Role of TfR2-Y250X and TfR1-rs3817672 Single Nucleotide Polymorphism on Pathophysiology of Iron Deficiency Anemia

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Abstract

Background: Transferrin receptor (TfR) is a carrier protein for transferrin. It is regulated in response to intracellular iron concentration and plays a role for the import of iron into the cell. The transferring receptor 2 (TFR2) gene showed homology to transferrin receptor 1 (TFR1) gene and encodes a transmembrane protein with a large extracellular domain, which is able to bind transferrin. Mutations in transferrin receptors (TFR2 and TFR1) may alter the pathophysiology of iron deficiency anemia. Alteration in genes encoding transferring receptor cause change in iron homeostatis and provides a tool for investigating the excess iron absorption and abnormal iron distribution in iron related disorders. However the clinical significance of the interaction of transferring mutations with iron deficiency anemia remains unclear. Thus, the objective of my study was to investigate the effect of TFR1 and TFR2 genotypes on pathophysiology of iron deficiency anemia.

Study Design: Study subjects were 460 iron deficiency anemia patients and 500 age and sex-matched healthy controls. Transferrin receptor, ferritin and CRP analysis was done by ELISA method while ESR analysis was done according to Wintrobe’s method. CBC analysis was done by auto-analyzer. TFR1-rs3817672 SNP and TFR2 (Y250X) mutation was analyzed by using PCR RFLP method.

Result: Amongst the iron deficiency anemia patients, 13 were heterozygous and five were homozygous for rs3817672 SNP. TFR2 (Y250X) mutation was detected in 6 patients with heterozygous conditions. None of the patients were presenting homozygous condition while four controls were presenting heterozygous and one with homozygous condition. Controls were presenting 3% and 0.6% of TFR1 rs3817672 SNP heterozygosity respectively.

Conclusion: TFR2-Y250X and TFR1-rs3817672 SNP showed clinical association with iron deficiency anemia and screening for mutations of TFR2 is a new diagnostic tool that can be offered to patients who do not have HFE mutations or who have incomplete HFE genotypes. This results may have practical implications for the molecular diagnosis of hemochromatosis. Genotyping the TfR gene should be included in the disease diagnostic protocols.

Introduction

Transferrin receptor is the main receptor for transferrin and allows transferrin bound iron uptake by the cell. Transferrin receptor gene expression is regulated by cellular iron requirements. TFR1 is a cell surface membrane protein and mainly involved in iron homeostasis by regulating cellular iron uptake in relations with the HFE protein.¹ A newly identified member of the transferrin receptor family is TFR2 which has moderate homology to TFR1.² The TFR2 gene organized into 18 exons and maps to 7q22, spans for approximately 21 Kb protein.³ Regulatory role for this molecule in iron absorption have been observed in patients with non-HFE haemochromatosis through mutational analysis of TFR2 gene.⁴⁻⁵ Both TFR1 and TFR2 are capable of transporting transferrin bound iron into the cell and supporting cell growth.⁶⁻⁷ However, their properties differ in several critical ways. TFR1 has a higher similarity for holotransferrin than does TFR2.²,⁴ The TFR2 mRNA expression prototype is quite different from TFR.⁹ In particular, TFR1 gene demonstrates minute hepatic expression whereas the TFR2 gene is expressed at much elevated levels in liver compared with other tissues. Both are fluctuate in their response to changes in cellular iron status. The TFR1 transcript contains multiple iron responsive elements in the untranslated regions which stabilize the TFR1 transcript under circumstances of low cellular iron whereas TFR2 transcript does not include these elements, and TFR2 message and protein levels vary mildly with changes in iron status.⁷⁻⁹ Since the initial report of the Y250X mutation, four additional TFR2-coding sequence mutations have been identified in patients with non-HFE-associated HH. These mutations are: E60X, M172K, AVAQ 594–597del, and Q690P. With the exception of Q690P, all of the recognized TFR2 mutations have been in pedigrees from Italy.¹⁰,¹¹ However the clinical importance of the interaction of transferring mutations with iron deficiency anemia leftovers ambiguous. Thus, the objective of my study was to investigate the effect of TFR1 and TFR2 genotypes on clinical and iron parameters in patients with iron deficiency anemia.

