SCREENING FOR C282Y, H63D AND S65C ALLELES BY HFE STRIP ASSAY TEST ON PATIENTS WITH ABNORMAL FERRITIN LEVELS, IRON OVERLOAD AND TRANSFERRINE LEVEL

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ABSTRACT

Hereditary haemochromatosis (HH) is common disease among Caucasians; reported disease frequencies vary from 0.3 to 0.8%. Genetic analysis of HH was performed by screening of HFE mutations C282X and H63D, as mutations causing the genetic disease. The incidence of HH in Albania is unknown, and clinical cases are rare. Studies in the region show that C282Y allele was detected in only 50% of HH patients in Greece, and in 60% of HH patients in Italy. Various methods are in use, but in the present paper the application of a Strip Assay protocol (Vienna Lab) that helps identifying three mutations in the same strip assay, C282X, H63D and S65C. In the present investigation 20 patients who were not diagnosed with hemochromatosis, but presented iron overload and abnormal serum ferritin levels are involved. The results reported that alleles frequencies for S65C, H63D and C282Y were 2,5%, 7,5% and 2,5%, respectively. S65C is a rare allele in European populations which givesa mild form of hemochromatosis. The high frequency of this allele suggests a large study of this mutation in our population. C282Y allele and H63D allele in heterozygotes patients with increased baseline of iron parameters suggested that other genetic factors linked to hemochromatosis might be present in Albanian population, other than C282Y and H63D mutations. We conclude that an elevated hepatic iron index should indicate the utility of searching for *HFE* mutations prior to other iron gene studies.
Keywords: HFE gene, hemochromatosis, ferritin, iron overload, C282Y, H63D, S65C

1. INTRODUCTION

Hereditary hemochromatosis (HH) is an inherited autosomal recessive disorder of iron metabolism. Due to excessive intestinal absorption, iron

accumulates in parenchymal cells of the liver, pancreas, heart and other organs with resultant damage to their structure and impairment of their function. It is one of the most common genetic diseases in Caucasians with a prevalence of nearly 1 in 300 (Merryweather-Clarke *et al*., 1997a). Although the symptoms of the disease are often nonspecific, much of the organ damage is irreversible once it has occurred. Early detection and therapy is therefore very important as a part of preventive medicine. The discovery of the responsible gene *HFE* in 1996 (Feder *et. al*., 1997) enabled molecular analysis to be included in the diagnostic strategy for HH (Merryweather-Clarke *et al*., 1997b). C282Y and H63D alleles were identified as contributing to the HH disease. The third common mutation of *HFE* is 193A→T substitution in exon 2 (S65C) and was shown to be generally benign, although a C282Y/S65C genotype may confer a slight increase in disease risk, contributing to a mild disease phenotype (Rochette *et al*., 1999; US PST2006; Oliveira *et al*., 2009). As clinical signs and symptoms are different, finding cases with hemochromatosis depends heavily on clinical observation and the reason why biochemical tests are requested. In this study we focused on the iron markers levels to established a DNA based method and optimized a protocol for screening mutation in the HFE gene.

2. MATERIALS AND METHODS

In the present investigation, 20 hospitalized patients from the Clinic of Gastro-Hepathology, University Hospital Center, Mother Theresa, Tirana, suffering from cirrhosis are involved. All the patients underwent serum iron load, ferritin and transferrin level test. The K3 EDTA was used to collected 5 ml blood from each patient for DNA test of HFE.

The QIAGEN DNA extraction kit was used for **DNA extraction** from EDTA blood.

Biochemical analysis was carried out for ferritin and transferrin blood content via VidasFerritin kit (an automated quantitative test using the ELFA technique Enzyme Linked Fluorescent Assay) and miniVidas equipment. Serum iron load was analyzed using Colorimetric Chromazurol B, End point method and Minitecno analyzer.

Regarding the **HH strip assay**, the protocol used for identification of three mutations causing HH, C287Y, H63D and S65C is based on a hybridization assay on nitrocellulose strips, where attached oligomeres have the wild type sequence and mutation sequencies for C287Y, H63D and S65C mutations (Vienna Lab). Once the DNA samples were amplified with primer mix supplied by the kit, the amplified DNA was used for the hybridization of the strips to find homologically the wild type sequence or mutation sequence for three well known HH mutations.

Identification of genotypes

Genotypes are identified as described by manufacturer. Heterozygotes are represented by two colored bands, one of wild type and another to the mutation position. Homozygotes are represented only by one colored band, in wild type position or in mutated position.

3. RESULTS AND DISCUSSIONS

Twenty patients were hospitalized at the Clinic of Gastrohepatology, University Hospital Center, Mother Teresa, Tirana, Albania, suffering from cirrhosis. The figure 1 depicts the mean values of serum iron indexes.

