Population-based analysis of the frequency of HFE gene polymorphisms: Correlation with the susceptibility to develop hereditary hemochromatosis

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Received March 22, 2016; Accepted May 10, 2016

DOI: 10.3892/mmr.2016.5317

Abstract. Hereditary hemochromatosis (HH) is an autosomal recessive genetic disease, characterized by increased dietary iron absorption. Due to the absence of an effective excretory mechanism, the excess iron in the body may accumulate resulting in toxic effects. The HFE gene also affects the activity of hepcidin, a hormone which acts as a negative regulator of iron metabolism. In this study, we performed a population-based analysis of the distribution of three hemochromatosis-related polymorphisms in the HFE gene (rs1800562, rs1799945 and rs1800730). DNA from 1,446 non-related individuals of Greek ethnicity was collected and analyzed, either from whole blood or buccal swabs. The frequency distribution of these HFE gene polymorphisms was then determined. The results revealed that in our Greek population cohort (gr) the frequencies of each polymorphism were as follows: rs1800562: GG (wild-type)=97.0%, GA=1.5%, AA=1.5%; rs1799945: CC (wild-type)=74.4%, CG=23.4%, GG=2.2%; rs1800730: AA (wild-type)=98.1%, AT=1.5% and TT=0.4%. No association between the HFE polymorphisms rs1800562, rs1799945 and rs1800730 and gender could be established. As regards the rs1800562 polymorphism, the A allele (mutant) was ~1.8-fold more frequent in the European population (eur) than in the Greek population ([gr]=1.2%<[eur]=2%). However, these pathogenic mutations were found more frequently in the Greek population compared to the global population (gl) [rs1800562: (gl)=1%<[gr]=2.3%; rs1799945: (gl)=7%<[gr]=13.9%; rs1800730: (gl)=<1%<[gr]=1.2%]. This suggests that the Greek population may differ genetically from the northern European population, due to influences from neighboring Asian and African populations. These findings also suggest that there is no gender-associated inheritance of these polymorphisms, and gender-specific symptoms appear as a result of independent biological processes. Thus, the early detection of the tendency towards iron accumulation may be achieved by the genotypic analysis of the polymorphisms that may contribute to the development of the hemochromatosis.

Introduction

Iron contributes in multiple ways to the physiological processes of the human body, both as a part of cytochrome heme, as well as a key component molecule in hemoglobin and myoglobin heme, which binds oxygen in red blood cells. Due to the absence of an effective excretory mechanism, the maintenance of normal iron levels is critical and is mainly succeeded by regulating intestinal absorption and by continuously recycling and reusing cellular iron (1,2).

The ability of iron to release free oxygen radicals can be a potential health hazard, while excessive iron levels may promote oxidative stress by increasing the steady-state concentration of intermediate oxygen radicals. This may lead to fibrosis and carcinogenicity, and may also deactivate essential metabolic enzymes (1,3).

Body iron is found mainly in the form of heme iron (organic iron) and non-heme iron (inorganic iron). Different absorption mechanisms are implemented for these two types of dietary iron. The total iron absorption is low, ~10% of the 10-20 mg obtained from diet. Heme iron is absorbed more efficiently than non-heme iron (1).

Heme iron enters the cell either by binding to a carrier protein of the cell membrane, or through an endocytotic
mechanism. Inside the cell, the enzymatic decomposition of the heme molecule is mediated by heme oxygenase, resulting in the release of Fe\(^{3+}\), CO and biliverdin. Fe\(^{3+}\) is then reduced to Fe\(^{2+}\).

