

Molecular Analysis of A1AT (S and Z) and HFE (C282Y and H63D) Gene Mutations in Egyptian Cases with HCV Liver Cirrhosis

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Abstract

Background. Alpha-1-antitrypsin (A₁AT) S and Z deficiency alleles and hemochromatosis (HFE) mutant C282Y, H63D alleles were reported to potentially affect the liver even if present in a heterozygous state. **Objectives.** This is a cross-sectional, randomized, case controlled study for evaluation of the frequency of these alleles in Egyptian patients with HCV liver cirrhosis and of their association with the disease. **Subjects.** This study included 48 cases with viral C cirrhosis recruited from the Hepatology Unit, Mansoura University Hospital, Egypt, and 70 unrelated healthy controls. **Methods.** PCR amplification of relevant gene segment followed by restriction enzyme digestion TaqI for detection of A1AT gene S & Z alleles, digestion with Rsa I and Bcl I for HFE gene C282Y and H63D alleles. These alleles were then characterized through analysis of resulting restriction fragment length polymorphism (RFLP). **Results.** Both heterozygous (MS) and homozygous (SS) genotypes were significantly more frequent in cases than in controls (P<0.05, RR= 2.23 and 2.17 respectively). Gene frequency of S allele was higher in cases than controls (P<0.05, RR=2.17). Homozygosity (ZZ) genotype, present only in cases (6.3% vs 0.0% in controls,) did not reach statistical significance. HFE gene heterozygosity for H63D allele was detected in 20.0% of cases and 21.4% of controls, whereas C282Y allele was detected neither among cases nor in controls. **Conclusion.** The presence of the relatively high frequency of A1AT S and HFE H63D allele carriers in Egyptian cases of HCV liver cirrhosis suggest the necessity to implement routine molecular analysis of these genes for detection of risk genotypes among affected families.

Key words

Alpha-1-antitrypsin deficiency - Pi alleles - hemochromatosis - mutations - liver cirrhosis - Egypt

Rezumat

Premize. Alelele S și Z ale deficitului de alfa-1-antitripsină (A1AT) și alelele mutante C282Y, H63D din gena hemocromatozei (HFE) au fost raportate a implica un risc potențial pentru hepatopatii chiar atunci când sunt prezente în stare heterozigotă. **Obiective.** Acest studiu randomizat, transversal, caz-controlat are drept scop evaluarea frecvenței acestor alelele la pacienții egipteni cu ciroză hepatică virală C și a asocierii lor cu afecțiunea hepatică. **Subiecți.** Studiul include 48 pacienți cu ciroză virală C recrutați din Departamentul Hepatologie, Spitalul Universitar Mansoura, Egipt, și 70 martori sănătoși, neînruțiți cu pacienții. **Metode.** Amplificarea PCR a segmentului relevant al genei a fost urmată de restricția digestiei enzimatică TaqI pentru detectarea alelelor S și Z ale genei A1AT, digestie cu Rsa I și Bcl I pentru alelele C282Y și H63D ale genei HFE. Aceste alele au fost ulterior caracterizate prin analiza polimorfismelor fragmentelor rezultate (RFLP). **Rezultate.** Atât genotipul heterozigot MS cât și cel homozigot SS au fost semnificativ mai frecvent prezente la pacienți decât la martori (P<0.05, RR= 2,23 și respectiv 2,17). Frecvența alelelor S a fost mai mare la pacienți decât la martori (P<0.05, RR=2.17). Genotipul homozigot ZZ prezent doar la pacienți (6,3% vs 0,0% la martori) nu a atins semnificație statistică. Heterozigotismul pentru alela H63D a genei HFE a fost detectat la 20,0% pacienți și la 21,4% martori, în timp ce alela C282Y nu a fost detectată nici la pacienți, nici la martori. **Concluzie.** Prezența unei frecvențe ridicate a purtătorilor alelelor S ale genei A1AT și H63D ale genei HFE la pacienții egipteni cu ciroză virală C sugerează necesitatea implementării unei analize moleculare de rutină a acestor gene pentru detectarea genotipului cu risc la membrii familiilor afectate.

Introduction

Alpha-1-antitrypsin (A₁AT) deficiency is a common inherited metabolic disorder characterized by retention of the liver-produced protein A₁AT in liver and low serum A₁AT level. This deficiency is common in adults with

decompensated liver disease and might be associated with premature development of pulmonary emphysema (1).