Material and Methods

Patient and Laboratory work

The subjects recruited for this study were presented Iron deficiency anemia and study was conducted in the department of Biotechnology APS University, India. Cases of IDA were diagnosed by means of iron profiling, whereas patients with thalassemia, hemoglobinopathies and other chronic disease were excluded from the study. Five ml venous blood sample were collected from the patients after they had signed an informed consent form. Complete blood count and red cell indices were measured by automated cell analyzer (SYSMEX K-4500 Kobe, Japan). Serum transferrin
Isolation of DNA was done by using a kit (Bioserve). TFR2-Y250X mutation was performed by PCR-RFLP method according to published literature.12 Identification of TFR1 SNP rs3817672 was done by using PCR-RFLP method. A 591 bp fragment containing rs3817672 SNP amplified using sense primer 5’CCAGGAGGACCTCTGGC and antisense primer 5’GATACTGACTGGCCTAGGG. Restriction enzyme (NlaIV) was selected with the help of NEB cutter software. Restriction enzyme was used according to the manuals of the manufacturer. Taq DNA polymerase and DNTPs were obtained from NEB, oligonucleotide were procured form Sigma Eldritch Company and used 25 pm concentration. 1.5U/µl Taq polymerase while 0.2mm/µl DNTPs each and 1.5mm/ µl mgcl2 were used for 50µl reaction. Amplification was performed using BIO-RAD thermocycler machine. A total of 35 cycle were performed using the following conditions; 94°C, 1 min.; 60°C, 1 min; and 72°C, 2 min. with an additional 5min. Extension at 72°C, in the final cycle. Agarose gel picture of amplified TFR1 gene and restriction digestion fragments are given in Figures 1 and 2 respectively.

Statistical analysis
Mean values, standard deviations, and frequency distributions were used to evaluate the biochemical and clinical data. Student’s t-test was used to compare the means of groups using GraphPad software (version 3.06). P value < 0.05 was considered statistically significant.

Result
Blood samples were collected and characterized from a total of 460 patients of Iron deficiency anemia and 500 age- sex matched controls. Patient and controls were predominantly belonging in tribal groups of central state of India. A total 289 male and 171 female with a mean age of 23.5±2.8 and 20.1±3.2 years respectively, were recruited for the study. Out of 171 female, 67 were in gestation and 104 were in non gestation period. A complete blood count, Iron profile, CRP sTfR and ESR were performed in all subjects as well as in controls (300 male and 200 female with mean age 22.7±3.1 and 19.3±2.8 years respectively). TFR1 SNP rs3817672 primers was designed and characterized by using of PCR-RFLP method. DNA of rs3817672 resulted in a PCR product of 591 bp when visualized in agarose gel. NlaIV Restriction enzyme was used for TFR1 SNP rs3817672. Digested product was checked in 3% ethidium bromide containing agarose gel. After restriction digestion of TFR1 SNP rs3817672 PCR product, various fragmented products were seen in agarose gel which was track through double digested bromophenol blue dye. Three genotypic patterns were seen in studied subjects. A 93, 151, 347 bp and 93,151, 244, 347 bp product size were representing homozygous mutant and heterozygous conditions respectively while 244,347bp restriction fragments denote wild type homozygous condition. Amongst the IDA patients, 13 were heterozygous and five were homozygous for rs3817672 SNP. TFR2 (Y250X) mutation was detected in 6 patients with heterozygous conditions. None of the patients were presenting homozygous condition while four controls were presenting heterozygous and one with homozygous condition. Controls were presenting 3 % and 0.6% frequency of TFR1 rs3817672 SNP heterozygous and homozygous respectively. Comparative Iron profile, value of sTfR, CRP and ESR of mutant and wild genotype IDA patients is given in Table 1. Comparative frequency of clinical symptom is given in Figure 3.