Iron absorption depends on the amount of stored iron in the body, which is reflected by transferrin saturation (TS) levels (3). Transferrin is expressed by immature enterocytes and binds to them, serving as an intestinal ‘iron probe’. Immature enterocytes are not actively involved in iron absorption, but they express transferrin receptors (TFRs) and the HFE protein. The HFE gene is responsible for the production of HFE protein, which is found on the cell surface, primarily in the liver and intestines, as well as in macrophages of the immune system. It activates the divalent metal transporter 1 (DMT-1)/ferroportin (FPN) system, which serves as a detector of iron levels in the body and prepares immature enterocytes for diversification to their mature absorptive form (3). HFE regulates the production of another major protein known as hepcidin, which is considered to be a specific regulatory hormone of iron levels. HFE also interacts with TFRs. However, the role of these interactions in iron regulation is still unclear (4).

Normally, the saturation of plasma transferrin regulates the expression of hepatic hepcidin via the HFE, TFR2 and hemojulvin (HJV) signaling pathway. Hepcidin is secreted into the blood, binds with FPN in the intestines and macrophages, and induces the degradation of FPN, thus reducing intestinal absorption and the recycling of iron from macrophages, to maintain the saturation of plasma transferrin. In the case of hereditary hemochromatosis (HH), mutations in the HFE, HJV and TFR2 genes prevent the synthesis of hepcidin, increasing FPN and iron release levels in intestinal cells and macrophages, thus leading to an increase in plasma TS levels, and ultimately causing iron deposition in the liver and other tissues.

A heterogeneous group of hereditary and idiopathic causes represent diseases characterized by iron overload (5). HH is an autosomal recessive genetic disease linked to iron metabolism and is a commonly inherited disorder in which iron obtained through food cannot be eliminated after absorption, as the body does not have an effective excretory mechanism to eliminate the excess iron. Over time, this excess iron leads to an accumulation state, which is toxic to cells. The clinical significance of this autosomal recessive disease is mainly observed in homozygotes for the mutated gene. If not treated in time, HH may prove to be fatal. In Northwestern Europe, 3-5 individuals in 1,000 are homozygous carriers (http://www.irondisorders.org/hemochromatosis). Of note, abnormalities in the regulation of iron levels, which may result in iron accumulation, occur gradually and patients remain asymptomatic up to the fourth decade of life, although total body iron may have already accumulated, reaching levels of 10-20 g, mainly deposited in the liver, heart and endocrine glands as hemosiderin (6).

HH occurs 2 to 10-fold more frequently in adult males than in females. Possibly in women, due to menstruation, monthly iron loss delays the accumulation of iron for approximately one decade and symptoms usually begin to appear after menopause. In men, clinical symptoms appear earlier, although rarely before the age of 40 or 50 (7).

The genotyping of HFE gene mutations may reveal individual excretory mechanism disorders, and may thus lead to the early initiation of treatment, long before the development of clinical symptoms. This would contribute significantly to the prevention of hemochromatosis-induced cirrhosis and to a normal life expectancy.

The most frequent polymorphism of the HFE gene is located in the short arm of chromosome 6 (6p), at position 845, where guanine (G) is replaced by adenine (A), resulting in the replacement of a cysteine by tyrosine at position 282 of the HFE protein sequence (C282Y), which leads to a non-functional protein (8). The C282Y polymorphism prevents HFE protein from reaching the cell surface, thus preventing the interaction with hepcidin and TFRs (4).

The second most frequent HFE gene polymorphism consists of a histidine to aspartic acid replacement at position 63 of the HFE protein sequence (H63D), due to a cysteine (C) to guanine (G) replacement at position 187. This polymorphism may disrupt iron homeostasis and cause iron accumulation when it occurs simultaneously with the C282Y polymorphism (8).

A further association that seems to play a significant role in the development of hemochromatosis is the S65C polymorphism, where serine is replaced by a cysteine at position 65 of the HFE protein sequence (9).

The HFE gene affects iron absorption by altering the expression of hepcidin (10). The C282Y polymorphism of the HFE gene is responsible, alone or in combination with H63D and/or S65C, for almost 90% of hemochromatosis cases among Northern European populations (11). All three HFE gene polymorphisms are associated with the disease (10). In this study, we performed a population-based frequency distribution analysis of the HFE gene polymorphisms, rs1800562 (C282Y), rs1799945 (H63D) and rs1800730 (S65C), and their susceptibility to developing HH. We examined samples obtained from 1,446 individuals of Greek ethnicity.