A₁AT gene is part of a gene cluster called SERPIN supergene (SERine Proteinase INhibitor) localized to chromosome 14q32.1. There are many inherited variants of A₁AT; Pi M and its subtypes is the most common allele, Pi S and Pi Z are deficiency alleles which vary widely in different populations. Genetic epidemiologic studies for carrier detection worldwide revealed a total population of 4.4 billion with at least 116 million carriers (Pi MS and Pi MZ) and 3.4 million deficiency allele combinations (Pi SS, Pi SZ and Pi ZZ)(2). Furthermore, A₁AT deficiency is found in different ethnics; Z allele is seen in 1-2% of US whites, 0.48% of Africa, 0.4% central Asia, 1.51% Australia and New Zealand and S allele in 2-4% of US whites, 3.1% of Africa, 0.43% in central Asia, 3.95% in Australia and New Zealand(3). Most adults with Pi ZZ genotypes presented with signs and symptoms of chronic obstructive pulmonary disease with evidence of cirrhosis in 10-40% of the affected especially among older men(4).

Hereditary hemochromatosis (HH) on the other hand, is not an uncommon disease, affecting approximately 1/200 Caucasians; its prevalence approaches 0.3-0.8% in some studies. In addition, carriers of one mutant allele may reach up to 12-15%(5). Mutations affecting the HFE gene (on chromosome 6) namely C282Y and H63D were recently reported to be mostly responsible for the disease phenotype although up to 37 allelic variants were detected(6). In non-Caucasian populations (African, Asian, South Pacific and Aboriginal Australians), C282Y mutation is either absent or has a low frequency. Regarding H63D mutation, it is not associated with the same degree of iron overload and its frequency in HH patients and general population may be identical (7).

There is presently insufficient information regarding the distribution of A₁AT and HFE gene mutations among Egyptian normal and diseased subjects. Taking into consideration the fact that liver cirrhosis represents a major health problem among Egyptians of all age groups and frequently is attributed to environmental factors such as malnutrition, HCV infection and shistosomiasis. This study was planned to assess the frequency of allelic mutations of these two metabolic disorders, namely S and Z alleles of A₁AT gene and C282Y and H63D alleles of HFE gene among Egyptian cases suffering from liver cirrhosis after HCV infection to test their association or contribution as a risk factor to the development of the disease.

Subjects and methods

This study was designed as a cross-sectional randomized case controlled study. A sample of randomly selected 48 cases with HCV liver cirrhosis were recruited from the cases admitted and followed in the Hepatology Unit, Mansoura University Hospital, the main referral site for the Nile Delta region of Egypt. Their age mean \pm SD was 49.4 \pm 4.24 years

and sex of 38 males and 10 females. They were diagnosed as HCV liver cirrhosis based on thorough history, clinical examination in addition to relevant investigations including liver function tests, virology and liver biopsy in addition to imaging studies. For genotype comparison 70 healthy unrelated adults were included as a control group. The controls as well as their families were healthy and free from any liver disease. This was mainly established through individual interview and not on documented laboratory investigation.

An informed written consent was obtained from all subjects after full explanation of procedures.

All subjects underwent A1AT and HFE genotyping as follows:

DNA Extraction and Purification. It was done using Generation Capture Column Kit (Gentra Systems, USA) where a sample applied directly to the purification matrix contained in a spin column; cells were lysed upon contact with matrix releasing DNA which is then captured by the matrix material allowing wash-out of any contaminants such as proteins, heme and RNA, leaving only DNA. Finally, DNA was released from the matrix using DNA Elution solution and heat without the need for precipitation.

Detection of A1AT gene mutations (8)

The Z (342Glu3Lys) and S (264Glu3Val) mutations in the α 1-antitrypsin gene were identified by multiplex PCR using the thermal cycler (Techne Genius, UK). 3 pmol of each primer (described below) and 0.5 U of Taq DNA polymerase (Qiagen, UK) were added to; 100 mg of DNA in 30 mL (final volume) of a solution containing 20mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM MgCl₂, and 200 mM each dNTP. Temperature cycling conditions were as follows: (a) initial 5-min denaturation at 94 °C; (b) 35 cycles of 1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C; and (c) a final extension for 10 min at 72 °C. The presence of either mutation destroys a TaqI restriction site in the respective PCR products. Fragments of (157+22) and (100+21) were detected for wild-M allele, 179 bp fragment for Z allele and 121 bp fragment for S allele. Fig.1 presents the classic wild A1AT genotype MM, as well as its heterozygote MS, double heterozygous SZ and homozygous ZZ cases compared to a DNA size marker.

