Evaluation of the Association of Transferrin Receptor Type 2 Gene Mutation (Y250X) with Iron Overload in Major β-thalassemia

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Abstract
Thalassemia is an inherited blood disorder in which the body produces defective hemoglobin. One of the important processes to reduce the complication of major β-thalassemia is blood transfusion that leads to elevated ferritin levels in the blood. Many patients who had major β-thalassemia may hold hemochromatosis conditions resulting from iron metabolism disorders. In the patients who have β-thalassemia, the mutation Y250X in the TFR2 gene may have a role in the incidence of hemochromatosis. This study was aimed to determine the relationship between ferritin levels and Y250X mutation in major β-thalassemia patients. In the current study twelve blood samples were divided into nine major β-thalassemia patients and three healthy controls. DNA was isolated from blood samples and the amplification of the target region was performed base on the specific primers. Sanger sequencing was used to find genetic SNPs associated to iron overload. Blood parameters such as hemoglobin, MCV, MCH, and serum ferritin levels were analysed and the recorded data showed the following results; 8.1 ± 0.8 g/dL, 84.6 ± 5.5 fL, 27 ± 0.7 pg, respectively. Recorded data showed that the mean serum ferritin level in major β-thalassemia patients was 1921.7±848 ng/mL. Y250X mutation was not found in major β-thalassemia patients and healthy controls.

Keywords: β-Thalassemia, Hepcidin, Mutation, Ferritin

1. Introduction
β-thalassemia is one of the most predominant form of thalassemia in which the production of hemoglobin β chain is impaired quantitatively. β-thalassemia is characterized by anemia and depending on the severity of the anemia. Iron metabolism is a multi factorial process. This complex procedure is controlled by several mechanisms mainly targeting intestinal absorption. A key element in iron hemostasis is called Hepcidin, which is a small antimicrobial peptide and encoded by the HAMP gene on 19q13. It contains 2637 bp and comprises three exons (1).

Transferrin receptor type 2 (TFR2) gene is located at 7q22.1 produce a single membrane protein type II. The function of TFR2 is regulating iron enter to cell by binding once with transferrin protein and hepcidin enzyme according to concentrations of iron (1).

The Y250X mutation (604720.0001) is occurred in a region shared by both the alpha and beta transcripts of TFR2. Also it has been associated with type III hereditary hemochromatosis which caused by a homozygous nonsense mutation in the TFR2 (2). A transversion C/G at position 13451 of Homo sapiens transferrin receptor 2 (TFR2) accession number (NG_007989.1), lead to amino acid change tyrosine (TAC) at residue 250 of the transcript. These mutation form stop sign (TAG) and symbolize by (Y250X) (3).

The mutations of TFR2 gene may have role in hemochromatosis in humans. The relation of TFR2 and differentiation of the erythroid are observations in two point: first, the adjacency of EPO and TFR2 genes that may contribute to joint regulation and second the Single nucleotide polymorphisms (SNP rs80338880) in the TFR2 gene have been correlated to erythroid quantitative characteristics, such as erythrocytes account and hematocrit (4). Despite the fact that TFR2 is necessary for effective erythropoiesis, Tfr2 null mice and TFR2 hemochromatosis patients have functional erythropoiesis and may sustain several phlebotomy procedures without developing anemia complication (5).

Therefore, the current study was design to analyze the association between ferritin levels and Y250X mutation in major β thalassemia.

2. Materials and Methods
2.1. Patients
Blood samples were obtained from nine major β thalassemia patients and three samples as healthy individuals, from Samawah city, Iraq. All participants signed informed consent. Inclusion criteria were considered as regular transfusion (2-4 week interval), regular iron chelating therapy, and mean age above 5 years. Iron overload status was defined as ferritin level more than 1000 ng/ml as explained by TIF.
The diagnosis of thalassemia was based on the clinical manifestation and hemoglobin electrophoresis. Iron level was estimated by serum ferritin, in addition to hemoglobin, mean corpuscular volume and mean corpuscular hemoglobin.

