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Original article

# Hemochromatosis C282Y gene mutation as a potential susceptibility factor for iron-overload in Egyptian beta-thalassemia patients

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ABSTRACT

*Background:* Hereditary hemochromatosis is the most frequent cause of primary iron overload that is associated with HFE gene's mutation especially the C282Y mutation. The interaction between hemoglobin chain synthesis' disorders and the C282Y mutation may worsen the clinical picture of beta-thalassemia major (β-TM).
*Aim:* To establish the prevalence of the C282Y mutations in Egyptian β-TM patients and to address its adverse effects.
*Methods:* Two-hundred and five β-TM patients were recruited and divided into two groups based on their serum ferritin (SF); group I (N = 125) (SF ≤ 2500 ng/dl) and group II (N = 80) (SF > 2500 ng/dl). All patients were subjected to clinical and laboratory assessment with special emphasis on iron overload complications. Genotyping was assessed by polymerase chain reaction for detection of C282Y mutation in HFE gene.
*Results:* The C282Y mutation was not detected in the studied β-TM neither in homozygous nor heterozygous state. There were several iron overload complications including cardiac complication (9.1%), liver disease (36.6%), delayed puberty (56.6%), primary (35.71%) and secondary amenorrhea (21.42%), short stature (27.3%), diabetes (3.4%), neutropenia (9.7%), arthralgia (10.2%), gastrointestinal (21.1%), depression (2.9%) and others (12.05%). Group I showed a statistically significant lower rate of taking iron-rich diet when compared to group II. Group II showed significant longer mean duration of disease, higher total transfusion rate per life, lower mean HbF% level, higher mean HbA% level, and higher rate of elevated liver enzymes than patients with SF ≤ 2500 ng/dl.
*Conclusion:* The C282Y mutation was not detected in the studied cohort of Egyptian β-TM patients neither in homozygous nor heterozygous state in spite of manifestations of iron overload complications.


## 1. Introduction

Primary hemochromatosis can stem from inherited abnormalities in iron transport and regulation [1]. Hereditary Hemochromatosis (HH) is a recessive autosomal disorder involving iron metabolism leading to chronic inappropriately high rate of intestinal iron absorption for the degree of systemic iron stores [2]. The HFE hemochromatosis (HFE gene mutation) is considered the most frequent form representing >90% of HH cases [3]. Approximately 80–90% of clinical cases are homozygous for a cysteine-to-tyrosine substitution at amino acid position 282 in the HFE gene (C282Y mutation) that disrupts the binding of the HFE gene with beta2 microglobulin and prevents its surface expression [4]. The frequency for the C282Y allele ranged from an average of 6% for European to virtually zero in Asian and African populations [3] but is responsible for 60% of the HH cases in the Mediterranean population [5].

In thalassemia major, HFE mutation could significantly increase or severely aggravate the iron overload [6]. In order to establish the prevalence of the C282Y mutation among the Egyptian β-TM patients and to address its adverse effects, we have studied the molecular background of HFE gene (C282Y mutation) mutation in 205 Egyptian β-TM patients.

## 2. Patients and methods

This cross-section study had been conducted, over a two-year period, at Hematology Oncology Department, Pediatric Hospital, Ain Shams University in Cairo, Egypt. Two-hundred and five

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patients with transfusion dependent β-TM, were recruited. The diagnosis of the recruited patients was confirmed by qualitative and quantitative analysis of hemoglobin using high performance liquid chromatography (HPLC) using D-10 (BioRad, Marnes La Coquette, France). They were 111 females and 94 males, their mean age was 12.42 ± 7.35 years, the youngest was two years and the oldest was 36 years. The details of the study design and laboratory investigations were explained to all individuals and/or their parents, and verbal informed consent were obtained from each patient or their legal guardians before enrolment in the study. The study was approved from the institutional regulatory board of Pediatric Hospital, Faculty of Medicine, Ain Shams University and were in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki) for experiments in humans, 1975.

All patients were subjected to retrospective review of the filing system (patient specific record-keeping system for Hematology Oncology Department) and self-reported history from the patients and/or their parents during their follow up visits emphasized on demographic data, dietetic habit of taking high iron containing diet [7], transfusion and chelation history. Thorough clinical assessment with special emphasis on any complications of iron overload was done.