Primers for S allele of A1AT gene:

PF (Forward) 5'-TGAGGGGAAACTACAGCACCTCG-3',
PR (Reverse) 5'-AGGTGTGGCAGCTTCTTGGTCA-3'

Primers for Z allele of A1AT gene:

PF (Forward) 5'-ATAAGGCTGTGCTGACCATCGTC-3'
PR (Reverse) 5'-TTGGGTGGGATTCACCACTTTTC-3'

Detection of HFE gene mutations (9)

Separate PCR reactions are conducted for the two mutations in a volume of 25 μ L containing 100 ng of each primer (described below), 1X manufacturer's PCR buffer, 200 μ mol/L each dNTP, 2 μ L (~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 (Techne, Genius, UK). Restriction digestion was performed directly

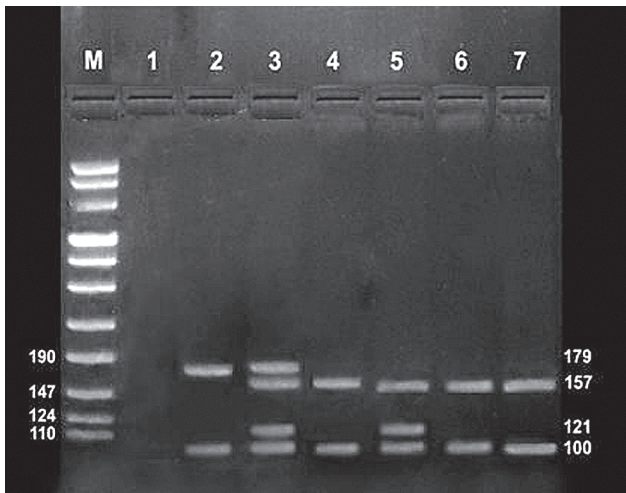


Fig.1 AIAT alleles S and Z genotyping using PCR-RFLP showing: Lane M: DNA Ladder as a marker, Lane 1: Water negative control, Lane 2: ZZ genotype (179 & 100bp band), Lane 3: SZ genotype (179, 157 & 121, 100bp bands), Lane 4, 6 & 7: MM genotype (157 & 100bp bands), Lane 5: SM genotype (157 & 121, 100 bp bands).

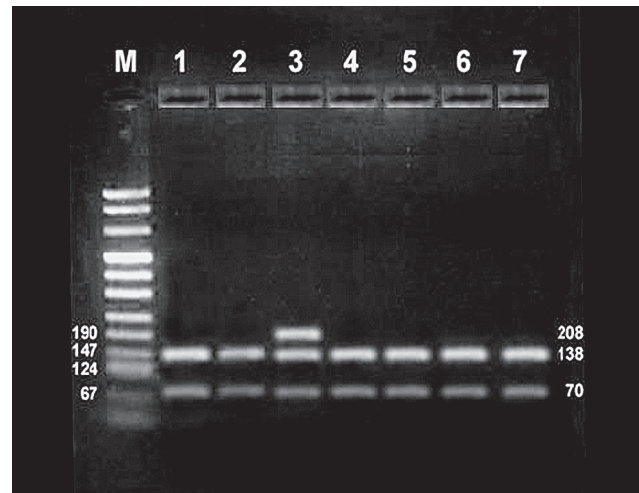


Fig.3 Detection of the HFE H63D mutation by PCR-RFLP with H63D primers and digested with Bcl I. Lane M; DNA marker, Lanes 1, 2, 4, 5, 6 7; Normal wild allele digested bands (138/70), Lane 3; Heterozygote for H63D mutant allele showing normal digested bands (138/70) as well as the undigested amplified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

