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Original Article

C282Y and H63D Haemochromatosis Alleles in Egyptian Patients with Cirrhosis

Ahmad Settin[1], Tarek Al-Dosokey[1], Mohammad Al-Haggar[1], Mahmoud El-Bendary[2], Mohammad Ezz[2], Rizk El Baz[1], Amr El-Shahed[1], Rabab Abo-Al-Kassem[1]

[1]Genetics Unit and Pediatric and [2]Adult Hepatology and Tropical Units, Mansoura University Hospitals, Mansoura, Egypt

Abstract

Background and Study Aims
Hereditary haemochromatosis (HH) is a common inherited autosomal recessive disorder of iron metabolism characterized by excessive body iron accumulation starting in the liver and later in other organs resulting in liver damage and other clinical disorders. The aim of this study is to determine the frequency of C282Y and H63D mutations of haemochromatosis (HFE) gene among Egyptian patients with liver cirrhosis. This may be important for assessing the significance of such mutations as a risk factor for the development and severity of disease.

Patients and Methods
In a cross-sectional, case-control study, 46 children (<18 years) and 48 adult patients (>18 years) with liver cirrhosis in addition to 33 unrelated healthy control subjects were included in the study. Cases were recruited from the Hepatology and Tropical Medicine Units, Mansoura University Hospitals, Egypt. Alleles whether normal (wild) or mutant (C282Y, H63D) were checked in all included subjects using PCR followed by restriction enzyme (Rsa I and Bcl I) digestion with analysis of RFLP (Restriction Fragment Length Polymorphism) in addition to iron status evaluation for levels of serum iron, serum ferritin and total iron binding capacity.

Results
Frequencies of C282Y and H63D heterozygosity were 2.2% and 15.2% in cirrhotic children, 0 and 20.0% in cirrhotic adults versus 0 and 21.2% in controls, respectively. No statistically significant difference in allele frequencies was detected among the 3 studied groups.

Conclusion
H63D mutations are common in cirrhotics whether children or adults and also among non-cirrhotic control subjects in contrast to C282Y mutations. The clinical significance of such mutations on the risk of development and severity of cirrhosis in Egyptian subjects needs to be assessed in further studies.

Key words
Hereditary haemochromatosis, mutant alleles, liver cirrhosis

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Corresponding author
Ahmad A Settin, MD, Padiatrics and Genetics Department, Faculty of Medicine, Mansoura University, Mansoura University Children Hospital, Mansoura, Egypt, Tel.: +20106161303, Fax: +20502234092,
E-mail: settin@mans.edu.eg

INTRODUCTION

Hereditary haemochromatosis (HH) is not an uncommon disease affecting approximately 1/200 of caucasians; its prevalence approaches 0.3-0.8% in some studies. Furthermore, carriers of one mutant allele may reach up to 12-15% of population[1]. Mutations affecting the HFE (on chromosome 6) namely C282Y and H63D were recently reported to be mostly responsible for the disease phenotype, although up to 37 allelic variants are so far detected[2]. Association of C282Y homozygosity with HH is population-dependent. In European studies it ranges between 64% in Italians, 80-90% in English and French. In non-caucasian populations (African, Asian, South Pacific and Aboriginal Australians), C282Y mutation is either absent or of low frequency. Regarding H63D mutation, it is not associated with the same degree of iron overload and its frequency among HH patients is lower than the C282Y mutation. H63D frequency in HH patients and general population may be identical[3]. H63D penetrance appears to be low in European population (16%), moreover, the incidence of compound heterozygous genotype C282Y/H63D is postulated to have a reduced penetrance (0.44-1.5%) but it is higher if H63D or a genetically linked modifier did not contribute to the phenotype[4-6].

HH is an autosomal recessive disease resulting from abnormally high intestinal iron absorption leading to its accumulation first in the liver and later in other organs. Morbidity and mortality could be improved if the disease is detected early before the development of cirrhosis, and treated by regular venesection and/or administration of iron chelating agent[7,8]. Definition of iron overload could be based on biochemical tests as well as hepatic histopathological changes. Genotype/phenotype correlation and phenotypic expression even in a homozygote or compound heterozygote states are not constant and may be affected by age, gender and other environmental conditions[9,10].