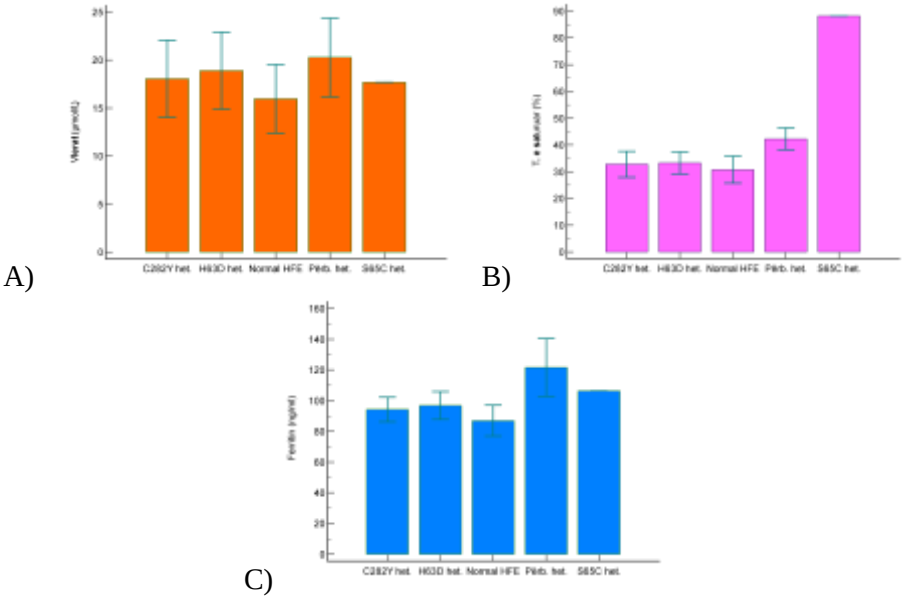

Fig. 1: A- the serum iron concentration compared with the HFE genotypes, B- the saturated transferrin compared with the HFE genotypes, C- the serum ferritin concentration compared with the HFE genotypes. Descriptive statistics of continuous variables are presented, which are summarized as mean ± standard deviation (SD). Categorical variables are presented as absolute frequencies and percentages.

Iron was found at high levels as reported by all iron markers parameters. Here, the mutation alleles could be noted when compared with the level of iron markers without mutations.

We used iron markers parameters for the selection of the samples undergoing further analyses employing molecular strips assay for the screening of HFE alleles, and the results are in the Figure 2 and 3 reported.

One out of 10 patients appeared to be S65C heterozygote as in Figure 2 depicted.

Fig. 2: Photo of 10 strips hybridized with 10 DNA samples amplified for 3 mutations of HH, C287Y, H63D, S65C. Sample HK8, first strip on the right part of the photo, was identified as S65C heterozygote genotype.

The Figure 2 depicts the hybridized and colored strips. All samples presented wild type mutations, except the sample HK8 showing a heterozygote genotype for S65C mutation. The presence of a colored band at position of S65C mutation and a band of lower intensity at wild type position of the above mutation means that this patient is a heterozygote genotype.

We set up this new protocol for the identification of three most frequent mutations causing HH. The 20 patients were equally divided into two groups. In the first group were selected patients with high ferritin level and iron load to find possible HH patients as suggested in (de Diego *et al*., 2004; Pedersen *et al*., 2008; Lee *et al*., 2009). The protocol was successfully applied, and the results showed the rare mutation S65C, in heterozygotic state, found in one patient. In the second group We continued to use this protocol in other 10 patients for increased parameters not only high levels of ferritin and iron load, but also high levels of ferritin level.

In the second group, 3 patients, carriers of H63D mutation, 1 patient carrier of C282Y mutation, and 6 wild-type patients— normal for C282Y/H63D— were found (Figure 2).

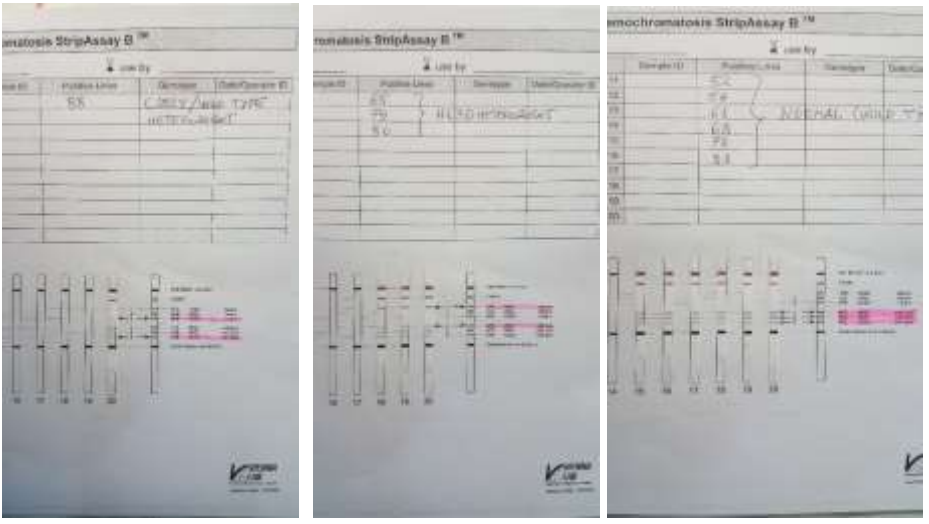

Fig. 3: From 10 patients analyzed, 6 resulted wild type, 3 resulted carriers of H63D mutation and 1 patient was found carrier of C282Y mutations.