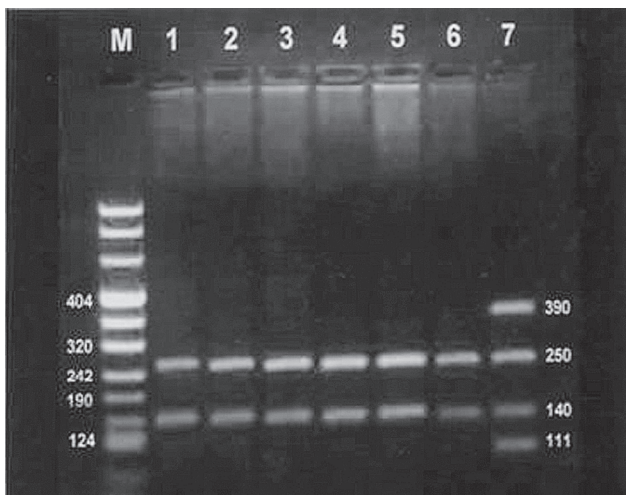


Fig.2 Detection of the HFE C282Y mutation by PCR-RFLP with C282Y primers and digested with Rsa1. Lane M; DNA marker, Lanes 1-6; Normal C282Y allele (250/140), Lane 7; Positive control mixed samples of undigested amplified fragment with its digested products showing normal wild allele digested bands (250/140) as well as band of digested mutant allele (111 bp) while the other 29 bp fragment is invisible.

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. The products are electrophoresed on a 3% agarose gel. Amplification with the primers for codon 282 produced a PCR product of 390 bp fragment which is digested into 250 and 140 bp fragments in the wild allele but the mutant allele will be digested into 250, 111 and 29 bp; however 29 bp band is always invisible being lost early in the run (Fig.2). On the other hand amplification for codon 63 gave a 208 bp product which is digested into 138 and 70 bp fragments in the wild allele

while the mutant allele will resist digestion giving the same size band (208 bp) (Fig.3).

Primers for Codon 282 of HFE gene:
 PF (Forward) 5'-TGGCAAGGGTAAACAGATCC-3',
 PR (Reverse) 5'-CTCAGGCACTCCTCTCAACC-3',
Primers for Codon 63 of HFE gene:
 PF (Forward) 5'-ACATGGTTAAGGCCTGTTGC-3',
 PR (Reverse) 5'-GCCACATCTGGCTTGAAATT-3'

Statistical analysis

Allele frequency was calculated 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, and relative risk ratio with 95% confidence interval using SPSS version 10.0 (10).

Results

Compared to controls, cases with HCV liver cirrhosis showed a significantly higher frequency of: a) SS homozygosity (10.4% vs 1.43%, RR=2.17, P<0.05); b) MS heterozygosity (31.3% vs 7.14%, RR=2.23, P<0.05); c) total heterozygous MS and MZ genotypes (33.3% vs 11.43%, RR=1.96, P<0.05); d) total homozygous and double heterozygous SS, ZZ and SZ genotypes (16.7% vs 2.86%, RR=2.16, P<0.05); e) S allele (26.04% vs 5.7%, RR=2.17, P<0.05) (Table I).

HFE gene analysis (Table II) showed that the abnormal C282Y was not detected among patients or controls (0%). Heterozygosity for H63D allele was noted to be similar in controls (21.2%) and in cases (20.0%).

Table I Frequency of the studied A1AT genotypes among cases of viral C liver cirrhosis as compared with controls

	Cirrhosis N (%)	Controls N (%)	RR (95% CI)
<i>Individual genotype frequency</i>			
Total	48 (100)	70 (100)	
MM	24 (50.0) *	60 (85.7)	0.4 (0.27-0.61)
MS	15 (31.3) *	5 (7.14)	2.23 (1.53-3.24)
MZ	1(2.1)	3 (4.28)	0.6 (0.11-3.36)
SZ	0 (0.0)	1 (1.43)	-
SS	5 (10.4) *	1 (1.43)	2.17 (1.42-3.33)
ZZ	3 (6.3)	0 (0.0)	2.56 (2.04-3.21)
<i>Total heterozygous</i>			
MS+MZ	16 (33.3) *	8 (11.43)	1.96 (1.31-2.92)
<i>Total homozygous and compound heterozygous</i>			
SS+ZZ+SZ	8 (16.7) *	2 (2.86)	2.16(1.45-3.21)
<i>Individual allele frequency</i>			
Total	96 (100)	140 (100)	
M	64 (66.7) *	128 (91.4)	0.46 (0.35-0.6)
S	25 (26.04) *	8 (5.7)	2.17 (1.66-2.84)
Z	7 (7.3)	4 (2.86)	1.61 (1.0-2.59)

