ularly in countries where a significant proportion of HH patients carry a wild type HFE gene (such as Italy and Greece), and for further characterizion of HH cases carrying only one mutated HFE allele.

Maria Pissia, Katerina Polonifi, Marianna Politou, Konstantinos Lilakos, Nikos Sakellaropoulos, George Papanikolaou

First Department of Internal Medicine, School of Medicine, National and Kapodistrian University of Athens

Funding: This study was supported by the National and Kapodistrian University of Athens (Grant 70/4/5901 to N.S.).

Keywords: iron, juvenile hemochromatosis, HFE 2.

Correspondence: Marianna Politou, MD, PhD, 23-27 Makrigianni st, 117-42 Athens, Greece. E-mail: mpolitou@hotmail.com

References

1. Cazzola M, Cerani P, Rovati A, Iannone A, Claudiani G, Bergamaschi G. Juvenile genetic hemochromatosis is clinically and genetically distinct from the classical HLA-related disorder. Blood 1998; 92:2979-81.
2. Papanikolaou G, Politou M, Roetto A, Bosio S, Sakelaropoulos N, Camaschella C, et al. Linkage to chromosome 1q in Greek families with juvenile hemochromatosis. Blood Cells Mol Dis 2001; 27:744-9.
3. Lanzara C, Roetto A, Daraio F, Rivard S, Ficarella R, Simard H, et al. The spectrum of hemojuvelin gene mutations in 1q-linked juvenile hemochromatosis. Blood 2004;[Epub ahead of print].
4. Lee PL, Beutler E, Rao SV, Barton JC. Genetic abnormalities and juvenile hemochromatosis: mutations of the HJV gene encoding hemojuvelin. Blood;2004 Feb 24 [published online].
5. Roetto A, Papanikolaou G, Politou M, Alberti F, Girelli D, Christakis J, et al. Mutant antimicrobial peptide hepcidin is associated with severe juvenile hemochromatosis. Nat Genet 2003; 33:21-2.
6. Papanikolaou G, Samuels ME, Ludwig EH, MacDonald ML, Franchini PL, Dube MP, et al. Mutations in HFE2 cause iron overload in chromosome 1q-linked juvenile hemochromatosis. Nat Genet 2004;36:77-82.
7. Papanikolaou G, Politou M, Terpos E, Fourlemadis S, Sakellaropoulos N, Loukopoulos D. Hereditary hemochromatosis: HFE mutation analysis in Greeks reveals genetic heterogeneity. Blood Cells Mol Dis 2000;26:163-8.
8. Beutler E, Felitti VJ, Koziol JA, Ho NJ, Gelbart T. Penetrance of 845G→A (C282Y) HFE hereditary haemochromatosis mutation in the USA. Lancet 2002;359:211-8.

Red Cell Disorders

Hb Gun Hill: a further *de novo* observation

Here we report the third observation (the second *de novo*) of unstable Hb Gun Hill or [β91(F7)-95(FG2)Leu-His-Cys-Asp-Lys→0]. The two-year old male carrier showed low mean corpuscular hemoglobin (MCH) and mean hemoglobim concentration (MCHC), 8.5% fetal hemoglobin and ≈30% variant hemoglobin. Mild hemolytic symptoms were detected seven years later. DNA sequencing and functional studies of mRNA and globin chains were performed.

haematologica 2004; 88:743-745
(*http://www.haematologica.org/journal/2004/6/743)*

Hb Gun Hill [β91(F7)-95(FG2)Leu-His-Cys-Asp-Lys→0] has been described at the protein level in a North-European family[1] and as a *de novo* mutation in a black American female.[2] The variant is due to the deletion of five amino acids from the final three residues of F-helix to the first two residues of the FG corner.[1] Because of the duplication of Leu-His at codons 91-92 and 96-97 the deletion breakpoints remain ambiguous. The five-residue deletion causes a profound rearrangement of the heme pocket, which impairs heme binding[1] and leads to molecular instability. The variant hemoglobin (Hb) shows lower absorption in visible wavelengths, and consequently a lower total Hb value,[1-3] because there are only two heme groups per molecule (linked to the α-chains). Moreover, the alteration of the molecular structure causes functional alterations such as increased oxygen affinity, decreased cooperativity and absence of the Bohr effect.

