visking 8/32), and the purity of each peak was tested on thin-layer starch gel electrophoresis as described. The chain from the purified hemoglobin was isolated by the procedure of Clegg et al.[11] with a column of carboxymethyl cellulose, 8 *M* urea, and 2-mercaptoethanol. Excess reagents were removed by passing the appropriate fractions through a column of Sephadex G-25 using 0.5% formic acid. The samples were not aminoethylated. The β-chains so obtained were hydrolyzed with trypsin (Trypsin-TCA Worthington) for 90 min in ammonium bicarbonate volatile buffer.[12]

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Tryptic peptides were separated by two-dimensional peptide mapping (fingerprinting) using the isoamylalcohol-acetic acid-water (35:35:30) solvent of Baglioni,[13] followed by electrophoresis at pH 6.4 with a pyridine-acetic acid-water (10:0.4:90) buffer[14] in a lucite tank containing Varsol Number 3 with a potential gradient of 50 V/cm. The dried peptide maps (fingerprints) were rendered visible with 0.2% ninhydrin in water-saturated butanol or by tert-butyl hypochlorite[15] and submitted to the usual color tests for arginine, tyrosine, tryptophan, histidine, and sulfur containing amino acid alone or in combinations.[16] Quantitative amino-acid analyses of the peptides were made after hydrolysis in an evacuated tube with constantly boiling HCl at 100°C for 20 hr with a Hitachi KLA-3A analyzer. Tryptophan was determined by the specific reaction for tryptophan and by measuring ultraviolet absorption spectra of the β-chain solutions between 270 and 300 mμ[17] with the Beckman DK-2 recording spectrophotometer.

Results

Family Studies

Figure 1 gives analyses of the hemoglobins obtained from the proposita and her relatives. It is noted that over three generations ten individuals of this kindred possess this hemoglobin trait which appears to be transmitted through the paternal side. The proposita is a 36-yr-old housewife who has apparently been in good health and showed no clinically significant abnormality.

Hematologic Findings

Red cell count: 480 $\times$ 10^4/cu mm; reticulocytes: 0.9%; hemoglobin: 14.4 g/dl; hematocrit: 43%; no morphologic abnormality of the red cells was noted; white cell count: 6400/cu mm; leukocyte analysis: normal; osmotic fragility: normal; thrombocytes: 16 $\times$ 10^4/cu mm; no hemorrhagic tendency was observed. Other family members possessing this hemoglobin showed no abnormal symptoms or findings (Table 1).

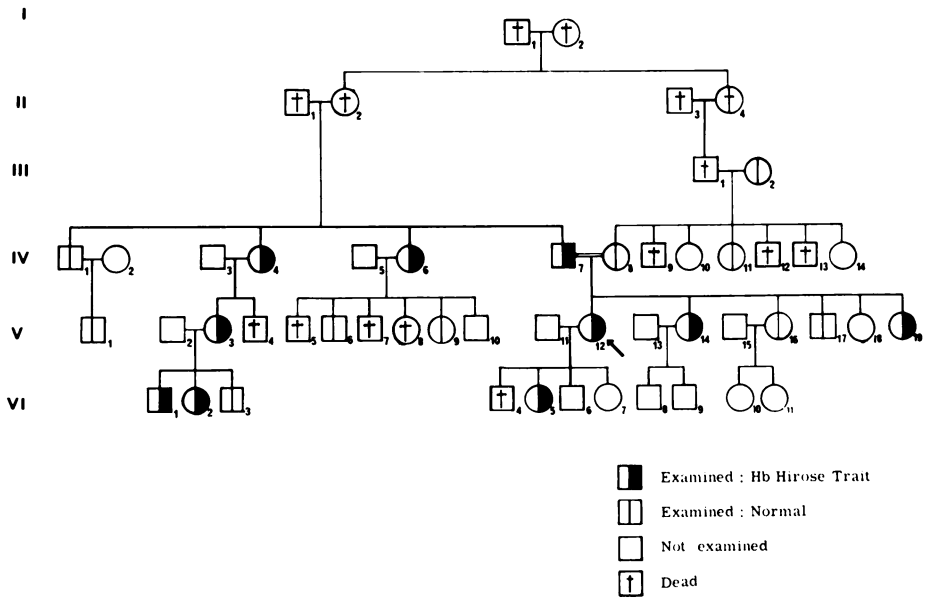

Fig. 1.—Pedigree of hemoglobin Hirose trait. Arrow indicates proposita.

