Analysis of HFE and TFR2 mutations in selected blood donors with biochemical parameters of iron overload

Marco De Gobbi, Filomena Daraio, Christian Oberkanins, Anne Moritz, Fritz Kury, Gemino Fiorelli, Clara Camaschella

Background and Objectives. Hereditary hemochromatosis is a recessive condition characterized by iron accumulation in several organs, followed by organ damage and failure. The disorder is prevalently due to C282Y and H63D mutations in the HFE gene, but additional HFE and TFR2 mutations have been reported. Early iron overload may be assessed by biochemical parameters such as increased transferrin saturation and serum ferritin.

Design and Methods. Taking advantage of the collection of 178 DNA samples selected for increased transferrin saturation (>50% in males and >45% in females) from a previous large scale screening of Italian blood donors, we simultaneously assessed the presence of 14 hemochromatosis-associated molecular defects (11 of HFE and 3 of TFR2) by a reverse hybridization-based strip assay.

Results. In the series studied the overall C282Y allele frequency was 9% and that of the H63D and S65C was 22.2% and 1.4%, respectively. One rare HFE allele (E168Q), but no TFR2 mutation was detected. When checked at a second examination, transferrin saturation was significantly higher in C282Y homozygotes, H63D/C282Y compound heterozygotes and H63D homozygotes as compared to wild-type subjects ($p<0.05$).

Interpretation and Conclusions. Our results confirm previous findings on C282Y and H63D mutations in Italy, show that the C282Y allele frequency is enriched in samples selected for altered iron parameters, and that a few rare genotypes are present in Northern Italy. None of the known TFR2 mutations was identified in this series confirming the preliminary indication of their rare occurrence. Subjects with hemochromatosis-associated genotypes show a persistently higher mean transferrin saturation than do those with wild type genotypes.

Key words: screening, hemochromatosis, HFE, TFR2.

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Hereditary hemochromatosis is a common autosomal recessive disorder of iron metabolism characterized by increased intestinal iron absorption, which leads to iron overload in several organs. If untreated by phlebotomy, hemochromatosis can result in the development of cirrhosis (and increased susceptibility to hepatocarcinoma), diabetes, cardiac failure, endocrine complications and arthritis. Untreated patients have a shortened life expectancy, whereas survival is normal in subjects diagnosed and treated in the pre-symptomatic stage. Most hemochromatosis patients of Northern European origin (from 80 to 100% in various series)[1] are homozygous for the C282Y mutation in the HFE gene.[2] Combining several studies a decreasing gradient of the C282Y mutation among patients is recognized from Northern to Southern Europe[1] in agreement with the decreasing allele frequency in healthy controls.[3] About 64% of patients are C282Y homozygous in Italy[4,5] and the percentage is even lower in other Southern European countries.[6-8] A second mutation (H63D) is common in the general population and its role in the pathogenesis of iron overload remains uncertain. However, H63D in association with C282Y,[2] and rarely in the homozygous state, may cause iron overload. Recently other missense, nonsense and splice site mutations have been identified in the HFE gene, usually in the compound heterozygous state with C282Y.[8-12] Moreover, it has been shown that hemochromatosis is characterized by genetic heterogeneity. Three other forms of the diseases have been identified. Type 2 or juvenile hemochromatosis differs from HFE-hemochromatosis because of an earlier symptomatic presentation and a more severe clinical course.[13,14] The type 2 gene is unknown although the locus maps to chromosome 1q[15] and rare cases are due to hepcidin mutation on chromosome 19q.[16] Type 3 results from mutations in transferrin receptor 2 (TFR2)[17-18] on 7q22 and type 4, an atypical dominant form, is due to mutations in ferroportin 1 (FPN1) gene.[19-21]

To obtain preliminary information on the prevalence of all the described HFE and TFR2 mutations in iron overload, we took advantage of the availability of a series of DNA samples, selected for increased transferrin saturation and or serum ferritin, from a previous large scale screening of Italian blood donors.[22] Samples with altered iron parameters, already typed for C282Y and H63D mutations, were re-analyzed by means of a strip assay for the simultaneous detection of 14 hemochromatosis-associated molecular defects.