Subjects and methods

In this study, samples from 1,446 healthy Greek (Caucasian) individuals, 670 men and 776 women of Greek origin were collected and analyzed. Samples were obtained from buccal swabs. The median age of the participants was 38 years their ages ranged from 18 to 98 years (Fig. 1). All volunteers provided written and signed informed consent. Following anonymization, genomic DNA was isolated from epithelial cells collected from the oral cavity with swabs, using nucleic acid isolation columns (Tissue Nucleospin; Machery-Nagel GmbH & Co. KG, Düren, Germany). Genotypes of HFE gene polymorphisms were determined by real-time polymerase chain reaction (PCR) using the Simple Probes commercial LightSnip kit and the LightCycler FastStart DNA Master HybProbe Kit (Roche Diagnostics, Penzberg, Germany). The reactions were performed on a LightCycler 480 Real-Time PCR system (Roche Applied Science, Mannheim, Germany) in accordance with the manufacturer's recommendations. Hybridization was analyzed using melting curve analysis software provided with the instrument. The genotypes were classified as homozygous for the wild-type allele, and heterozygote and homozygous for the polymorphism allele.

Contingency tables 2x2 (1 degree of freedom) were designed and odds ratios (ORS), as well as the corresponding confidence intervals were calculated. Statistical analysis was performed at a significance level of a=0.05 and a statistically significance P-value was calculated by Fisher's exact test. All
Results

The majority (97%) of the 1,446 Greek volunteers analyzed for polymorphism rs1800562 was homozygous wild-type (G:G). Of the volunteers, 1.52% were heterozygous (G:A), and only 1.48% were homozygous for the mutant allele (A:A). The frequency of the wild-type G alleles was 2,828 (97.7%) and that of the mutated A allele was only 64 (2.3%). A Hardy-Weinberg disequilibrium was detected for polymorphism rs1800562 in the volunteer Greek population in our study ($\chi^2=608.06$) (Table I and Fig. 2).

As regards polymorphism rs1799945, data were obtained only from 1,429 volunteers. Of these volunteers, 74.4% were homozygous for the wild-type genotype (C:C). In addition, 23.4% were heterozygous (C:G) and 2.2% were homozygous for the mutant allele (G:G). The frequency of the wild-type allele was 2,462 (86.1%) and that of the mutated allele was 396 (13.9%). HWE was detected for polymorphism rs1799945 in the volunteer Greek population of our study ($\chi^2=0.62$) (Table II and Fig. 3).

Finally, we obtained data for polymorphism rs1800730 from 1,219 volunteers. The vast majority (98.1%) of the volunteers was homozygous for the wild-type genotype (A:A). Of these volunteers, 1.5% were heterozygous for the mutant allele (A:T) and only 0.4% were homozygous for the mutant allele (T:T). The frequency of the wild-type allele was 2,410 (98.8%) and that of the mutated allele was only 28, which represented only 1.2% of the examined volunteers. Hardy-Weinberg disequilibrium was detected for polymorphism rs1800730 in the volunteer Greek population of our study ($\chi^2=149.05$) [Table III and Fig. 4].

Table I. Genotype and allele distribution of the rs1800562 in polymorphism in 1,446 volunteers.

| rs1800562 | No. (%) |
|-----------|---------|
| Genotype frequency |
| G:G | 1,403 (97.0) |
| G:A | 22 (1.5) |
| A:A | 21 (1.5) |
| Total | 1,446 (100.0) |
| Allele frequency |
| G | 2,828 (97.7) |
| A | 64 (2.3) |
| Total | 2,892 (100.0) |

Table II. Genotype and allele distribution of the rs1799945 polymorphism in 1,429 volunteers.