To the best of our knowledge, no sufficient information was so far reported concerning haemochromatosis genotyping among Egyptian population. Taking into consideration the fact that liver

cirrhosis constitutes a major health problem among Egyptians of all age groups and is frequently attributed to environmental factors like malnutrition, hepatitis C virus (HCV) and bilharziasis, this work was planned to estimate the frequency of HFE gene common mutant alleles (C282Y and H63D) among Egyptian cirrhotic patients in both children and adult samples compared to healthy unrelated controls (in a cross-sectional case control study).

PATIENTS AND METHODS

A group of 94 cases with chronic liver disease ending in liver cirrhosis were enrolled: 46 cases aged less than 18 years (mean age 8.5 ± 4.4 years) and diagnosed as childhood cirrhosis and 48 cases aged more than 18 years (mean age 49.4±4.24 years). They were recruited from patients admitted and followed up at the Hepatology and Tropical Units in Mansoura University Hospitals which serve the central area of the Nile Delta region in Egypt.

Cases were diagnosed as liver cirrhosis based on thorough history and clinical examination, in addition to relevant investigations including liver biopsy pathology. Accordingly, cases with childhood cirrhosis were diagnosed as having cryptogenic cirrhosis (13 cases), autoimmune hepatitis (AIH, 24 cases) and metabolic liver cirrhosis (9 cases). On the other hand, all adult patients with cirrhosis had HCV infection in common (28 cases with pure HCV, 8 cases with hepatocellular carcinoma (HCC) on top, 7 were diabetics and 6 had hepatic schistosomiasis). Thirty three normal adult unrelated healthy subjects with negative family history of liver disease were enrolled as a control group. An informed written consent was obtained from all subjects after full explanation of procedures. A peripheral blood sample of 6 ml was taken; half left to clot to obtain serum for biochemical tests and the other half was put on EDTA for DNA extraction and purification.

Serum iron studies were done, namely, total serum iron (MMB-IRON kit TPTZ, Marmar Bio, INC, St. Petersburg, U.S.A), serum ferritin (IMX®SYSTEM FERRITIN kit Abbott Laboratories, Diagnostics Division, USA), and total iron binding capacity (IRON TIBC kit (Marmar Bio, INC, St. Petersburg, U.S.A) using the instructions recommended by the manufacturer, while transferrin saturation was calculated according to the formula: (serum iron / TIBC) X100.

DNA Extraction and Purification was done using Generation Capture Column Kit (Gentra Systems, USA) where a sample is applied directly to the purification matrix contained in a spin column. Cells were lysed upon contact with matrix releasing DNA which were then captured by the matrix material allowing wash of any contaminants like protein, haeme and RNA leaving only DNA. Finally DNA was released from the matrix using DNA elution solution and heat without the need for precipitation[11].

Characterization of C282Y and H63D alleles of HFE gene was done using the technique described by Lynas[12]. DNA amplification: Separate PCR reactions are conducted for the two mutations using the primers described by Feder et al.[2] A total PCR volume of 25 µL contains 100 ng of each primer (P1 and P2, 10 pomles/µl), 1× manufacturer's PCR buffer, 200 µmol/L each dNTP, 2 µL (~50 ng) DNA, and 0.4 U Taq polymerase enzyme (Qiagen, UK). After 2 minutes of initial denaturation at 94°C, 35 cycles of just 1 minute at 94°C and 1 minute at 58°C are conducted in a thermal cycler (Genius, Techne, UK).

Primers for Codon 282:

P1 (sense) 5'-TGGCAAGGGTAAACAGATCC-3'

P2 (antisense) 5'-CTCAGGCACTCCTCTCAACC-3'

Primers for Codon 63:

P1 (sense) 5'-ACATGGTTAAGGCCTGTTGC-3'

P2 (antisense) 5'-GCCACATCTGGCTTGAAATT-3'

Digestion of amplified PCR product using restriction enzymes: Restriction digests are performed directly in the PCR mixes by addition of 5 U Rsa I (codon 282 reactions) or Bcl I (codon 63 reactions) and incubating for 2 hours at 37°C or 50°C, respectively.