Design and Methods

Samples

DNA was available from 178 Italian blood donors selected from a large scale screening and already typed for C282Y and H63D mutations.[22] Selection was based on transferrin saturation (TS) > 45% in females and > 50% in males and/or serum ferritin (SF) > 300 μg/L in males and > 250 μg/L in females at a single determination.[22] Repeated fasting transferrin saturation was obtained in 169 cases.

Molecular analysis

For the simultaneous detection of 14 known HFE and TFR2 mutations a reverse hybridization assay (Haemochromatosis StripAssay, ViennaLab Labordiagnostika GmbH – Austria) was used. HFE gene exons 2, 3 and 4 and TFR2 exons 2, 4 and 6 were amplified in a single, multiplex polymerase chain reaction (PCR), using a set of 5'biotinylated primers. A thermocycling program of 35 cycles (94°C for 15 seconds, 58°C for 30 seconds and 72°C for 30 seconds) with a final extension at 72°C for 3 minutes, was performed on a Perkin Elmer GeneAmp®, PCR System 2400 (Perkin Elmer Biosystem, Foster City, CA, USA). PCR products were hybridized to a teststrip presenting a parallel array of allele-specific probes, and detected by enzymatic color reaction as previously described.[11] The following mutations were investigated: V53M, V59M, H63D, H63H, S65C, Q127H, E168Q, E168X, W169X, C282Y, Q283P of HFE and E60X, M172K, Y250X of TFR2.

Control DNA samples, previously typed by restriction enzyme analysis for mutations of HFE[4,10] and by direct sequencing for mutations of TFR2,[17] were available.

Statistical analysis

We calculated allele and genotype frequencies for HFE mutations in the selected series. The χ^2 test was used to compare allele frequencies between blood donors of different geographical origin and between our results and those of other studies. Enrichment factors for C282Y and H63D allele frequencies in selected blood donors were calculated using frequencies from neonates and unselected blood donors for comparison.

Differences among TS according to HFE genotypes were evaluated by the non-parametric Mann-Whitney test (GraphPad Prism 3.0, GraphPad Software, Inc).

Results

The strip assay method was first validated by testing available samples with known HFE and TFR2 mutations, among these homozygotes or heterozygotes for H63D, S65C, E168X, W169X, C282Y, Q283P, E60X, M172K and Y250X. Correct and unequivocal results were obtained for all these controls (see examples in Figure 1, right panel).

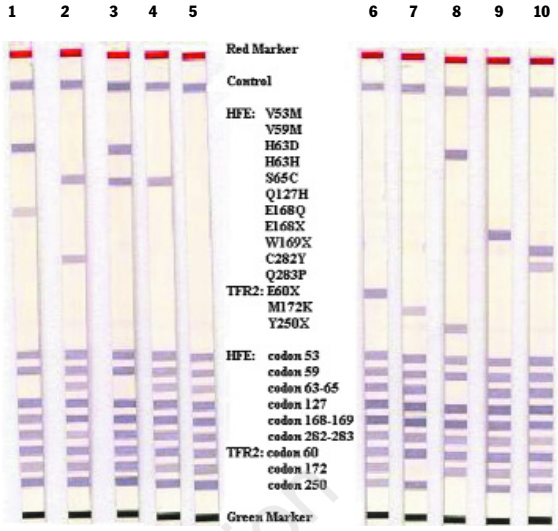

Figure 1. The following samples are shown: Left, lane 1: H63D homozygote with E168Q heterozygosity; lane 2: C282Y/S65C compound heterozygote; lane 3: H63D/S65C compound heterozygote; lane 4: S65C heterozygote; lane 5: wild type; Right: lane 6 E60X heterozygote; lane 7: M172K homozygote; lane 8: Y250X homozygote (with coexisting H63D homozygosity); lane 9 E168X heterozygote; lane 10: C282Y/W169X compound heterozygote.