| rs1799945 | No. (%) |
|-----------|---------|
| Genotype frequency |
| C:C | 1,064 (74.4) |
| C:G | 334 (23.4) |
| G:G | 31 (2.2) |
| Total | 1,429 (100.0) |
| Allele frequency |
| C | 2,462 (86.1) |
| G | 396 (13.9) |
| Total | 2,858 (100.0) |

Table III. Genotype and allele distribution of the rs1800730 polymorphism in 1,219 volunteers.

| rs1800730 | No. (%) |
|-----------|---------|
| Genotype frequency |
| A:A | 1,196 (98.1) |
| A:T | 18 (1.5) |
| T:T | 5 (0.4) |
| Total | 1,429 (100.0) |
| Allele frequency |
| A | 2,410 (98.8) |
| T | 28 (1.2) |
| Total | 2,438 (100.0) |
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No association between the HFE polymorphisms rs1800562, rs1800562 and rs1800562 and gender could be established. The OR of the male and female volunteers exhibited no statistically significant difference (Table IV).

Data for all three polymorphisms of the HFE gene were from 1,205 volunteers. No volunteer carried all three polymorphisms simultaneously. Moreover, 871 (73%) individuals carried the wild-type alleles in all three polymorphisms, and only six volunteers (0.5%) were heterozygous for two polymorphisms and homozygous for the wild-type allele of the third polymorphism. Over a quarter of the 1,205 volunteers (327 individuals, 27.2%) carried a single mutant allele of one of the investigated polymorphisms, while the other two were wild-type (Fig. 5).

Discussion

HH is an autosomal recessive genetic disorder that is associated with iron metabolism and is among the most common genetic disorders observed in individuals of European descent, characterized by excessively increased dietary iron absorption (12).

Non-heme iron exists either in a trivalent (Fe$^{3+}$) or in a divalent (Fe$^{2+}$) form. The Fe$^{3+}$ ion is poorly absorbed as it tends to form salt complexes with anions and at a pH value >3 is insoluble. On the contrary, Fe$^{2+}$ hardly forms complexes and is soluble at high pH values ≤8 (1). Non-heme iron is absorbed almost exclusively as Fe$^{2+}$. Fe$^{2+}$ can enter the cell either by binding with an intraluminal form of transferrin followed by endocytosis or via the co-transport of Fe$^{2+}$ and H$^{+}$ through DMT-1 (3). Regardless of the route followed by ferrous iron (Fe$^{2+}$) into the cell, cytoplasmic Fe$^{2+}$ binds to mobilferrin to reach the basolateral membrane and then enters the plasma through FPN, an exporter of divalent metals. Once in the circulation, Fe$^{2+}$ is once again reduced to Fe$^{3+}$ and is transferred to other organs bound to plasma transferrin, the major iron transport protein (1).

Iron can accumulate and become toxic to the body, due to the inability of the body to effectively eliminate this excess. If left untreated, HH can lead to morbidity, including liver cirrhosis, hepatocellular carcinoma (HCC), diabetes, heart disease and even death (13). Iron overload leads to the development of histological lesions in many vital organs. In the liver, iron is deposited inside hepatocytes, Kupffer cells and biliary ductal cells, and iron accumulation leads to fibrosis, which gradually progresses to cirrhosis. HCC manifests in 20-30% of patients with cirrhosis. Iron deposition in the myocardium causes progressive heart failure, dilation of the heart and disturbances in the cardiac conduction system. Hemosiderin accumulation in the pancreas can induce damage to the islets of Langerhans, which may lead to a subsequent development of diabetes mellitus, particularly in patients with genetic predisposition and a family history of diabetes. Other endocrine glands may also be affected, such as the thyroid and gonads. Almost all patients who develop cirrhosis develop bronze skin pigmentation due to hemosiderin accumulation in skin macrophages and increased melanin in the epidermis. Finally, the deposition of hemosiderin in the joints may cause damage due to advanced synovial calcification and secondary lesions of the adjacent bone (3).