A two-year old boy from Catania, Sicily, was found to have a low MCH and MCHC associated with slight anisocytosis, slight reticulocytosis but without microcytosis (Table 1). Hematologic and metabolic parameters were normal. Cation-exchange high performance liquid chromatography (HPLC) (Diamat, Bio-Rad, Richmond, CA, USA) revealed increased levels of Hb F (8.5%) and the presence of an abnormal Hb absent in the parents, which was eluted after the Hb A2 and constituted about 11% of the total Hb (Figure 1A and Table 1). On cellulose acetate electrophoresis at pH 8.4 the Hb variant was slightly slower than Hb A2 (Figure 1B). Indeed, reverse-phase HPLC[4] and detection at 220 nm showed that the β^{var} accounted for about 30% of total β-chains. Heat and isopropanol tests were positive. A high number of inclusion bodies in the erythrocytes were detected after 24 hrs of exposure to brilliant cresyl blue at 37°C, but were absent after two hours of incubation. These findings suggested the hypothesis of an unstable Hb.

DNA sequencing of the β-globin gene revealed heterozygosis for Hb Gun Hill due to a deletion of 15 nucleotides (Figure 1C) resulting in a β-globin chain variant with the deletion of five amino acids. To evaluate the possible role of mRNA defects in the reduction of Hb variant (30% vs. 50%), β-globin mRNA was analyzed with semi-quantitative reverse transcription polymerase chain reaction (RT-PCR). The cDNA labeled with (α-^{32}P)dCTP was separated by polyacrylamide gel electrophoresis; normal or mutated homodimers and two heterodimers were obtained. The radioactivity of the mutated and wild type homodimer bands was comparable (Figure 1D).

In vitro biosynthesis of the globin chains with ^{3}H-leucine[4] was carried out at 30, 60, 90 and 120 minutes. The β and α chain biosynthetic ratio was balanced, (cpm β^{A}+ β^{var}/mg β^{A}+ β^{var})/(cpm α/mg α)= 1.06±0.11, but the specific activity of $\beta^{Gun\ Hill}$ was about 2.3 times that of β^{A}. This finding was explainable only by instability of the variant and its rapid breakdown, as reported for several unstable β-chain variants.[5,6]

At the age of nine years the propositus presented with moderate hemolysis (very low haptoglobin) and reticulocytosis. The Hb variant with cation-exchange HPLC was about 16%, Hb F was 5.5% and the bilirubin level was normal. The patient showed reticulocytosis, high levels of lactate dehydrogenase and low levels of haptoglobin but had no jaundice, splenomegaly or hepatomegaly (Table 1). These symptoms were milder than those described in the two other patients with Hb Gun Hill[3] who had hemolytic anemia with increased indirect bilirubin and one also had scleral jaundice and splenomegaly (Table 1). This difference could be because our patient is younger than the other two. The mutation in our patient was *de novo*. Paternity was confirmed by analysis of blood groups and RFLP haplotypes[7] in the β-globin gene cluster (Table 1). Two duplicated

Table 1. Hematological data of the family under study and of the patients reported in literature.[2,3]