Restriction fragment length polymorphism (RFLP) analysis on agarose gel 3% digested DNA fragments were separated by electrophoresis, stained with ethidiome bromide and visualized with ultraviolet transilluminator. Amplification with the primers for codon 282 produced a PCR product of 390 bp fragment which was digested into 250 and 140 bp fragments in the wild allele but the mutant allele (Cys to Tyr) would be digested into 250, 111 and 29 bp; however 29 bp band was always invisible being lost early in the run (Figure 1).

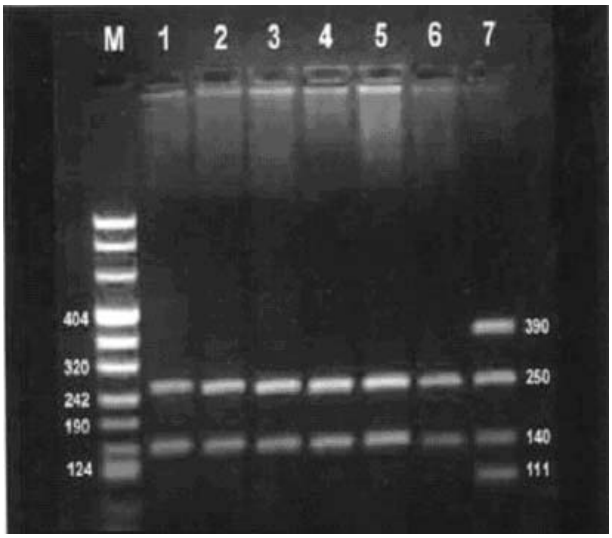

Figure 1: Detection of the HFE C282Y mutation by PCR-RFLP with C282Y primers and digested with Rsa1. Lane M; DNA marker, Lanes 1-6; Normal C282Y allele (250/140), Lane 7; Mixed samples of undigested amplified fragment with its digested products showing normal wild allele digested bands (250/140) as well as band of digested mutant allele (111 bp) while the other 29 bp fragment is invisible.

On the other hand amplification for codon 63 gave a 208 bp product which was digested into 138 and 70 bp fragments in the wild allele while the mutant allele (His to Asp) would resist digestion so its size remained as it is (208 bp) (Figure 2).

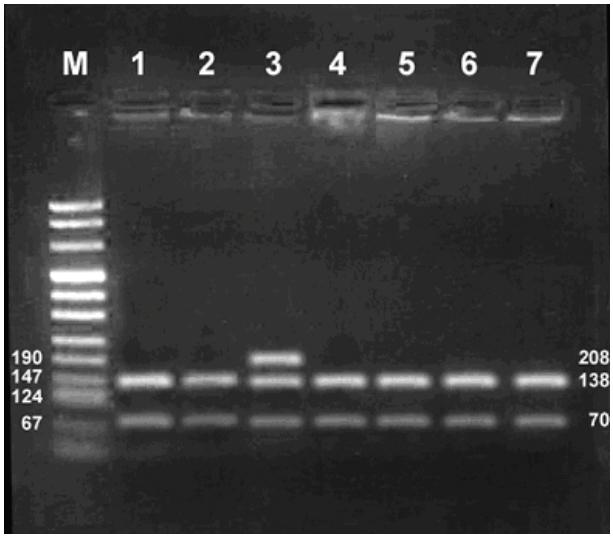

Figure 2: Detection of the HFE H63D mutation by PCR-RFLP with H63D primers and digested with Bcl 1. Lane M; DNA marker, Lanes 1, 2, 4, 5, 6 7; Normal wild allele digested bands (138/70), Lane 3; Heterozygote for H63D mutant allele showing normal digested bands (138/70) as well as the undigested amplified fragment (208).

Statistical analysis: Data were analyzed using SPSS version 10.0. Allele frequency was done using gene counting method (each individual is represented by 2 alleles). Genotype and allele frequency among different studied categories was assessed using Fisher's exact test, Chi square test Odds ratio and relative risk with 95% confidence interval. Analysis of difference between patients' groups and control regarding the iron status parameters was done using Mann Whitney U test.