Table 1.—Hematologic Findings of Hemoglobin Hirose's Family*

Pedigree Carriers of Hb Hirose	Sex	Age	Hemoglobin (g/dl)	RBC (× 10^4/cu mm)	Hematocrit (%)
IV-4	F	71	9.9	308	38
IV-6	F	67	10.2	404	35
IV-7	M	64	13.8	452	46
V-3	F	50	10.5	328	38
V-12	F	36	14.5	480	43
V-14	F	34	12.0	310	37
V-19	F	20	Not examined		
VI-1	M	26	12.8	410	38
VI-2	F	24	12.9	490	39
VI-5	F	13	Not examined		
Mean			12.1	398	39
Normal complement of Hb A					
III-2	F	79	12.9	460	40
IV-1	M	75	13.7	465	37
IV-8	F	58	11.8	371	42
IV-11	F	46	12.7	475	37
V-1	M	17	13.8	495	38
V-6	F	45	13.0	436	46
V-9	F	35	12.0	345	37
V-16	F	32	10.9	380	37
V-17	M	28	12.7	478	44
VI-3	M	16	14.5	495	40
Mean			12.8	440	40

* No significant differences between hemoglobin Hirose carriers and noncarriers. All are in good health.

Characterization of Hemoglobin

Figure 2 shows the abnormal component of this hemoglobin on thin-layer starch gel electrophoresis at pH 8.6 in comparison with hemoglobin A, cord blood hemoglobins, and hemoglobin S. Hemoglobin Hirose migrates slower than hemoglobin A and hemoglobin F but faster than hemoglobin S. The

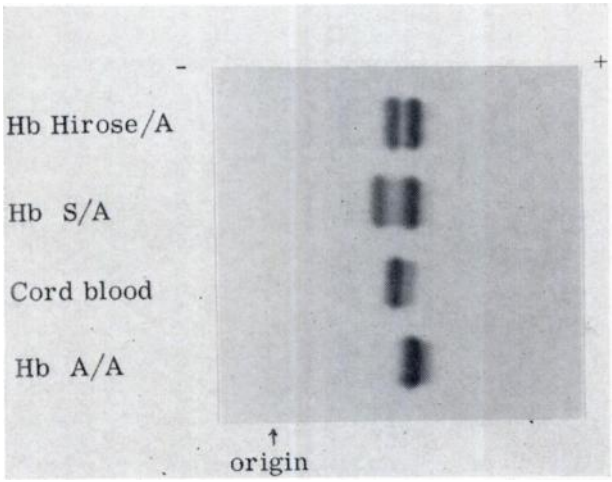

Fig. 2.—Thin-layer starch-gel electrophoresis of hemolysates of hemoglobin Hirose, hemoglobin S, cord blood and normal adult hemoglobin, tris-EDTA-borate buffer, pH 8.6, stained with Amido Black 10B.