	Father	*Mother*	*Son (proband)*		*Patient*[2]	*Father*[3]	*Daughter*[3]
Age (years)	52	47	2	9	25	41	10
RBC ($\times 10^{12}$/L)	5.06	4.64	5.36	5.18	5.30	5.68	4.44
Hb (g/L)	154	127	116 (136[a])	117	114 (137[a])	135 (162[a])	126 (151[a])
MCV (fL)	91	85	81	82	76	85	101
MCH (pg)	30	27	22 (26[a])	22	nr	24 (28[a])	29 (34[a])
MCHC (g/L)	333	322	267 (314[a])	270	nr	280 (340[a])	280 (340[a])
Hb A2 (%)	2.2	2.2	3.0	4.3	nr	nr	nr
Hb F (%)	0.3	0.8	8.5	5.5	2.8	2.4	2.4
Hb Gun Hill (%)	–	–	11 (30[b])	16	22-25 (31-35[a])	21 (30[a])	20 (29[a])
Retic. ($\times 10^9$/L)	81	130	260	518	nr	227-341	311-444
Iron (μg/dL)	100	46	78	75	nr	nr	nr
Ferritin (μg/L)	193	4.1	25	32	nr	nr	nr
Transferrin (mg/dL)	373	504	300	290	nr	nr	nr
LDH (IU/L)	159	177	180	729	nr	nr	nr
Bil. ind. (mg/dL)	0.92	0.85	0.30	0.76	nr	2.7	2.0
Bil. total (mg/dL)	0.98	0.94	0.55	0.94	nr	2.8	2.3
Haptogl. (mg/dL)	323	88	57	<5.8	nr	24	nr
F.E.P. (μg/g Hb)	1.4	2.8	1.8	1.2	nr	nr	nr
S. T. R. (μg/mL)	1.5	3.1	3.3	nd	nr	nr	nr
Heinz body	–	–	nd	+[c]	+	+	nr
Urinary pigments	nd	nd	–	–	nr	–	nr
Blood group	0 Rh+	A Rh +	A Rh+				
Haplotypes[d]	I/I	III/R[e]	I/III				
Spleen	nor	nor	nor	nor	nr	3 cm[f]	nor
Scleral icterus	–	–	–	–	nr	+	-

[a]At 540 nm, the Hb Gun Hill had an extinction coefficient about half that of the Hb A because of the lack of the heme groups. The numbers in parentheses indicate approximate Hb values after correction for the variant Gun Hill percentage. [b]This value was obtained with reverse-phase HPLC and detection at 220 nm.[c]In the sample but not in one control Heinz bodies were detected in large numbers after 24 hrs of exposure to brilliant cresyl blue at 37°C, but were absent after two hours of incubation. [d]The RFLP analyzed in the β-globin gene cluster are Hind III/$^{G}\gamma$, Hind III/$^{A}\gamma$, Hinc II/ψβ and 3' ψβ, Ava II/β and Bam HI/3'β. Haplotypes were determined through family linkage analysis and classified according to Orkin et al.[7] [e]The haplotype indicated with R was (+ - - - /+ +). [f]Below the costal margin Bil.: bilirubin; Bil. Ind.: indirect biluribin; Haptogl.: haptoglobin; F.E.P.:free erythrocyte protoporphyrin; L.D.H.: lactate dehydrogenase; S.T.R.: soluble transferrin receptor; nd: not done; nr : not reported; nor: normal; -: absent; +: present.

sequences at codons 90/92 and 95/97 (5'-AGCTGCAC-*TGTGACA*-AGCTGCAC-3') can mediate a slipped-mispairing,[6,8,9] or less probably an unequal crossing over leading to the deletion. Moreover, semi-palindromic sequences such as that of 6 nucleotides with 1 mismatch at codons 92/98 (5'-CAC(T)GTG-*ACAAGCTG*-CACGTG-3') could favor the formation of an intrastrand stem-loop structure, followed by the synthesis of anomalous sequences.[10]

To conclude, of the three known cases of the unstable Hb Gun Hill this is the second due to a *de novo* mutation. The high frequency of *de novo* events leads us to define the mutation as *recurrent* and indicates that the mutation occurs in a region definable as a deletion hot spot. It is likely that the Hb Gun Hill as well as other variants which impair binding with the heme group should be hypothesized in all patients showing a low MCH and MCHC (due to decreased absorption of the Hb variant) associated with a normal mean corpuscular volume (MCV) and mild hemolytic symptoms.

Maria De Angioletti, Giuseppina Lacerra,* Rosario Testa,° Angela Flagiello,# Gino Schilirò,° Clementina Carestia**

**Istituto di Genetica e Biofisica "A. Buzzati Traverso", Naples; °Dipartimento di Pediatria, Università degli Studi di Catania; #Dipartimento di Chimica Organica e Biochimica, Università di Napoli Federico II, Naples, Italy*

Key words: Hb Gun Hill, unstable hemoglobin, de novo mutation, β-globin gene.