RR (95% CI) : relative risk and 95% confidence interval

*p significant < 0.05 by Fisher's exact test

Table II Frequency of the studied HFE genotypes among cases with viral C liver cirrhosis as compared with controls

Genotype/ allele frequency	Cirrhosis N (%)	Controls N (%)	RR (95% CI)
<i>Individual genotype frequency</i>			
Total	48 (100)	70 (100)	
N/N	40 (83.6)	55 (78.6%)	1.21 (0.66-2.22)
N/C282Y	0 (0.0)	0 (0.0)	-
N/H63D	8 (20.0)	15 (21.43%)	0.83(0.45-1.52)
<i>Individual allele frequency</i>			
Total	69 (100)	66 (100)	
N (Wild allele)	88(91.6%)	125 (89.3%)	1.19 (0.66-2.13)
C282Y	0 (0.0)	(0) (0.0)	-
H63D	8 (8.33)	15 (10.71)	0.84 (0.47-1.51)

RR (95% CI) : relative risk and 95% confidence interval

Discussion

Presently, chronic HCV infection is considered as an additional major health problem to that of schistosomiasis in Egypt. The co-existence of the two diseases represents a significant morbidity and mortality in tropical settings leading to chronic liver disease and cirrhosis(11-13). Only 15–20% of people infected with HCV have an acute viral hepatitis episode, but the majority develop chronic hepatitis that is usually asymptomatic and undetected for many years. Over a course of 20–40 years, 20% of those with HCV chronic hepatitis progress to cirrhosis, and a proportion of these (2–3% per year) die as a result of complications of cirrhosis or hepatocellular carcinoma.

It had been documented in nationwide prospective screening studies that only 10-15% of Pi ZZ population develop clinically significant liver disease in the first 20 years of life, however 85-90% had elevated serum transaminases in infancy without any obvious liver injury by age of 18, while heterozygous patients of Pi Z allele still bear an increased risk for chronic liver disease (14,15). An significantly increased prevalence of MZ in patients with cryptogenic cirrhosis and with chronic active hepatitis was also reported. Therefore, authors recommended serum analysis for the MZ phenotype and meticulous examination of biopsy specimens as serum levels of A1AT may be unreliable for identification of the subgroup of patients with chronic active hepatitis or cryptogenic cirrhosis (16). An interactive and synergistic role of A1AT heterozygosity for Z allele (MZ) and HCV infection causing enhanced liver damage was also documented (17,18). In another study, it was demonstrated that hepatic dysfunction could develop as early as 6 month of age among MS heterozygotes with subsequent development of cryptogenic cirrhosis between ages of 1 month and 18 years(19).

Regarding distribution of A1AT alleles in the Egyptian population, there are no large Egyptian studies published sofar. Nevertheless, the rates we found among normal controls were more or less conforming with those reported by DeSerres et al (2) in his worldwide study of A1AT alleles including our Mediterranean Region.

In the present study, cases with post HCV liver cirrhosis showed a significantly higher frequency of S allele homozygosity (SS genotype) and also S and Z heterozygosity (MS and MZ). On the other hand, homozygosity for Z allele (ZZ genotype) although did not reach statistical significance, probably due to the small number of patients, was detected only among cases and not in controls.

Analysis of HFE gene mutations among cases with liver disease is recommended because evaluation of iron status parameters among carriers of mutant alleles may fail to give significant high levels (20,21).

Studies in Africa for HFE gene C282Y and H63D allele frequencies 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 found in about 9% of the chromosomes genotyped among the Central Ethiopians and Algerians(22). In agreement with these results, our study revealed absence C282Y mutation among our cases and controls while H63D mutation has been detected in the heterozygous state among ~20 % of them. Our result as well as other reports from Africa concluded that H63D allele is not restricted to European populations.

Recently in a study in Taiwan, H63D heterozygote and homozygote frequency was found in less than 5% of the general population and tended to be associated with liver cirrhosis irrespective of viral etiology; thus suggesting the necessity for H63D mutation screening among chronic HCV patients (23,24).

In conclusion, the increased frequency of mutant A1AT deficiency alleles together with the existence of HFE mutant alleles among HCV liver cirrhosis cases may warrant us to do further studies assessing their relevance for risk stratification for the development of liver cirrhosis. For mutation positive cases we would recommend genotyping of other family members for adequate prophylaxis regarding life style - exercise, healthy food etc - and environmental sanitation.

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