Laboratory investigations included complete blood count (CBC) with examination of Leishman-stained smears for red blood cell (RBC) morphology and differential white blood cell (WBC) count, with calculation of the mean pre-transfusion hemoglobin level over the last six months prior to the study, kidney function test (serum creatinine), liver function tests (alanine transaminase, total and direct bilirubin), and random blood sugar were performed. Serum ferritin (SF) concentration (chemiluminescence principle (Im – Multie)) was performed and patients were divided into two groups according to their SF level; Group I: βTM had SF levels ≤2500 ng/dl and Group II: βTM had SF levels > 2500 ng/dl.

Five milliliters of blood were withdrawn on five milliliters EDTA vacutainer (BDH) under complete aseptic technique and stored at −20 c until genotyping was performed for C282Y mutation detection as follow:

- Genomic DNA was extracted from peripheral leukocytes by using (Wizard genomic DNA purification kit, Promega). According to manufacturer manual 300 μl of blood sample and 900 ml of lysis solution were incubated for 10 min at room temperature, centrifuged at 13,000–16,0000 rpm for 20 s. The supernatant was discarded and 300 ml nuclei lysis solution were added and were mixed by inversion. Then protein precipitation solution was added and vortex mixed for 20 s, was centrifuged at 13.000–16.000 rpm for 3 min.
- To precipitate the DNA, the supernatant was transferred to a new tube containing 300 ml iso-propanol and mixed gently. The mixture was centrifuged 13.000–16.000 rpm and the ethanol was aspirated followed by air drying of the pellet (10–15 min). DNA was rehydrated in the 100 ml of DNA rehydration solution for 1 h at 65 °C. The dissolved DNA was stored at −20 °C till the next step.
- PCR amplification of targeted DNA fragment was done using ready Mix ™ Taq PCR reaction mixture (Sigma–Aldrich Biotechnology LP.) as follows:
  - PCR Master Mix was thawed at room temperature, vortex mixed and then centrifuged briefly and the PCR mix was prepared on ice according to manufacturer. Two primers sets were used for DNA amplification according to Oliveira et al., 2006 [8]: 5′GGGTATTT CCTTCCTCCAACC3′ and 5′CTCAGGCACTCCTCTCAACC3′
  - The amplification PCR program (PCR Hypaid Sprint thermal cycler), was performed according to manufacturer manual, and generated an amplified PCR fragment of 441 bp in length for the fragment harboring C282Y gene mutation. The amplified PCR products was then digested using RsaI restriction enzyme (Promega Corporation kit, Part# 9PIM712, USA) and the C282Y gene mutation created a new RsaI site. The digested PCR products were cut into two fragments (145 and 296 bp) in the normal allele, while in the mutated DNA allele three fragments (29, 116, and 296 bp) were generated [8].
  - The digested PCR products were analyzed on 3% agarose gel electrophoresis and the samples were run at 80 V for 45 min. Afterward, the digested PCR products were examined under ultraviolet trans-illuminator and the size is recorded in relation to the ladder and to the bands co-running samples. Photography of the gel was done using digital camera for demonstration of the results and for future follow up.

*Statistical analysis*

Analysis of data was done using IBM Statistical Program for Social Science for Windows, Version 20 (Armonk, NY: IBM Corp 2011). Quantitative variables were described as mean, standard deviations (SD), median, and range, and qualitative variables were described as number and percentage. Comparison between two independent groups with quantitative data and parametric distribution was done by using Independent *t*-test. The $\chi^2$ test was used to detect the difference in proportions between the groups. Pearson correlation coefficients were used to assess the relation between two studied parameters in the same group. The confidence interval was set to 95% and consequently the p-value was considered significant at the level of <0.05.

## 3. Results

As regards the demographic data of the studied cohort, 30.2% were third in order of birth or higher owing to the national high birth rate, moreover there was a high prevalence of consanguineous marriage (61.5%) and a high rate of positive family history (59.5%) among the studied patients. The age distribution of the studied patients relatively represent non-homogenous pattern in the various age group and most of patient's age were between ten and twenty years (39%).

There was a positive correlation between patient's age and both total transfusions rate given per life (r = 0.88, $p < 0.0001$) and transfusion index (r = 0.14, $p < 0.04$) as illustrated in (Fig. 1). As regards the different type of chelating agents used during the cohort, the main chelating agent used during the study was oral iron chelator deferiprone (DFP) (65.3%), 102 patients treated with dose of 50 mg/kg/day, 21 patients were on dose of 75 mg/kg/day and ten patients were on dose of 100 mg/kg/day. Deferoxamine subcutaneous (DFO SC) on dose of 40 mg/kg/day for five days per week was used as a single iron chelator agent in 27.3% of patients, and in combination with deferiprone in 3.9% of patients. Only three of our patients (1.5%) did not receive chelation therapy.