The data for the 20 patients are as following: alleles frequencies for S65C, H63D and C282Y were 2,5%, 7,5%, and 2,5%, respectively.

S65C is a rare allele found in European populations which gives a mild form of hemochromatosis. Similar alleles frequency is found in other European population studies. In the German population, the allele frequencies for C282Y and H63D were 1.9%, and 18.9%, respectively. In the Italian population, the alleles frequencies for C282Y and H63D were 0.5% and 12.6%, respectively. In the Greek population, allele frequencies for C282Y and H63D were1.4% and 11.9%, respectively (de Diego *et al*., 2004; Ferreira *et al*., 2008; Pedersen *et al*., 2008; Lee SH *et al*., 2009; Neghina *et al*., 2009).

The high allele frequency is a means to address a larger study of this mutation in the Albanian population. C282Y/H63D wild-type individuals has an increased baseline of iron parameters, probably due to genetic factors unrelated to the C282Y/H63D mutations (Ferreira *et al*., 2008; Pedersen *et al*., 2008; Neghina *et al*., 2009). Strip assay seems to be an efficient method which, combined with serum iron level markers, can be used to screen patients for the HFE gene. This method, combined with iron parameters, helps to identify the target group for molecular analysis to address mutations in the HFE gene and diagnose inherited hemochromatosis.

We conclude that an elevated hepatic iron index should indicate the utility of searching for *HFE* mutations prior to other iron gene studies.

REFERENCES

Merryweather-Clarke AT, Liu YT, Shearman JD, Pointon JJ, Robson KJ. 1997a. A rapid non-invasive method for the detection of the haemochromatosis C282Y mutation. *British Journal of Haematology*, **99 (2):** 460.

Merryweather-Clarke AT, Pointon JJ, D Shearman J, Robson KJ. 1997b. Global prevalence of putative haemochromatosis mutations. *Journal of Medical Genetics*, **34(4):**275-8. doi: 10.1136/jmg.34.4.275.

Feder JN, Gnirke A, Thomas W, Tsuchihashi Z, Ruddy DA, A Basava, Dormishian F, Jr Domingo R, Ellis MC, Fullan A, Hinton LM, Jones NL, Kimmel BE, G S Kronmal, Lauer P, Lee VK, Loeb DB, Mapa FA, McClelland E, Meyer NC, Mintier GA, Moeller N, Moore T, Morikang E, Prass CE, Quintana L, Starnes SM, Schatzman RC, Brunke KJ, Drayna DT, Risch NJ, Bacon BR, Wolff RK. 1996. A novel MHC class I-like gene is mutated in patients with hereditary haemochromatosis. *Nature Genetics*, **13 (4):** 399.

Rochette J, Pointon JJ, Fisher CA, Perera G, Arambepola M, Arichchi DS, De Silva S, Vandwalle JL, Monti JP, Old JM, Merryweather-Clarke AT, Weatherall DJ, Robson KJ. 1999. Multicentric origin of Haemochromatosis gene (HFE) mutations. *The American Journal of Human Genetics*, **64 (4):** 1056-1062.

Oliveira VC, Caxito FA, Gomes KB, Castro AM, Pardini VC, Ferreira ACS. 2009. Frequency of the S65C mutation in the hemochromatosis gene in Brasil. *Genetics and Molecular Research*, **8 (3):** 794-798.

US preventive Services Task Force. **2006**. Screening for hemochromatosis recommendation statement. *Annals of* **Internal Medicine**, **145 (3):** 204-208.

Pedersen P, Melsen GV, Milman N. 2008. Frequencies of the haemochromatosis gene (HFE) variants C282Y, H63D and S65C in 6020 ethnic Danish men. *Annals of Hematology*, **87 (9):** 735-740.

de Diego C, José Murga M, Pedro Martínez-Castro P. 2004. Frequency of HFE H63D, S65C, and C282Y mutations in patients with iron overload and controls from Toledo, Spain. *Genetic Testing*, **8 (3):** 263-267.

Lee SH, Lee SH, Kim JW, Shin SH, Kang KP,Choi HC, Choi SH, Park KU, Kim HY, Kang W, Jeong S-H. 2009. HFE gene mutations, serum ferritin level, transferrin saturation, and their clinical correlates in a Korean population. *Digestive Diseases and Sciences*, **54:** 879-886.

Ferreira ACS, Oliveira VC, Caxito FA, Gomes KB, Castro AM, Pardini VC. 2008. Prevalence of C282Y and H63D mutations in the HFE gene of Brazilian individuals with clinical suspicion of hereditary haemochromatosis. *Revista brasileira de hematologia e hemoterapia*, **30**: 379-383.

Neghina AM, Anghel A, Sporea I, Popescu A, Neghina R, Collins A, Thorstensen K. 2009. Mutant HFE genotype leads to significant iron overload in patients with liver diseases from western Romania. *Journal of Applied Genetics*, **50 (2):** 173-176.