Funding: The study was supported by grants from the Ministero dell' Istruzione, Università e Ricerca (MIUR), Law 488/92 Cluster C02, PE2.

Correspondence: Clementina Carestia, MD, Istituto di Genetica e Biofisica "Adriano Buzzati Traverso", CNR, Via G. Marconi 10-12, 80125, Naples, Italy. Phone: international +39.081.7257241. Fax: international +39.081.7257243. E-mail: carestia@iigb.na.cnr.it

References

1. Bradley TB Jr, Wohl RC, Rieder RF. Hemoglobin Gun Hill: deletion of five amino acid residues and impaired heme-globin binding. Science 1967;157:1581-3.
2. Murari J, Smith LL, Wilson JB, Schneider RG, Huisman TH. Some properties of hemoglobin Gun Hill. Hemoglobin 1977;1:267-82.
3. Rieder RF, Bradley TB Jr. Hemoglobin Gun Hill: an unstable protein associated with chronic hemolysis. Blood 1968;32:355-69.
4. Pagano L, Lacerra G, Camardella L, De Angioletti M, Fioretti G, Maglione G, et al. Hemoglobin Neapolis, β126(H4)Val→Gly: a novel β-chain variant associated with a mild β-thalassemia phenotype and displaying anomalous stability features. Blood 1991; 78:3070-5.
5. Rieder RF. Synthesis of hemoglobin Gun Hill: increased synthesis of the heme-free β^{GH} globin chain and subunit exchange with a free α-chain pool. J Clin Invest 1971;50:388-400.
6. Bunn FH, Forget BG. Human hemoglobin variant. In Dyson J, editor. Hemoglobin: molecular, genetic and clinical aspects. Philadelphia PA: WB Saunders Company; 1986. p. 412-5.
7. Orkin SH, Kazazian HH, Antonarakis SE, Goff SC, Boehm CD, Sexton