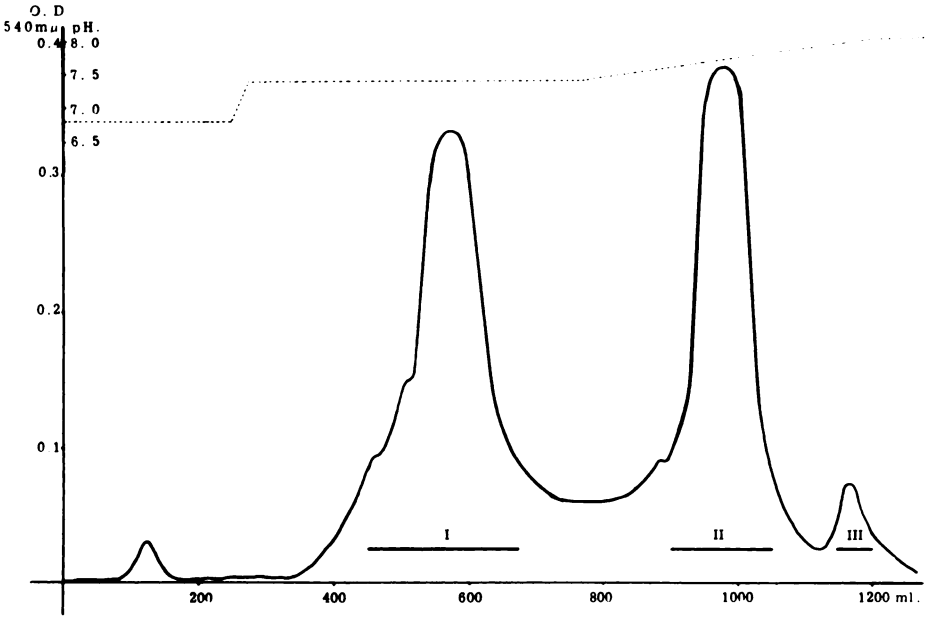

Fig. 3.—Separation of hemoglobin A (peak I), A_2 (peak III), and hemoglobin Hirose (peak II) on column chromatography of carboxymethyl cellulose with 0.01 *M* phosphate buffer.

abnormal component comprised 41.4% of the total hemoglobin, and A_2 fraction was estimated to be 2.3% on the chromatogram of hemoglobin Hirose (Fig. 3).

Hybridization tests of purified hemoglobin Hirose with canine hemoglobin revealed that the electrophoretic mobility of this hemoglobin can be attributed entirely to a charge difference in the β-chain. The ratio of alkali denaturation test and Heinz body formation test was within normal limits. The heat denaturation test incubating the hemolysates at 50°C for 3 hr or 2 min at several temperatures from 37°C to 100°C showed no differences between hemoglobin Hirose and normal adult hemoglobin. The ratio of heme to globin 540/280-mμ ratio of each hemolysate before and after heat denaturation was normal. The α- and β-chains of purified hemoglobin Hirose were separated clearly on a carboxymethyl cellulose column chromatography (Fig. 4).

Peptide Analysis

The peptide map of the β-chain of hemoglobin Hirose differed from that of the β-chain of hemoglobin A only in the following points (Fig. 5): the peptide β Tp IV (Ingram 14) is missing and an unusual spot is observable anodally to peptide β Tp II (Ingram 12) in hemoglobin Hirose. Because the normal peptide β Tp IV spot is frequently undetectable by ninhydrin straining, the presence of β Tp IV of hemoglobin A and the absence of β Tp IV of hemoglobin Hirose were ascertained by the reaction of tert-butyl hypochlorite which is more sensitive with peptides of larger molecular weight.