The diagnosis of hemochromatosis is based on a combination of clinical, laboratory and genetic findings. Diagnosis requires confirmation of elevated serum ferritin and TS levels, with or without symptoms. The measurement of serum ferritin
leads to a defect in the mechanisms regulating iron levels and populations was also performed and possible gender specific frequencies for all three polymorphisms versus other based study that has taken place in Greece to date, analyzing To the best of our knowledge, this is the largest population-chromatosis (16), are distributed in a Greek population sample. nucleotide polymorphisms of the \textit{HFE} gene, related to hemochromatosis (17), have been shown to be associated with iron overload and have been used in genetic predisposition analysis for hemochromatosis (14). Genotypic analysis of iron overload has become crucial (14). Genotypic analysis of \textit{HFE} gene polymorphisms rs1799945 (H63D) also causes the malfunction of hepcidin, resulting in an increased probability of developing type 1 HH. The mutant allele (A) occurs in 1% of the global population, and in 4% of the European population. This study revealed the A allele in 2% of the Greek study population. The mutant allele appeared twice as often in our study population as compared to the global population. Furthermore, the rs1800562 (C282Y) polymorphism in the Greek study population appeared half as often as in the European population (Table V).

As regards the rs1800562 (C282Y) polymorphism, the genetic predisposition of developing HH was higher for individuals homozygous for the mutant A:A genotype, who are more prone to develop symptoms. These symptoms seem to be more severe in men or women after menopause, and appear in 0.1% of the global population and in 0.2% of the Northern European population. In the Greek population in this study, the results revealed an increased prevalence of the homozygous mutant genotype A:A, which was present in 1% of the volunteers. Individuals heterozygous for this mutation, i.e., those with the G:A genotype, are usually not affected unless carrying the H63D mutation simultaneously (13). Genetic testing confirms the diagnosis (3).

In the present study, we examined how three single nucleotide polymorphisms of the \textit{HFE} gene, related to hemochromatosis (16), are distributed in a Greek population sample. To the best of our knowledge, this is the largest population-based study that has taken place in Greece to date, analyzing these specific mutations. A comparison of the study population frequencies for all three polymorphisms versus other populations was also performed and possible gender specific differences were investigated.

The rs1800562 (C282Y) polymorphism of the \textit{HFE} gene leads to a defect in the mechanisms regulating iron levels and in the malfunction of hepcidin, resulting in an increased probability of developing type 1 HH. The mutant allele (A) occurs in 1% of the global population, and in 4% of the European population. This study revealed the A allele in 2% of the Greek study population. The mutant allele appeared twice as often in our study population as compared to the global population. Furthermore, the rs1800562 (C282Y) polymorphism in the Greek study population appeared half as often as in the European population (Table V).

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The rs1800562 (C282Y) polymorphism of the \textit{HFE} gene leads to a defect in the mechanisms regulating iron levels and
The rs1799945 (H63D) polymorphism of the HFE gene-occurred more frequently in individuals of Northern European descent. The susceptibility to develop hereditary hemochromatosis seems to be enhanced in the Northern European population as compared to the Southern European population. This mutation, i.e., those carrying the T:T genotype in the global population is <1%, in the European population it is 0.2% and in the Greek population it is 0.4%. These individuals are predisposed to present with very mild symptoms of hemochromatosis, particularly if they also carry the rs1800562 polymorphism. Heterozygous individuals, i.e., those carrying the T:A genotype are found in 0.7% of the global population, in 2.8% of the European population (17) and in 1.5% of the Greek population (Table X).