One hundred and seventy-eight DNA samples from blood donors with abnormal serum iron parameters were re-analyzed for multiple HFE and TFR2 mutations. One hundred and forty samples were from blood centers located in Northern Italy and 38 from Central-Southern Italy.

The results obtained, reported as genotypes observed and allele frequencies of the different mutations, are summarized in Tables 1 and 2, respectively. Results for C282Y and H63D mutations were in agreement with those of previous typing performed with an endonuclease-based approach.[22] Three subjects (1.7%), all from the North, were homozygotes for C282Y mutation. Sixteen (9%), 14 from North Italy and 2 from South Italy, were C282Y heterozygotes, while 10 (5.6%) were homozygotes and 49 (27.5%) heterozygotes for H63D mutation. Nine C282Y/H63D compound heterozygotes were identified (5.1%). The overall C282Y allele frequency in this population selected for biochemical alterations of iron parameters was 9.0% and that of the H63D 22.2%. As H63D, S65C and C282Y variants have (with very rare exceptions) never been found on the same chromosome but occur in *trans*, the total allele frequency was corrected for linkage disequilibrium (24.8% for H63D and 2.0% for S65C).

Considering the geographical origin, a signifi-

Table 1. HFE genotypes in blood donors with altered iron parameters at a single determination.

	North (n=140)		Center-South (n=38)		Total (n=178)	
	N. of subjects	% of subjects	N. of subjects	% of subjects	N. of subjects	% of subjects
H63D/WT	36	25.7	13	34.2	49	27.5
C282Y/WT	14	10	2	5.3	16	9
H63D/H63D	7*	5*	3	7.9	10	5.6
C282Y/H63D	8	5.7	1	2.6	9	5.1
C282Y/C282Y	3	2.15	0	0	3	1.7
S65C/WT	3	2.15	0	0	3	1.7
C282Y/S65C	1	0.7	0	0	1	0.6
H63D/S65C	1	0.7	0	0	1	0.6
WT/WT	67	47.9	19	50	86	48.3

**including one H63D homozygous and E168 heterozygous subject.*

Table 2. HFE allele frequencies in blood donors with altered iron parameters.

(n=280)	North (n=76)		Center-South (n=356)		Total	
	N. of chr.	% of chr. (± 95% CI)	N. of chr.	% of chr. (± 95% CI)	N. of chr.	% of chr. (± 95% CI)
H63D	59	21.1±4.7	20	26.3±9.8	79	22.2±4.3*
C282Y	29	10.3±3.6	3	4±4.4	32	9.0±3.0
S65C	5	1.8±1.5	0	0	5	1.4±1.2*
WT	187	66.8±5.5	53	69.7±10.3	240	67.4±4.9

**The allele frequency corrected for linkage disequilibrium, considering that H63D and S65C variants have (with very rare exceptions) never been found in C282Y chromosomes, was: H63D 24.8 and S65C 2.0%. chr.: chromosomes.*

cantly higher frequency of C282Y alleles was found in subjects from Northern Italy, than in subjects from Central-Southern Italy (10.3% vs. 4%, p=0.017). Contrariwise, the H63D allele frequency was higher in samples from Central-South Italy than from samples from the North (26.3% vs. 21.0%), but the difference was not statistically significant, even when the percentages were corrected for disequilibrium.

Rare genotypes were found in patients from North Italy: three subjects were S65C heterozygotes, and one subject each was S65C/C282Y and S65C/H63D compound heterozygote. Considering only subjects from Northern Italy, the S65C allele frequency was 1.4% (2.0% when corrected for disequilibrium). The E168Q mutation was identified in a single individual who was an H63D homozygote. Examples of the rare genotypes observed in blood donor samples are shown in Figure 1 (left panel).

S65C and E168Q mutations were not found in the limited series examined from Central-Southern Italy. The other investigated mutations of HFE (V53M, V59M, H63H, Q127H, E168X, W169X, Q283P) and of TFR2 (Y250X, E60X, M172K) were not detected in the samples analyzed.

The comparison of these data with those obtained in unselected Italian blood donors[27,28] and with results of a previous study on neonates[26] are reported in Table 3. There are significant differences for H63D and C282Y allele frequencies between unselected neonates or unselected blood donors and our selected subjects with increased iron parameters (p<0.001).