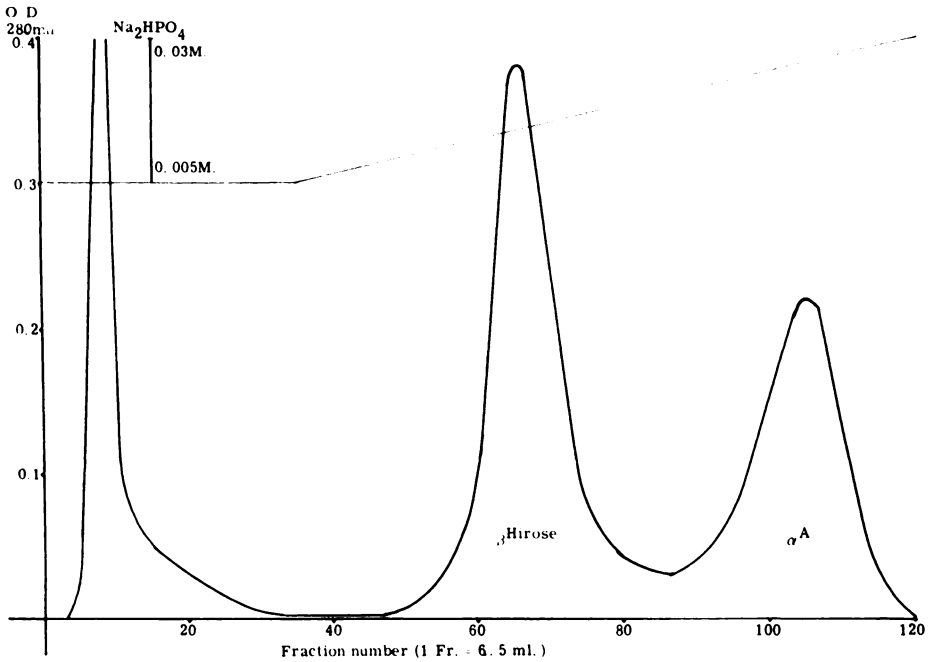

Fig. 4.—Separation of α-chain and β-chain from globin of hemoglobin Hirose on column of carboxymethyl cellulose with 8 *M* urea-2-mercaptoethanol buffer (pH 6.7).

Table 2 gives the amino acid analysis of the abnormal peptide and β Tp IV expected in numbers of residues of hemoglobin A. The amino acid sequence of the latter β^A Tp IV is Leu (31)-Leu-Val-Val-Tyr-Pro-Try-Thr-Gln-Arg(40). In the abnormal peptide, on the other hand, one serine residue appears and the other amino acid composition is identical with that of β^A Tp IV. Since the tryptophan residue of β-37 is completely destroyed during acid hydrolysis of the peptide, this point should be confirmed by the specific reaction for tryptophan. Proser's evaluation of the staining for tryptophan in the new spot of hemoglobin Hirose was negative; β Tp IV of hemoglobin A, however, was apparently positive for tryptophan. Absence of one of the two tryptophan residues in the β-chain of hemoglobin Hirose was recomfirmed by the ultraviolet absorption spectra. It may thus be noted that the tryptophan fine structure band at 289 mμ of the affected β-chain is not prominent as compared with that of hemoglobin A (Fig. 6). These findings suggest that only a single tryptophan residue is present in hemoglobin Hirose, compared with two residues in the normal β-chain.

Discussion

Hemoglobin Hirose is characterized by the replacement of tryptophan by serine located at the third residue of C-helix of the β-chain. The formula of hemoglobin Hirose can therefore be designated as $\alpha_2\beta_2$37(C3)Try→Ser (Fig. 7).

The substitution of serine for tryptophan has not been described before.