Group II (βTM with SF levels > 2500 ng/dl) showed a statistically significant longer mean duration of disease and higher total transfusion rate per life when compared to group I as illustrated in Table 1. Group I (βTM with SF levels ≤2500 ng/dl) showed a statistically significant lower rate of taking iron rich diet (62.4%) when compared to group II (86.3%) ($x^2 = 13.68$, $p = <0.0001$). Group II showed a statistically significant lower mean hemoglobin F% level and higher mean hemoglobin A% level when compared to group I. Furthermore, group II showed statistically significant higher rate of elevated liver enzymes ($P < 0.05$) and higher random blood sugar level.

As regards the frequency of observed iron-overload complications; twenty patients had cardiac complications in the form of

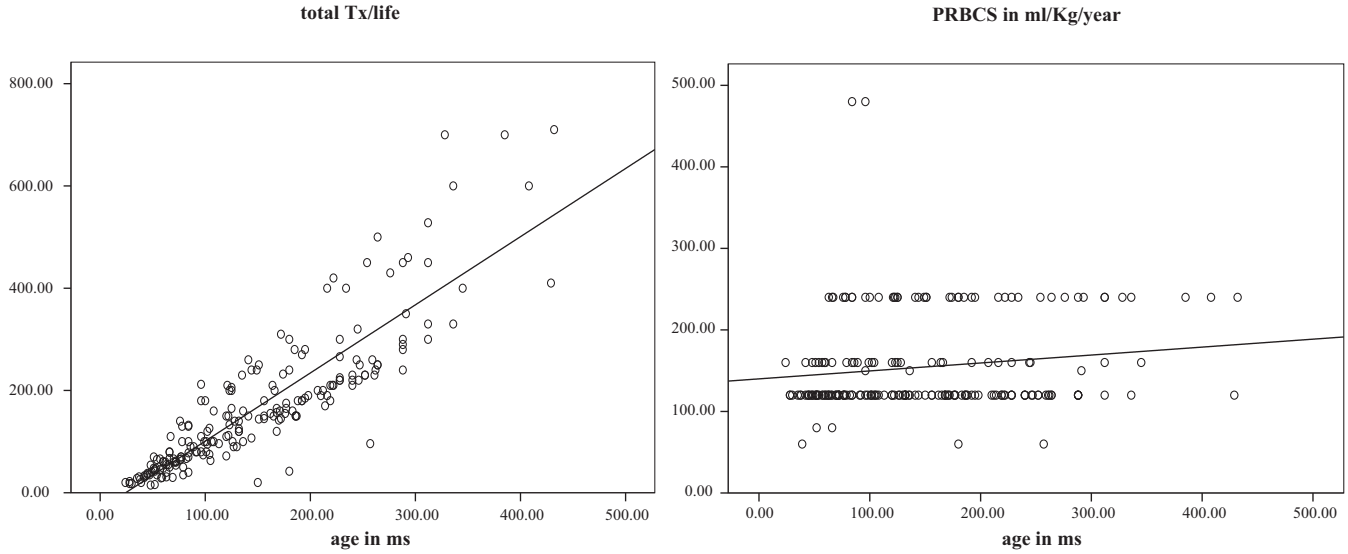


**Fig. 1.** Scatter diagram of the correlation between the age of the studied patients and [A] total transfusions per life and [B] transfusion index.