Discussion
The role of TFR1 gene mutation in iron deficiency anemia was unclear. The role of TFR2 as a substitute for TFR1 in its lack is still vague. Due to severe iron deficiency, knockout of TFR1 causes embryonic lethality in mice, while TFR2 mutations in humans and targeted mutagenesis or failure of TFR2 in mice result in iron excess.13-16 These studies propose that TFR1 and TFR2 have different functions and that TFR2 is not capable to substitute for the absence of TFR1. It has been established that targeted deletion or mutationesis of the TFR2 gene in mice recapitulates the human iron overload disorder type-3 hereditary hemochromatosis.15,16 First relationship between TFR2 mutations
and iron overload was established in a study of Italian patients with non HFE linked HH.16 My study report significant elevation of serum ferritin and haemoglobin level in TfR(Y250X and rs3817672 SNP) heterozygous and homozygous genotype while decreasing level of CRP and ESR were reported. Value of these parameters is statistically significant. Level of sTfR and TIBC were elevated in TfR(Y250X and rs3817672 SNP) wild type genotype while % transferrin saturation level was elevated in mutant and p-values were statistically significant. In a study of united state of America have failed to identify patients with mutations in TfR2 coding sequences in patients with non HFE linked HH and suggested that the largely contribution of TfR2 mutations to the total number of cases of HH is stumpy.17 TfR2 mutations manifest the augmented transferrin saturations, elevated serum ferritin levels and ultimately periporal hepatic iron loading in patients which is also observed in the mutant mice.18 Prolonged iron overload manifest the end organ damage included liver cirrhosis, arthritis, diabetes, and hypogonadism. The normal erythroid parameters in the TfR2 mutant mice might be explained by the activity of TfR1, which also is highly expressed in erythroid precursors.19 Patients with homozygous for TfR2 (Y250X) truncation mutation had a clinical picture similar to HFE related HH, including hepatic iron loading.20 My study report 15 heterozygous and 3 homozygous of TfR1rs3817672 SNP genotype in controls while 0.91% and 0.22% control were presenting TfR2 (Y250X) heterozygous and homozygous conditions respectively. Amongst IDA patients frequency of heterozygous and homozygous was 2.98% and 1.14% respectively. Frequency of TfR2 (Y250X) heterozygous genotype was 1.36 % in iron deficiency anaemia. This observation suggested that frequencies of these mutations are higher in patients than controls. We observed higher frequency of TfR1rs3817672 SNP in patients as well as in controls groups in compression of TfR2-Y250X mutation. Various TfR2 gene mutations have been observed since in patients with iron overload and suggested that the iron homeostasis abnormalities are caused by functional loss of TfR2.13,14 These studies revealed that the roles for TfR1 and TfR2 in iron homeostasis are not superfluous due to TfR1 forms a complex with HFE, it seemed plausible that TfR2 may connect also with HFE. The practice of using a battery of assays improves the precision of defining iron nutrition in a population; however, 2 pitfalls persist to confound this issue: the complexity in precisely detecting mild iron deficiency and the identification of inflammation as a cause of changes in laboratory test results that are not due to iron deficiency.20 These pitfalls can be clarified by the serum transferrin receptor (TfR) assay thus it may act the predator of disease anemia and showed positive correlation. It may act the predator of disease severity. Data of the study provide a genotype-phenotype correlation of TfR gene mutation with iron deficiency anemia. Identification of TfR2 as the HFE3 gene is of relevance regarding modifier genes in hemochromatosis. The presents of modifier genes that modulate the disease severity, can be used a diagnostic tools in Iron deficiency anemia.

Table 1: Comparative iron profile, inflammatory marker and hematological profile of TfR (Y250X and rs3817672 SNP) genotype

<table>
<thead>
<tr>
<th>IDA patients (N=460) Mean ±SD</th>
<th>TFR2 - Y250X and TfR1 rs3817672 Genotype (+/- or -/+ N= 24)</th>
<th>TFR2 - Y250X and TfR1 rs3817672 SNP Genotype (+/-) N=436</th>
<th>SE</th>
<th>95% CI</th>
<th>P value</th>
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<tbody>
<tr>
<td>Serum Ferritin µg/L</td>
<td>12.2 ± 1.8</td>
<td>10.5 ± 1.7</td>
<td>0.358</td>
<td>0.997 to 2.403</td>
<td>&lt;0.001</td>
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<tr>
<td>TIBC µg/dl</td>
<td>455.3 ± 17.6</td>
<td>489.5 ± 81.4</td>
<td>16.653</td>
<td>-66.926 to -1.474</td>
<td>0.04</td>
</tr>
<tr>
<td>Transferrin saturation %</td>
<td>14.3 ± 2.9</td>
<td>11.4 ± 3.1</td>
<td>0.648</td>
<td>1.227 to 3.773</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>ESR mm/hr</td>
<td>18 ± 4</td>
<td>24 ± 3</td>
<td>0.641</td>
<td>-7.26 to -4.74</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>CRP mg/L</td>
<td>1.2 ± 0.7</td>
<td>1.8 ± 0.6</td>
<td>0.127</td>
<td>-0.849 to -0.351</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>HGB g/dl</td>
<td>81 ± 1.2</td>
<td>72 ± 1.5</td>
<td>0.312</td>
<td>0.28757 to 1.51243</td>
<td>0.004</td>
</tr>
<tr>
<td>sTfR mg/L</td>
<td>9.3 ± 3.3</td>
<td>11.02 ± 2.3</td>
<td>0.492</td>
<td>-2.867 to -0.933</td>
<td>&lt;0.001</td>
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SE = Standard Error, CI = Confidence interval

References