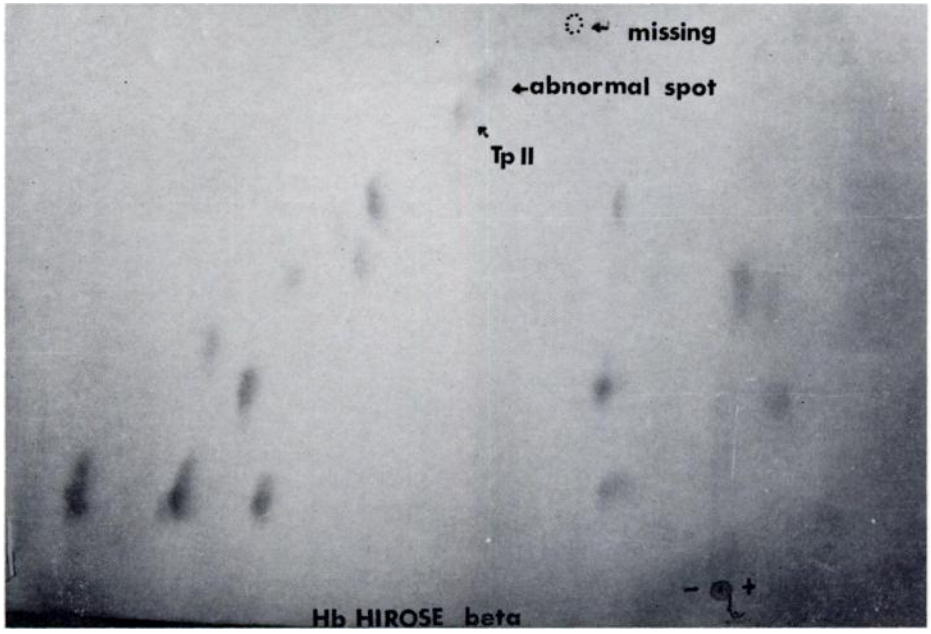

Fig. 5.—Peptide chromatogram of isolated hemoglobin Hirose β-chain with 0.2% ninhydrin staining. Peptide β Tp IV (dotted line) was missing and new abnormal spot (arrow) was visible anodally to peptide β Tp II.

Just as the amino acid substitution variants that have been previously reported from a single location involve single nucleotide substitutions, this structural alteration can also be explained by the substitution of a single nucleotide base in the DNA or mRNA codon.

Hemoglobin Hirose is clearly distinguishable from hemoglobin A by electrophoresis even though the substitution involves uncharged amino acids of the β-chain. This might either be a result of the loss of heme from the affected chain or the result of changing the polarity, size, and ionization of various

Table 2.—Comparison of Amino Acid Sequence of β Tp IV of Hemoglobin A With That of Abnormal Peptide of Hemoglobin Hirose

Amino Acid	Hb Hirose Abnormal Peptide		Hb A β Tp IV Expected Number of Residues
	μmoles	Number of Residues	
Leu	0.027	1.9	2
Val	0.024	1.7	2
Tyr	0.013	0.9	1
Pro	0.014	1.0	1
Try	*	*	1
Ser	0.014	1.0	0
Thr	0.014	1.0	1
Glu	0.015	1.1	1
Arg	0.009	0.7	1

* Tryptophan residue is completely destroyed in acid hydrolysis. Presence of tryptophan residue in β Hirose Tp IV could not be detected by Ehrlich's reagent or by ultraviolet absorption spectra.

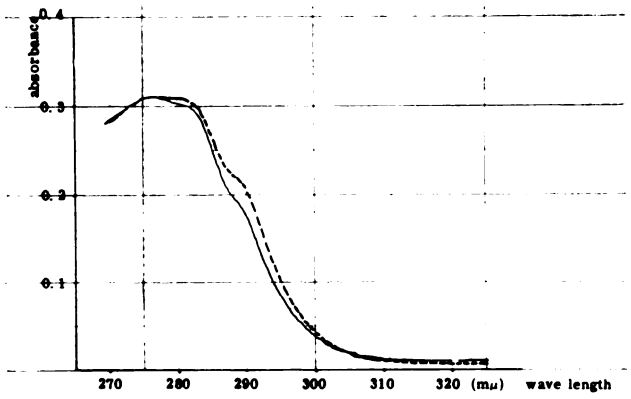

Fig. 6.—Ultraviolet absorption spectra of β-chain in 0.5% formic acid solution. Tryptophan notch at 289 mμ compared in β Hirose (solid line) and β A (dotted line).

adjacent amino acid residues. The loss of heme, however, is not observed in hemoglobin Hirose. The polarity of an amino acid side chain is one of the principal factors determining the conformation of the globin molecule. The size of an amino acid side chain may also be responsible for the configuration and stability of hemoglobin. Configuration and stability could thus depend on the molecular weight or volume of the side chain. According to Epstein,[18] the polarity values of side chains of serine and tryptophan redidues are assigned as 0.8 and 0.2, respectively, and the relative size of side chain of serine residue is 0.10 compared to the tryptophan value of 0.50. The substitution of serine for tryptophan at the third residue of C-helix of the β-chain seems to cause a stereochemical change and a relative change in polarity, resulting in the difference of +2 net charge per molecule.