**Table 1**
Comparison between group I and group II according to the studied parameters.

| Variables; Mean ± SD | Group I (Serum Ferrtin ≤ 2500 ng/dl) (N = 125) | Group II (Serum Ferrtin > 2500 ng/dl) (N = 80) | *t* | *p* |
|---|---|---|---|---|
| Age (months) | 139.70 ± 86.71 | 163.73 ± 88.94 | 1.92 | 0.06 |
| Age at first transfusion (months) | 15.92 ± 14.33 | 17.76 ± 19.56 | 0.78 | 0.44 |
| Duration of disease (months) | 122.40 ± 82.96 | 147.07 ± 85.39 | 2.05 | 0.04 |
| Frequency of transfusions (months) | 1.24 ± 0.46 | 1.36 ± 0.53 | 1.75 | 0.08 |
| Total transfusions/life | 150.46 ± 126.13 | 191.24 ± 142.87 | 2.14 | 0.03 |
| Transfusion index (ml/kg/year) | 148.72 ± 55.72 | 163.50 ± 63.61 | 1.75 | 0.08 |
| Age at start chelation (months) | 50.14 ± 40.11 | 53.05 ± 48.99 | 0.46 | 0.64 |
| Duration of chelation (months) | 31.04 ± 36.57 | 40.05 ± 48.17 | 1.52 | 0.13 |
| Hb F level (%) | 50.16 ± 23.12 | 43.33 ± 24.31 | 2.02 | 0.04 |
| Hb A level (%) | 44.50 ± 22.60 | 52.31 ± 23.52 | 2.38 | 0.02 |
| Hb A2 level (%) | 3.80 ± 2.47 | 3.28 ± 1.96 | 1.58 | 0.11 |
| Alanine aminotransferase (IU/L) | 44.88 ± 45.56 | 71.80 ± 65.48 | 3.47 | 0.001 |
| Total bilirubin (mg/dl) | 2.65 ± 2.14 | 2.14 ± 1.48 | 1.86 | 0.06 |
| Direct bilirubin (mg/dl) | 0.34 ± 0.23 | 0.34 ± 0.25 | 0.08 | 0.94 |
| Indirect bilirubin (mg/dl) | 2.31 ± 2.08 | 1.77 ± 1.38 | 2.05 | 0.04 |
| Random blood sugar | 85.74 ± 14.32 | 93.57 ± 24.10 | 2.92 | 0.004 |
| Serum creatinine (mg/dl) | 0.34 ± 0.14 | 0.32 ± 0.11 | 1.33 | 0.18 |
| Mean pre-transfusion hemoglobin (mg/dl) | 7.59 ± 1.40 | 7.38 ± 1.33 | 1.11 | 0.27 |

heart failure [18(7.79%)] and two patients (0.98%) had arrhythmia. Viral hepatitis markers in the form of HBs Ag & HCV Ab were assessed in 176 patients only out of the 205 studied patients, seven patients tested positive for HBs Ag and 68 tested positive for HCV Ab, furthermore seventy-three patients had hepatic complications in form of mild (3-fold), moderate (≥3-10-fold), severe (≥3-10-fold) increase liver enzymes [22(10.73%), 33(16.10%), 18(8.78%) respectively].

Seventy-six patients were eligible for assessment of puberty; of them 43 had delayed puberty (56.58%). Ten female patients out of the 28 who were supposed to have their menstruation had primary amenorrhea (35.71%). Ten patients had endocrinal complication; one patient (0.49%) had hypothyroidism, two patients (0.98%) had hyperparathyroidism and seven (3.41%) had diabetes mellitus. Fifty-six patients (32%) had short stature [their height were below the third percentile].

As regards the complications th7at might be due to iron chelation therapy; twenty patients had neutropenia in form of mild (ANC < 1500–>1000), moderate (ANC < 1000–>500), severe (ANC<5 0 0) [5(2.43%), 5(2.43%), 10(10.22%) respectively] and arthralgia were noticed in 23 patients (10.22%) and gastrointestinal tract upset were observed in 43 patients (21.1%).

As regards the hemochromatosis gene, the C282Y mutation was not detected in βTM patients neither in homozygous nor heterozygous state as all patients showed normal allele as shown in Fig. 2.

## 4. Discussion

The hereditary hemochromatosis is a recessive autosomal disorder involving iron metabolism and resulting from hemochromatosis gene (HFE) mutation [9]. It is considered the most frequent form representing >90% of HH cases [10] and approximately 80–90% of clinical cases are homozygous [3] and 3.6% are heterozygous for C282Y mutation [11]. In βTM, HFE mutation could significantly aggravate the iron overload [6]. In order to establish the frequency and address the adverse effects of the C282Y mutation, we have studied the molecular background of HFE gene (C282Y mutation) in 205 Egyptian βTM patients and we found that the C282Y mutation was not detected in our studied population neither in homozygous nor heterozygous state.