A correlation between TS and HFE mutations was possible in 169 subjects who had TS checked at a second test. Twenty subjects (16 males and 4 females) had an increased TS at the second test. In this group the C282Y allele frequency was 35% and that of the H63D was 30% (*data not shown*).

Table 3. Comparison of HFE allele frequencies in 4 different Italian studies.

Ref.	Population analyzed	H63D		C282Y	
This work	178 blood donors with increased TS and or SF (see text)	22.2%	–	9.0%	–
Restagno *et al.*[26]	1331 consecutive newborns	11.8%*	p<0.001	2.1%	p<0.001
Cassanelli *et al.*[27]	2100 plasma-platelet donors	13.3%	p<0.001	1.6%	p<0.001
Barosi *et al.*[28]	1050 potential blood donors	15%	p<0.001	2.2%	p<0.001

**Calculated only on 55 C282Y heterozygote neonates; χ^2 test for significance; p calculated by comparison with blood donors studied in this work.*

Subjects with C282Y homozygosity, H63D/C282Y compound heterozygosity and H63D homozygosity showed a mean TS (88.3%, 52.2% and 42.7%, respectively) significantly higher than that of wild type subjects (34.9%) (Table 4).

Table 4. Mean transferrin saturation according to HFE genotypes.

Genotypes	*Subjects (n=169)*	*Mean age (range)*	*Mean TS% (range)*	
C282Y/C282Y	3 (F=3)	30.7 (23-38)	88.3 (82-94)*	*p*=0.0036
C282Y/H63D	9 (M=7; F=2)	40.3 (18-63)	52.2 (32-68)°	*p*=0.004
H63D/H63D	9 (M=7; F=2)	40.7 (21-60)	42.7 (34-62)#	*p*=0.027
C282Y/WT	14 (M=12; F=2)	39.9 (24-65)	38.9 (18-80)	
H63D/WT	49 (M=43; F=6)	40.6 (18-64)	35.3 (18-59)	
S65C/WT	3 (M=3)	41.3 (22-64)	33 (22-39)	
S65C/C282Y	1 (M=1)	53	45	
S65C7H63D	1 (M=1)	29	38	
WT/WT	80 (M=71; F=9)	41.6 (18-65)	34.9 (15-65)	

**, °, # statistically different as compared to TS% in subjects with wild type genotype.*

Discussion

Hemochromatosis is considered a typical disease candidate for population screening programs, although the real need for screening and the best screening strategy (phenotypic or genotypic) are still controversial. Several elements support the need for screening for hemochromatosis: the disease frequency, the long-lasting pre-symptomatic stage, the effective treatment and the availability of simple diagnostic tools. However, the genetic defect in HFE is common, but the clinical penetrance of the disease is variable and in some series rather low,[23-24] posing problems for population screening programs. In addition, there is no agreement on the best screening strategy and on the population to be screened.[24] Biochemical screening is based on transferrin saturation, but with the proposed cut-off of 45-50% has the drawback of identifying all the other cases of secondary iron overload.[25] The availability of tests to identify the HFE gene mutations has introduced the possibility of genetic screening. Pilot genetic screens have been performed in neonates and blood donors; all were based on identification of C282Y and H63D mutations without analyzing additional genetic defects.