According to the literature, the rs1799945 (H63D) and rs1800730 (S65D) polymorphisms, in order to be held responsible for any abnormal iron levels, they should 'coexist' with at least one mutant allele of the rs1800562 (C282Y) polymorphism. There are indications that these polymorphisms, even in homozygosity, are not sufficient to cause HH (http://browser.1000genomes.org/Homo_sapiens/Variation/Population?v=rs1799945;%20vdb=variation). The absence of such conditions can cause changes in the gene pool frequencies, namely evolution. Violation of the HWE conditions may lead to population stratification, and genotyping errors could be reflected as significant deviations from HWE predictions (19). In the present study, HWE was detected only for the rs1799945 (H63D) polymorphism. This could be interpreted as a stability of this particular polymorphism regarding the mechanisms of evolution mentioned above, which means that evolution did not occur, and theoretically the gene pool frequencies remained unaltered. However, we cannot safely assume that the next generations will also follow the HWE because evolution is an inevitable result. The rs1800562 (C282Y) and rs1800730 (S65D) polymorphisms are in disequilibrium which means that, as regards these polymorphisms, the population studied has evolved due to several factors.

The results indicated that the investigated Greek population had a greater similarity to the global population (Table X). In this study, from 1,460 samples genotyped for both the C282Y and H63D polymorphisms, only two (2) were found to be compound heterozygotes (G:A/C:G), that is only 0.1% of the sample population. In addition, among the 1,256 volunteers analyzed for both the C282Y and the S65D polymorphisms, none of them were compound heterozygotes (G:A/A:T) i.e., 0% of the sample population. Based on these results we can conclude that the levels of compound heterozygosity in the Greek population are infinitesimal (zero).

According to the HWE law, genotype frequencies are functions of allele frequencies and a large random mating population is at equilibrium given that there is no migration, natural selection, or genetic drift (18). The absence of such conditions can cause changes in the gene pool frequencies, namely evolution. The proportion of homozygous carriers relative to this mutation, i.e., those carrying the T:T genotype in the global population is <1%, in the European population it is 0.2% and in the Greek population it is 0.4%. These individuals are predisposed to present with very mild symptoms of hemochromatosis, particularly if they also carry the rs1800562 polymorphism. Heterozygous individuals, i.e., those carrying the T:A genotype are found in 0.7% of the global population, in 2.8% of the European population (17) and in 1.5% of the Greek population (Table X).

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The results indicated that the investigated Greek population had a greater similarity to the global population. It seems that, in relation to the Northern European population, the Greek population varies considerably: all three investigated polymorphisms occur more frequently in individuals of Northern European descent. The susceptibility to develop hemochromatosis seems to be enhanced in the Northern European population as compared to the Southern European population.
population (http://www.irondisorders.org/hemochromatosis). The geographical position of Greece, on the crossroad between three continents, may genetically influence the Greek population. Despite the fact that the clinical manifestation of HH appears 2-10-fold more frequently in adult men than in women, no statistically significant difference could be demonstrated in the distribution of the HH-associated polymorphisms analyzed between the two genders. Other biological functions, such as menstruation, may contribute to regular iron loss, which delays iron accumulation for approximately a decade. HH symptoms usually appear after menopause.

Therapeutic phlebotomy is the primary treatment for hemochromatosis (20). It seems to stabilize the situation and contribute to the prevention of the progression to cirrhosis, which adversely affects the long-term survival of patients (http://www.snpedia.com/index.php/Rs1800730). Orthotopic liver transplantation is performed in patients with advanced cirrhosis. Moreover, the administration of chelating agents, such as deferoxamine helps in iron elimination. A series of hydroxyxypiridinone dendrimers are believed to possess a high affinity and selectivity for Fe$^{3+}$, which reduces absorption from the duodenum (21). A recent publication demonstrated that proton pump inhibitors may inhibit iron absorption, restricting its accumulation by increasing gastric pH. This prevents iron absorption, which mainly exists in the divalent form (17).

The three HFE gene polymorphisms investigated in the present study may lead to disturbances in the iron level adjustment mechanisms, as well as in the impairment of hepcidin, resulting in the increased probability of developing type 1 HH.

The genetic status of the HFE gene polymorphisms rs1800562, rs1799945 and rs1800730 seems to be an important preventive tool for delaying the development of the clinical symptoms of HH. In particular, for individuals with a family history of HH, genetic analyses and frequent iron and transferrin blood level measurements will prove beneficial and may help maintain a better quality of life and increase the average life expectancy.

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