2.2. Molecular analysis
Three mL of EDTA-blood was lysed with 12 mL of lysing buffer (NH4CL 150 mM, NaHCO3 10 mM, EDTA 1 mM) and centrifuged at 3000×g for 10 min. The supernatant was discarded and the WBC pellet was washed with normal saline twice. Genomic DNA was extracted from 0.2 mL of WBC with a commercial assay (GeneAll® Exgene™ Blood SV mini, Seoul; Korea) according to the manufacturer’s instruction.
Analysis of TFR2 gene Y250X mutation, twelve blood samples were subjected to DNA extraction using DNA Wizard Genomic Purification Kit (Promega, USA). PCR amplification of specific target region by GoTaq® G2 Master Green Mix (Promega, USA). The primers were used 1 μl of forward sequence 5’TGCACTGGGGTCGATGAG’3 and reverse 3’CTCAAGCCCTCCCTCCTCT’5. The thermal profile included one cycle at 95°C for 2 minutes (initial denaturation), and 35 cycles at 95°C for 60 seconds (denaturation steps), 56°C for 60 seconds (annealing steps), and 72°C for 60 seconds (synthesis), and one cycle at 72°C for 2 minutes (final synthesis).
The purification of PCR products were performed using Clean-Up system for PCR products (Wizard SV Gel, Promega, USA) in accordance with the manufacturer’s instruction manual. Genetic Analyzer 3137xl (Thermo Fisher Scientific, USA) was used to electrophoretic separated and detected of the purified PCR products in accord with the manufacturer's instruction. The DNA sequences quality had been manually inspected in the chromatogram, all samples.
The reference sequence of human transferrin receptor 2 (TFR2) accession number NG_007989.1 (www.ncbi.nlm.nih.gov) was aligned to the sequencing data by using Sequencer software v5.4.6 (Gene Codes, USA).

**Statistical analysis**

Statistical analysis was performed on IBM* SPSS* STATISTIC, 23.0 using independent t-test for the numeric variables and Fisher test for the nominal variables in which some categories had less than 5 cases. Any value less than 0.05 was considered statistically significant.

3. Results

The results of blood parameters, the mean serum ferritin levels were (1921.7 ± 848 ng/ml). HB, MCV and MCH were (8.1 ± 0.8 g/dL), (84.6 ± 5.5 fL), (27 ± 0.7 pg) respectively. The PCR product of Y250X mutation in TFR2 gene was selected on gel electrophoresis, the product size was 355 bp.

DNA sequencing was performed to the PCR products. The result of the sequence showed by alignment with the sequence with homo sapiens transferrin receptor 2 (TFR2), reference sequence gene at 7q22, accession number (NG_007989.1) indicated Y250X mutation was not found in major β-thalassemia patients and healthy control (all sample sequence wild type).

4. Discussion

Iron overload cause by defect in iron absorption pathway. Several studies reported the mutations in TFR2 gene induces increased intestinal iron absorption by increased iron uptake during diet and macrophage iron release leading to tissue iron overload (7, 8). Some study refer to effect of Y250X mutation on TFR2 gene may elevate iron level in blood (9). In major β thalassemia patients, the blood transfusion dilutes some complications of thalassemia syndrome while iron level raised (10). This mutation have important role in iron hemostasis in the body (2).

In this study, the Y250X mutation was absent in all samples. This result was agreement with study of Sun, Guo (11) that reported Y250X mutation in TFR2 gene does not occur in Tibetan patients with iron overload. Santos, Cancado (12) were described Y250X mutation not
detected of iron overload Brazilian blood donors. Moreover, Y250X mutation effect on clinical manifestations of iron overload. This document searched for the Y250X mutation in 63 individuals who have hereditary hemochromatosis. The results were most of patient have C282Y and H63D mutation but the Y250X mutation was not found in any of the 63 individuals that were tested (13).

In other side several studies reported Y250X mutation of TFR2 gene has a role in homeostasis of iron and the abnormal iron absorption, study of Kawabata, Fleming (14) found that homozygous TFR2 (Y245X) mutant mice (mirror to the TFR2(Y250X) mutation in humans) showed a hereditary hemochromatosis phenotype. Fleming, Ahmann (15) affirms the essential role for Y250X mutation in iron homeostasis and may consider a prognostic marker of abnormal iron absorption. The study of Piperno, Roetto (16) was two Italian patients who carried homozygous Y250X mutation, both cases have increased iron concentration in hepatic cells and elevate of serum ferritin.

In conclusion, from the above results it is revealed that there is not any association between transferrin receptor type 2 gene mutation (Y250X) with iron overload in major β-thalassemia patients.

Reference