Disorders of Iron Metabolism

Prevalence of the G320V mutation of the HJV gene, associated with juvenile hemochromatosis, in Greece

Mutations of the HJV gene, which maps on chromosome 1q21, underlie most cases of juvenile hemochromatosis. We evaluated the frequency of the most common mutation (G320V) of the HJV gene in the Greek population, since 50% of cases of hereditary hemochromatosis in Greece carry mutations of the HJV gene.

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Juvenile hemochromatosis (JH) is a rare autosomal recessive disorder of iron overload. JH is genetically and clinically distinct from HFE-related hereditary hemochromatosis (HH). Juvenile hemochromatosis is characterized by severe iron accumulation early in life (second decade). Hypogonadotrophic hypogonadism and cardiac failure are predominant complications.[1] The majority of the reported cases of JH originate from Greece, Italy and Saguenay-Lac-Saint-Jean (Quebec, Canada).[2,3] JH is genetically heterogeneous. The genetic defect that underlies a small subset of JH cases is mutations of the HAMP gene (the gene encoding the iron regulating peptide, hepcidin), while the majority of the cases are due to mutations of the HJV gene on chromosome 1q21.[5,6] The HJV gene encodes hemojuvelin, a protein of unknown function expressed in the liver, cardiac and skeletal muscles. Mutation analysis of 14 Greek patients with 1q linked JH, revealed six different mutations (I222N, G320V, I281T, C361fsX366, G99V and R326X).

The majority of the mutations appear to be private but, exceptionally, the G320V mutation was observed in 10 of 14 patients analyzed. The mutation was identified in probands deriving from different ethnic backgrounds (Greek, French and Canadian).[6] Recently,the G320V mutation was found in an Italian homozygote, likely with Greek ancestry, originating from a restricted geographical area in Southern Italy, where Greek traditions and language are still alive.[3] Whether this could be attributed to the historically documented migration of Greeks in Southern Italy remains an issue for further exploration.[2] The G320V mutation accounted for all cases of JH in Saguenay-Lac-St-Jean, due to a founder effect and inbreeding.[3] Furthermore, G320V was found in compound heterozygosity with I222N mutation in a JH patient originating from central Alabama. The national origins of the grandparents of this patient were stated to be Irish and native American.[4] Comparative haplotype analysis of Greek patients indicates that the G320V mutation occurred in a common ancestral haplotype.[6]

Given the wide geographic distribution of the Greek families carrying the mutation and the exclusion of consanguineous marriages in some of them, we suggested that G320V mutation might be more common among Greeks than initially thought. We, therefore, performed a genotype screening of 200 unrelated apparently healthy blood donors of Greek origin.

The 959 G⟶T transvertions, that leads to a G to V substitution at position 320 of the encoded protein (G320V), abolishes a recognition site for BanI. Therefore, G320V mutation was detected by a simple polymerase chain reaction (PCR)-restriction fragment length polymorphism (RFLP) method. A part of exon 4 of the HJV gene was amplified by PCR using the following primers: forward: 5'-AAC CAT GTG GAG ATC CAA GC-3', reverse: 5'-CTC CAG TGC TGC CTG AGC-3', generating a 333 bp product. Digestion of the normal product with BanI generates two fragments, 146bp and 187 bp. As expected, homozygosity for the mutant G320V allele results in only one fragment (333bp), while heterozygosity results in three (333, 187, 146bp) (Figure 1). Unfortunately, the genomic sequence of HJV lacks a second, in proximity, recognition site for BanI to serve as an internal control. Thus, it is advisable that positive results are confirmed by a second method (i.e. direct sequencing) to avoid misinterpretation of partially digested fragments.

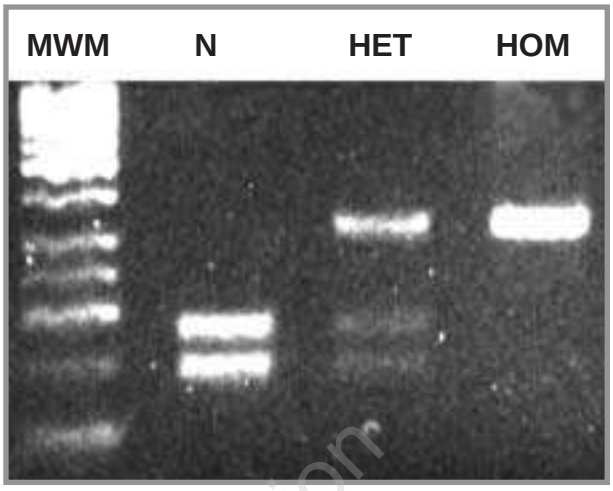

Figure 1. Detection of the G320V mutation of the HFE2 gene after digestion of the PCR product with BanI. MWM: molecular weight marker (50bp DNA ladder); N: normal; HOM: homozygous; HET: heterozygous.

None of the 400 chromosomes studied carried the G320V mutation, indicating that the frequency of the mutant G320V allele in the Greek population is lower than that (0.004) of the C282Y mutant allele of the HFE gene.[7]

Interestingly, we have previously reported that almost 50% of hereditary hemochromatosis patients of Greek origin have 1q linked juvenile hemochromatosis.[7] G320V homozygous HJV-associated JH is represented almost equally with C282Y homozygous HFE-related hemochromatosis in Greek patients, despite the much lower frequency of the former mutant allele in the control group. The penetrance of C282Y homozygosity is estimated to be very low.[8] Though the number of HH patients in this report is limited, our observation could be attributed to the incomplete penetrance of C282Y homozygosity compared to the high penetrance of the G320V homozygous genotype. Accordingly, we failed to identify non-expressing G320V homozygous subjects through genotype screening of relatives of 6 unrelated families with at least one G320V homozygous affected proband, an observation indicating that the penetrance of G320V homozygosity is high.

The PCR-RFLP assay described here for the detection of the G320V mutation of the HJV gene could prove a valuable supplement in the molecular diagnosis of HH, partic-

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.

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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.

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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