The current finding is in agreement with a previous Egyptian study on 59 βTM [12], and with others studies on 400 Iranian populations [6], limited group from Tehran region [13], and fifty Tunisian beta thalassemia patients [14], the authors did not find any

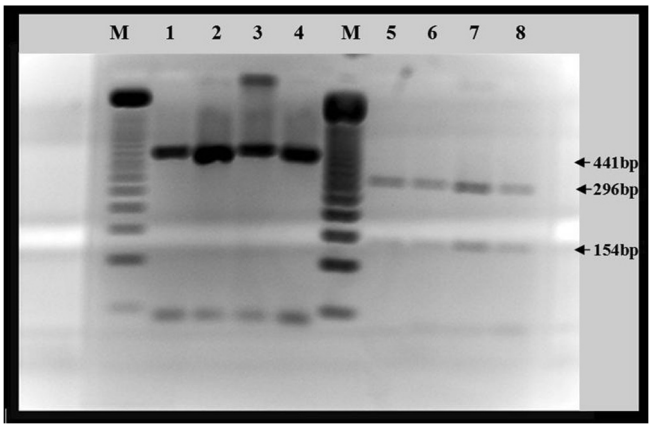


**Fig. 2.** Agarose gel electrophoresis for detection of the C282Y gene mutation yielded [Normal allele] M: Markers of 50 base pair (bp) ladder; Lanes 1–4: PCR products (441 bp) before digestion; Lanes 5–8: Digested PCR products yielded two fragments 145 bp and 296 bp [Normal allele].

case with C282Y mutation in the studied populations. Tunisian study [9] found one heterozygous case in 57 Tunisian subject and Arand et al., [11] reported 0% homozygous but 2% heterozygous state in 812 Spanish populations. This is possibly due to ancestral gene flow suggesting either a Celtic or Viking origin of the C282Y mutation [15] as the estimating allele frequencies of the C282Y mutation in the North European population is between 5 and 10% [16]. Oliveira and her colleagues [8] reported that the allele frequency of the C282Y mutation among 168 β T heterozygote and 173 normal individuals were 2.38 and 0.29% respectively and that high frequency of inheritance of this mutation among BTM patients may worsen their clinical picture

B-thalassemia, the most common genetic disorder in Egypt represents a major health problem with an estimated carrier rate of 9–10% [17]. It constitutes 39% of total hematologic patients and 84% of chronic hemolytic anemia in pediatrics patients attending Hematology and Oncology Clinic Children Hospital, Ain Shams University [18]. The age distribution in the study cohort relatively represent non homogenous pattern in the various age group and those older than 20 years has grown considerably (16.6%) this corresponds to the increasing life expectancy of the thalassemia patients worldwide. High consanguinity, high birth rate, together with limited prevention programs indicated by high incidence of positive family history and relatively high number of newly diagnosed cases aged 5 years or less (16.1%) representing the main factors contributing to relative high incidence of haemglobinopathies in Egypt. This is in the general agreement about factors contributing to high incidence of haemglobinopathies in Middle East Countries [19].

We reported high incidence of iron overload complications in this cohort without inheritance of the tested gene. This could be explained by poor compliance and irregularity of iron chelation therapy due to limited resources. We found high prevalence of cardiac events [9.76%]; heart failure was observed in 90% of them however five patients (27.8%) only need treatment with antifailure measures whereas the rest of them were suffered from anemic heart failure. Furthermore, liver disease of varying severity was noted in 36.6% of our patients and most of them had mild (29.3%) and moderate (44%) elevation of liver enzyme.

Serum ferritin was used for monitoring the efficacy of iron chelators and assessing the degree of iron overload. We found that patients with SF > 2500 ng/ml showed a statistically significant longer disease duration and higher total transfusion per life than patients in group I (SF < 2500 ng/dl) indicating that the hemosiderosis correlated positively with iron input. Patients with serum ferritin ≤2500 ng/dl had low incidence of taking rich iron diet. Consequently, the following manipulation of the diet to reduce the risk of iron loading were recommended and the diet should be varied and be rich in bread and cereals, and fruits and vegetables, with tea or coffee with meals (will reduce iron absorption) and with limited amount of meat (iron supplemented) and avoidance of ascorbic acid [20].

The rates of survival and complication-free survival continue to improve, due to better treatment strategies [21]. During the study period, we had only one patient died due to liver cirrhosis and liver cell failure; he was 14 years old with disease duration about 140 months that was not covered almost with effective chelation.

## 5. Conclusion

The C282Y mutation was not present in a cohort of studied Egyptian β-TM patients neither in homozygous nor heterozygous state in spite of manifestations of iron overload complications that warrant searching for other hidden causes.

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