Hemoglobin Hirose B37(C3) tryptophan→serine involves a replacement at the $\alpha_1\beta_2$ contact. In the atomic model of hemoglobin, the tryptophan residue at the third residue of C-helix of the β-chain contacts an arginyl residue at the fourth position of FG-helix of the α-chain which affects heme-heme interaction via the $\alpha_1\beta_2$ contact.[19-21] Therefore the mutational alteration as seen in hemoglobin Hirose may result in aberrations in the function of the hemoglobin molecule.

Fujita[22] determined the oxygen equilibrium of hemoglobin Hirose at pH 6.0, 6.5, 7.0, 7.4, and 7.9 by means of the oxygen electrode and continuous recording spectrophotometry.[23] The principal results showed that P_{50} for isolated hemoglobin Hirose was 2.6 mm Hg compared with 11.0 mm Hg for hemoglobin A at pH 7.0. The Hill constant, n, was 1.49 for hemoglobin Hirose compared with 2.90 for hemoglobin A at pH 7.0 and 20°C. Hemoglobin Hirose is thus

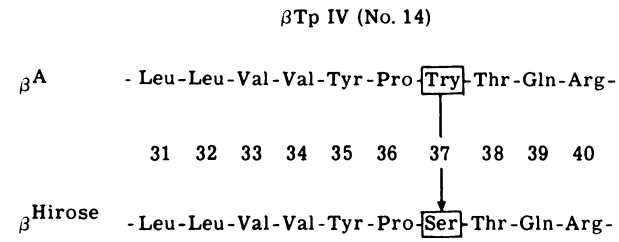

Fig. 7.—Amino acid sequences of β Tp IV (residue 31–40) of hemoglobin A and hemoglobin Hirose.

Table 3.—Hemoglobin Variants Characterized by Substitution of $\alpha_1\beta_2$ Contact, Increased Oxygen Affinity and Decreased Heme-Heme Interaction

Designation	Amino Acid Substitution	Oxygen Affinity	Heme-Heme Interaction	Bohr Effect	Contact Affected	Clinical Symptoms
Chesapeake	FG4(92)α Arg → Leu	High	n = 1.3	Normal	α_1-β_2	Polycythemia
J Cape Town	FG4(92)α Arg → Gln	High	n = 1.9	Normal	α_1-β_2	Mild polycythemia
Yakima	G1 (99)β Asp → His	High	n = 1.0	Normal	α_1-β_2	Polycythemia
Kempsey	G1 (99)β Asp → Asn	High	n = 1.1	Normal	α_1-β_2	Polycythemia
Hirose	C3 (37)β Try → Ser	High	n = 1.49	Decreased	α_1-β_2	None

characterized by a high oxygen affinity, decreased heme-heme interaction, and reduced Bohr effect. Hemoglobin Chesapeake[11,24] and hemoglobin J Cape Town[25,26] each involve a substitution at the fourth residue of the FG-corner of the α-chain; both involve changes in the $\alpha_1\beta_2$ contact. In individuals having these hemoglobins characterized by a high oxygen affinity and decreased heme-heme interaction, a polycythemic syndrome is manifested. Polycythemia was also observed in individuals with hemoglobin Yakima[27,28] and hemoglobin Kempsey.[29] This resulted from substitutions in the first residue of the G-helix also involving the $\alpha_1\beta_2$ contact (Table 3).

The fact that no clinical or hematologic symptoms are present in persons having hemoglobin Hirose may imply that there are other unknown factors in the manifestation of polycythemic syndromes. Because the structure of normal human hemoglobin results from a long evolutionary process, it is presumed that structural mutations, particularly those showing physicochemically significant abnormalities such as hemoglobin Hirose, tend to lead more or less to morbid conditions if any unfavorable changes occur in the environment.

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