RESULTS

The abnormal C282Y was not detected among adult patients or controls but in only a single cirrhotic child (2.2%) in a heterozygous state. Heterozygosity for H63D allele was noted to be higher in adult patients and controls (20 and 21.2% respectively) compared to paediatric cases (15.2%), without reaching statistical significance. Moreover, gene frequency of H63D allele was higher among controls (21.2%) compared to cases whether children or adults (7.6 and 8.3% respectively) (Tables 1 and 2).

Parameters of iron status showed no significant difference among the different studied groups (Table 3). However, the case with heterozygosity to C282Y allele showed high total iron binding capacity with normal serum iron, serum ferritin and transferringsaturation. The other cases with childhood cirrhosis showed no increase in serum iron, iron binding capacity or transferrin saturation. On the other hand, adult cirrhotic cases with heterozygosity to the allele H63D showed only increased serum ferritin.

Table 1: Frequency of haemochromatosis genotypes among cases of childhood liver cirrhosis compared to controls

Genotype/ Allele Frequency	Childhood Cirrhosis (n = 46)	Control (n = 33)	OR (95% CI)	RR (95%CI)
Individual genotype frequency				
N/N	38 (82.6%)	26 (78.8%)	1.28 (0.4-3.9)	1.11 (0.67-1.86)
N/282	1 (2.2%)	0 (0.0%)	2.21 (0.09-55.9)	1.73 (1.43-2.1)
N/63	7 (15.2%)	7 (21.2%)	0.67 (0.21-2.13)	0.89 (0.51-1.58)
Individual allele frequency				
N	84 (91.3%)	59 (89.4%)	1.25 (0.43-3.62)	1.1 (0.67-1.8)
282	1 (1.08%)	0 (0.0%)	2.0 (0.08-54.4)	1.72 (1.51-1.97)
63	7 (7.6%)	7 (11.2%)	0.69 (0.23-2.08)	0.85 (0.49-1.46)

N: Wild allele, 63:H63D allele, 282:C282 allele of HFE gene.
OR (95% CI): odds ratio and 95% confidence interval
RR (95% CI): relative risk and 95% confidence interval

Table 2: Frequency of studied haemochromatosis genotypes among cases of adult liver cirrhosis compared to controls

Genotype/ Allele Frequency	Childhood Cirrhosis (n = 46)	Control (n = 33)	OR (95% CI)	RR (95%CI)
Individual genotype frequency				
N/N	40 (83.6%)	26 (78.8%)	1.28 (0.41-3.9)	1.14 (0.68-1.9)
N/282	0 (0)	0 (0)	-	-
N/63	8 (20.0%)	7 (21.2%)	0.67 (0.21-2.13)	0.88 (0.53-1.49)
Individual allele frequency				
N	88 (91.6%)	59 (89.4%)	1.31 (0.44-3.79)	1.12 (0.69-1.84)
282	0 (0)	(0)	-	-
63	8 (8.3%)	7 (21.2%)	0.77 (0.26-2.23)	0.89 (0.54-1.46)

N: Wild allele, 63:H63D allele, 282:C282 allele of HFE gene.
OR (95% CI): odds ratio and 95% confidence interval
RR (95% CI): relative risk and 95% confidence interval

Table 3: Iron parameter in cases and control

	Iron (µg/d)	TIBC	Ferretin	TS (%)
Mean	88.2708	239.8750	230.8333	40.7071
Std. deviation	26.1320	48.1494	248.9380	17.3173
Control				
Mean	72.9091	251.5152	87.5758	32.9788
Std. deviation	12.9503	79.9657	12.6936	13.8672

TS: Transferrin Saturation

DISCUSSION

Cirrhosis is a dynamic process involving a combination of complex and intricate events that end in a common irreversible pathway for a variety of liver diseases. Unfortunately, cirrhosis may show a diagnostic difficulty[13] and is associated with high morbidity and mortality affecting persons during the most productive years of life[14].