The frequency of the C282Y genotype in Italy is low in different studies.[5] As expected, the C282Y allele frequency is higher in subjects with increased iron parameters than in controls[1,5] Although in this study the number of samples from South Italy was limited, a C282Y decreasing gradient from north to south was confirmed and the few C282Y homozygotes identified were all from the North. By comparing genotype and allele frequencies of mutations in this selected sample with those of unselected neonates[26] we found an enrichment of C282Y homozygotes and heterozygotes in the former group. We could not compare the frequency of H63D homozygotes because this was not tested in the neonates, but 5.1% of our selected sample were C282Y/H63D compound heterozygotes whereas only 1% of the neonates were.[26] One potential bias of this comparison is the difference in the geographical origin of the compared samples. However, when we compared blood donors from our study *vs* neonates all from North Italy,[26] the C282Y allele frequency was enriched approximately 4-fold. A comparison with other screening studies carried out in Northern Italy[27,28] (Table 3) confirms that there is 4.2-5.8-fold enrichment of the C282Y allele frequency among selected vs unselected blood donors,[27,28] confirming results in neonates. This enrichment is significant considering that it is related to increased iron parameters at a single determination. As expected, the allele frequency in the group with a confirmed increased TS at a second check is still further enriched. Indeed in a similar study of a French-Canadian population there was an even more significant enrichment of hemochromatosis-related genotypes (C282Y homozygotes, H63D homozygotes and compound heterozygotes) when comparing neonates with patients referred for iron overload.[29]

For the S65C allele, which is associated with low iron loading, the prevalence in our series is slightly higher than that previously observed in controls (2.0 vs 0.9%, if corrected for disequilibrium).[30] We found one H63D homozygote who was also heterozygous for E168Q in the HFE gene. To our knowledge E168Q had been previously identified in only two cases: in one Caucasian female from South Africa, with moderately elevated serum ferritin, who also was heterozygote for H63D[11] and in one C282Y/H63D hemochromatosis patient from the same northern Italian area as our case.[31] In the latter subject intra-familial segregation studies

demonstrated that E168Q was in *cis* with H63D, as was the case in our blood donor, making it difficult to speculate about individual contributions of H63D and E168Q to the hemochromatosis phenotype. It is likely that the H63D-E168Q allele can cause a hemochromatosis phenotype only if co-inherited with C282Y.[31] However further reports of E168Q mutants are needed to establish the role of this mutation. Except for H63D, S65C, E168Q and C282Y, no other HFE or TFR2 mutation was detected, suggesting that most mutations are rare, possibly confined to restricted geographical areas, as shown for Y250X[32] (*and unpublished results*), E168X and W169X.[33] Our data for Y250X are in agreement with published negative results, obtained when searching for this mutation in different series of patients.[34-36] Since the frequency of C282Y decreases in Southern Italy, we would expect that in this area other mutations contribute more significantly to the hemochromatosis phenotype. Although in our study the small sample size from the South did not allow us to identify any uncommon alleles, methods such as the strip assay used here, which enable the simultaneous identification of a large panel of known mutations, should be particularly useful in areas with a relatively low C282Y frequency such as the South of Italy or Greece.[7]

In our whole series 85% of the subjects studied had none of the well-known hemochromatosis-associated genotypes and about 50% were wild-type. These data are in agreement with a recent Italian study in which only 3/36 potential blood donors with biochemical evidence of iron overload and exclusion of secondary iron overload had a hemochromatosis-associated genotype.[28] When we considered the second determination of TS, 11/20 subjects with increased TS had a genotype at risk for iron overload. Indeed, transferrin saturation is significantly higher in C282Y homozygotes, H63D/C282Y compound heterozygotes and H63D homozygotes. Our findings highlight the limited specificity of altered iron parameters at a single determination for purposes of hemochromatosis screening in Italy, suggesting the existence of other genetic or acquired determinants of iron overload in this country.

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Pre-Publication report & Outcomes of Peer Review

Contributions

All the authors contributed to the design of the study and to the conceptions of the experimental work. All reviewed the manuscript and approved the final version.

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Disclosures

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In the following paragraphs, the Editor-in-Chief summarizes the peer-review process and its outcomes.

What is already known on this topic

HFE mutations are associated with HLA-related genetic hemochromatosis. Most of these patients are homozygous for the C282Y mutation, or C282Y/H63D genetic compounds. Evaluation of HFE mutations is currently a routine procedure in the differential diagnosis of parenchymal iron oveload.

What this study adds

This study confirms previous findings and provides the following new observations. It shows that rare genotypes may be associated with iron overload, and that several subject with evidence of iron overload do not have any known hemochromatosis genotype. In Southern Europe, genetic diagnosis of hemochromatosis requires great expertise.