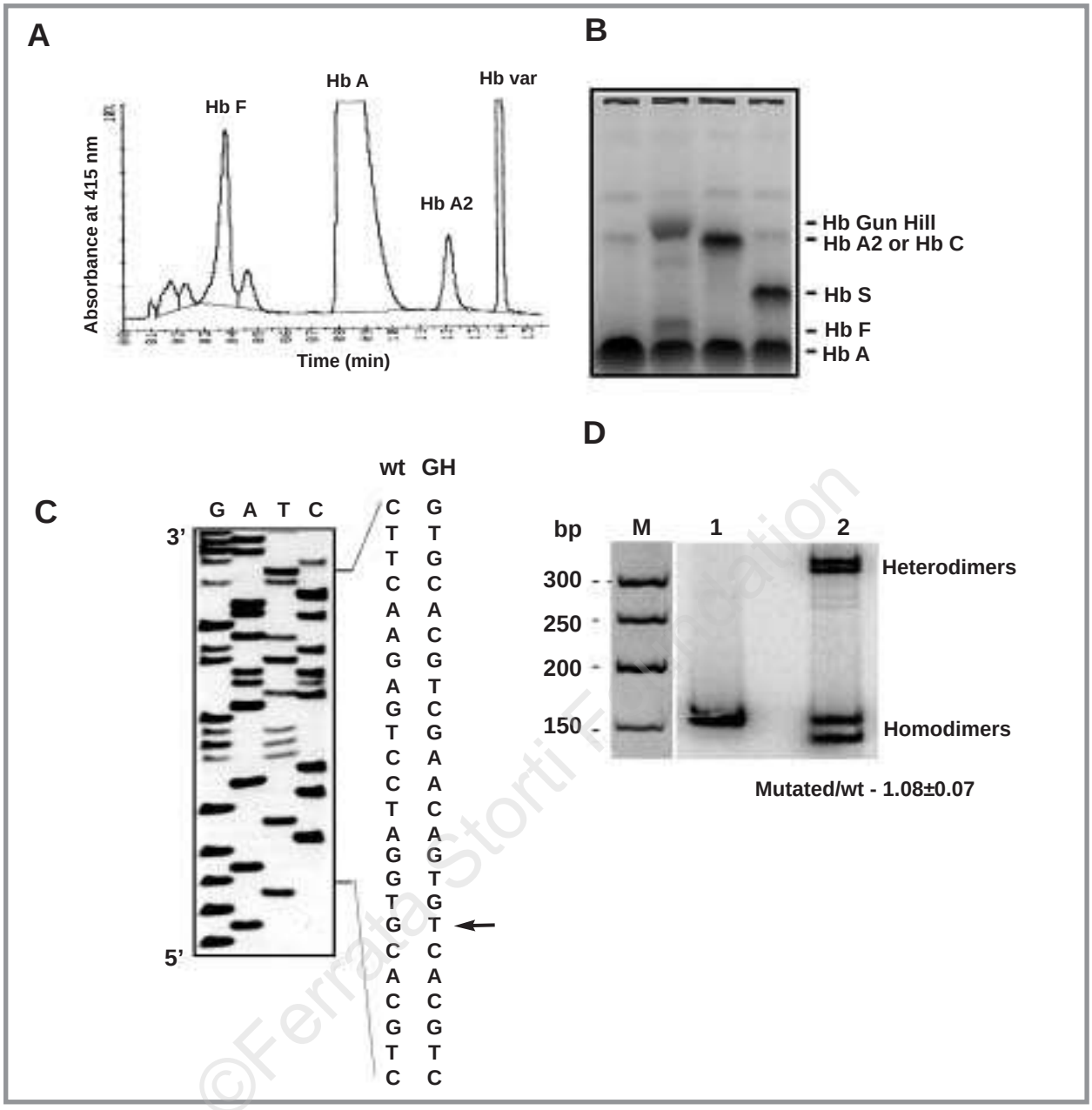

Figure 1. A) Cation exchange HPLC of the hemoglobins in the proband. B) Cellulose acetate gel electrophoresis: 1: father; 2: patient; 3: Hb C carrier; 4: Hb S carrier. C) Sequence analysis of the coding strand of the β-globin gene of the patient. The wild type (wt) and mutated (GH) DNA sequences are indicated. An arrow indicates the first nucleotide in heterozygosis. D) Semi-quantitative analysis of the mRNA by radioactive RT-PCR. Total RNA was isolated from reticulocyte-enriched peripheral blood cells with Triazol (Life Technologies, NY, USA). The cDNA was synthesized from the RNA using Moloney Murine Leukemia Virus Reverse Transcriptase and an Oligo d(T) primer (GeneAmp, Perkin Elmer, and Foster City, CA, USA). cDNA was amplified with 20 PCR cycles using (α-^{32}P)dCTP and the following primers: GTGCCTTTAGTGATGGCCTG and CTTTGCCAAAGTGATGGGCCAGC (+390/+409 and +1394/+1372 relative to the β-globin cap site). The cDNA samples were run on a 7.5% polyacrylamide gel at 150 V for 1.5 hours. Four bands were obtained and identified by sequencing as normal (156 bp) or mutated (141 bp) homodimer or heterodimer bands. The ratio between radioactive mutated/wild type (wt) homodimer bands was quantified using a phospho-imager with ImageQuant software (Molecular Dynamics, Sunnyvale, CA, USA). A 50bp ladder is shown. 1: normal control; 2: patient.

JP, et al. Linkage of β-thalassaemia mutations and β-globin gene polymorphisms with DNA polymorphisms in human β-globin gene cluster. Nature 1982;296:627-31.
8. Streisinger G, Okada Y, Emrich J, Newton J, Tsugita A, Terzaghi E, et al. Frameshift mutations and the genetic code. Cold Spring Harb Symp Quant Biol 1966; 31:77-84.
9. Efstratiadis A, Posakony JW, Maniatis T, Lawn RM, O'Connell C, Spritz RA, et al. The structure and evolution of the human β-globin gene family. Cell 1980;21:653-68.
10. Glickman BW, Ripley LS. Structural intermediates of deletion mutagenesis: a role for palindromic DNA. Proc Natl Acad Sci USA 1984;81:512-6.