In the past, schistosomiasis was the major public health problem in Egypt and nowadays chronic HCV is considered an additional major problem; the co-existence of the two disease processes constituted a significant morbidity and mortality in tropical settings leading to chronic liver disease and cirrhosis[15]. Epidemiologic study of HCV infection in the Nile delta region of Egypt showed that it is one of the highest prevalence rates of HCV worldwide since its discovery in 1989, with seroprevalence rates of up to 30-40% in villagers over the age of 30 years[16,17]. Risk of cirrhosis in HCV-infected was found four folds higher than non-infected persons (up to 52% of cirrhosis could be attributed to HCV), moreover, HCV proved to be a leading cause of liver cancer. To our knowledge only a few data exist about the contribution of genetically based metabolic diseases in chronic liver affection and liver cirrhosis among Egyptians raising the need for a correlative research study between these disorders and this growing national problem.

HH which is usually associated with HFE gene homozygous mutations is characterized by high intra-hepatic iron leading to accelerated liver injury, fibrosis and cirrhosis. In Germany, chronic HCV patients heterozygous for HFE mutations usually have high hepatic iron stores and advanced stages of fibrosis, therefore HFE mutations should be considered as important co-morbidity factors in chronic HCV infection[18].

Prevalence of C282Y homozygosity in patients with HH has been reported to be markedly lower in the Mediterranean basin than in northern Europe. Significant difference in mutation frequency among different Italian regions was noted with no difference in H63D allele frequency[19]. In Spain, no significant differences in phenotypic expression or in C282Y homozygosity frequency between patients born in northern and southern Spain were detected. Moreover genotypic/phenotypic expression of HH is similar to that reported in northern Europe[20].

Studies in Africa for C282Y and H63D gene frequency showed absence of C282Y mutation in Algeria, Ethiopia and Senegal supporting the Celtic origin of the disease, but H63D mutation although absent in Senegalese, was yet found in about 9% of the chromosomes genotyped among the central Ethiopians and Algerians[21]. In agreement with these results, our study revealed absence of C282Y mutation among our controls while H63D mutation has been detected in the heterozygous state among 21.2% of them. Our result as well as other reports from Africa concluded that H63D is not restricted to European populations.

In USA, C282Y and H63D heterozygotes were found among 5% and 15% of HH cases, respectively, similar to those found in control population and it was concluded that this finding should not be considered representative for HFE or iron overload prevalence in the general population[2]. Similarly in our study, 15.2% of childhood and 20.0% of adult liver cirrhosis cases were found to be H63D mutation carriers, while no adult case but only one cirrhotic child (2.2%) had C282Y mutation. These frequencies were not so far from that of the randomly selected unrelated healthy controls (21.2% and 0.0% for H63D and C282Y, respectively). Recently, H63D heterozygote and homozygote frequency was found in less than 5% of the general population and tend to be associated with liver cirrhosis irrespective of viral aetiology; thus suggesting the necessity for H63D mutation screening among chronic HCV patients in Taiwan[22].

Also in our study among patients diagnosed as HCV or AIH, C282Y mutation was not detected. It was found only in one child with cryptogenic liver cirrhosis, whereas the same percent (16.7%) of HCV and AIH were carriers for H63D mutation (H63D/wild allele genotype). In agreement with the cohort study done in Washington, USA on chronic HCV patients having end-stage liver disease and undergone transplantation, HFE mutations (C282Y or H63D) were associated with more advanced fibrosis and cirrhosis than in those with the wild-type HFE[23].

Analysis of iron status parameters among our cases, carriers of HFE mutant alleles failed to give significant high levels giving the impression of the importance of HFE genotyping regardless the iron studies as also concluded by other authors[24-26] who reported that the C282Y mutation alone only leads to a mild increase in iron accumulation in the majority of the patients, with the exception of H63D/C282Y compound heterozygotes.

The presence of C282Y allele mutation even in the heterozygous state could be considered as a risk for liver cirrhosis especially in absence of other hepatopathic factors. However, the almost equal prevalence of H63D mutant allele among patients and control should suggest only a partial role for this allele that may be augmented by other genetic and environmental factors. Further studies are required to assess the clinical significance of such mutations on the risk of development and severity of cirrhosis in Egyptian subjects.

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