Comunicación breve

Intronic SNP rs3811647 of the human transferrin gene modulates its expression in hepatoma cells

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Abstract

Introduction: Transferrin (Tf) exerts a crucial function in the maintenance of systemic iron homeostasis. The expression of the Tf gene is controlled by transcriptional mechanism, although little is known about the influence of genetic factors.

Objective: To study the role of rs3811647 in Tf expression using an in-vitro assay on hepatoma cells.

Design and Methods: Hep3B cells were co-transfected with constructs containing A (VarA-Tf-luc) and G (VarG-Tf-luc) variants of rs3811647, using luciferase as a surrogate reporter of Tf expression.

Results: Luciferase assays showed a higher intrinsic enhancer activity (p < 0.05) in the A compared with the G variants. In silico analysis of SNP rs3811647 showed that the A allele might constitute a binding site for the transcription factor glucocorticoid receptor (GR).

Conclusion: The A allele of SNP rs3811647 increases Tf expression in a manner that might underlie inter-individual variation in serum transferrin levels observed in different population groups.

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Abbreviations

Tf: Transferrin.
GR: Glucocorticoid receptor.

Introduction

Transferrin (Tf) is an iron-binding plasma protein that delivers iron to cells via the transferrin receptor pathway.1 A molecule of Tf can bind two atoms of ferric iron with high affinity. Iron chelation by transferrin serves three main purposes: to maintain ferric iron in a soluble form under physiologic conditions; to facilitate regulated iron transport and cellular uptake, and to maintain ferric iron in a redox-inert state, avoiding the generation of free radicals.2 Moreover, diferric Tf stimulates hepcidin expression, the central regulatory molecule of systemic iron homeostasis, through a TIR2/HFE mediated pathway.3
The expression of the Tf gene is controlled by transcriptional mechanisms and is tissue-specific. Many environmental factors are known to affect plasma Tf levels: in iron deficiency, the rate of Tf synthesis in the liver increases significantly, whereas inflammatory or immunologic stimuli may decrease the levels of circulating Tf. Recent studies observed increased Tf levels under hypoxia, a response that may facilitate iron supply for erythropoiesis. Nevertheless, little is known about the genetic factors that influence Tf levels in humans, although its expression pattern appears to show sexual dimorphism. Our research group recently published that only a few SNPs could explain a large percentage of the heritable variation of serum transferrin levels: in iron deficiency, the rate of Tf synthesis in the liver increases significantly, whereas inflammatory or immunologic stimuli may decrease the levels of circulating Tf. Based on these studies, we hypothesized that SNP rs3811647 increases transferrin expression. Here we show that this SNP constitutes an intronic enhancer that modulates Tf expression in hepatoma cells.

**Design and methods**

**Plasmid constructs**

A fragment of approximately 500 bp of intron 11 of the human transferrin gene (Tf), encompassing the SNP rs3811647, was amplified from placental genomic DNA with the following primers: sense, CATGCTAGCGGCTTGACACACGGATTTTT; antisense, CATCTCAGATACTCAGTGGGAGTGGCAAGG. The XhoI and NheI restriction sites are underlined. The cycling parameters were: 95°C for 5 minutes, then 95°C for 5 minutes (denaturation), 62°C for 1 minute (annealing), and 72°C for 1 minute (extension); 35 cycles of PCR were performed with a final extension at 10 minutes at 72°C. The PCR product was subcloned into pGEM-T Easy vector (Promega, Southampton, United Kingdom) and sequenced for verification of the nucleotide sequence (MWG Biotech, Ebersberg, Germany) and to confirm the presence of the A allele. The construct was digested with NheI and XhoI (New England Biolabs, Hitchin, United Kingdom), and the insert was purified with GeneClean (BIO101; Anachem, Luton, United Kingdom) and ligated into the NheI and XhoI sites of pGL3Promoter (Promega) to generate VarA-Tf-luc.

**Site-directed mutagenesis**

VarA-Tf-luc was subjected to site-directed mutagenesis using the QuikChange Multi Site-Directed Mutagenesis system (Stratagene, Amsterdam, The Netherlands) as instructed by the manufacturer. To change the A to G alleles we synthesized a mutagenic primer (mutations in lowercase) as follows: GGGAGTTTACAGACA-GATCGTCTAGGATTATACATCTAGGAAGG.

After initial denaturation for 5 minutes at 95°C, PCR cycling parameters were 95°C (5 minutes), 55°C (1 minute), and 65°C (11 minutes and 12 seconds), for a total of 30 cycles. Plasmids were sequenced to verify that the intended mutation had occurred. The resulting construct was designated VarG-Tf-luc.

**Cell culture, transfection and luciferase assay**

The human hepatoma cell line Hep3B was obtained from Antonello Pietrangelo and cultured in Dulbecco’s Modified Eagle Medium (DMEM), supplemented with 10% foetal calf serum (FCS) and antibiotics/antimycotics (Invitrogen). Cells were grown under standard cell culture conditions of 37°C and 5% CO2; for transfection, cells were seeded in 24-well plates at densities of approximately 104 cells/well. Cells were transfected with 100 ng/well of VarA-Tf-luc or VarG-Tf-luc with Lipofectamine 2000 (Invitrogen), as instructed by the manufacturer. As internal control, 50 ng of pSV gal vector (Promega) was included in all transfections to normalize transfection efficiencies. Cells were harvested after 48 hours for reporter assays; luciferase activities were determined with the luciferase assay reagent and β-galactosidase (βgal) activity was measured using the Beta-Glo reagent (both from Promega). Luminescence was measured in a Tropix TR717 microplate luminometer (Applied Biosystems); luciferase levels were normalized with respect to βgal activity in the samples.

**In silico analysis**

For the prediction of putative transcription factor binding sites, a sequence of 25 bases either side of the SNP rs3811647 was submitted to a net-based search tool Patch 1.0 (http://www.gene-regulation.com/cgi-bin/pub/programs/patch/bin/patch.cgi).

Settings for core and pair similarities, matrix conservation, and factor class levels were adjusted according to factors predicted.

**Statistical analysis**

Pairwise comparisons of control and SNP constructs were made using ANOVA test. A P value of 0.05 was considered significant. Graphs were plotted with the GraphPad Prism software and data were analysed using the SPSS statistical package for Windows (version 19.0; SPSS Inc., Chicago, IL, USA).

**Results**

Using reporter assays in which fragments of the intron encompassing SNP rs3811647 were ligated to
firefly luciferase as Tf surrogate, we found that the A allele enhanced gene expression compared with the G allele. The fold activation of the VarA-Tf-luc construct was significantly higher (p < 0.05) than that of the VarG-Tf-luc construct (fig. 1). In other words, the A allele of the SNP rs3811647 supports higher Tf expression than the G allele in hepatoma cells.

In silico analysis of this SNP showed that the A allele in rs3811647 could theoretically constitute a binding site for the glucocorticoid receptor (GR), also known as nuclear receptor subfamily 3 group C member 1 (NR3C1), whereas this binding site is lost in the G allele (fig. 2).

Discussion

The obtained results can be validated by the findings that our research group obtained in a group of menstruating women. We found that serum transferrin was significantly higher in AA homozygous women than in AG heterozygous and GG homozygous (p < 0.01), and serum transferrin saturation was significantly higher in GG than in AG and AA women (p = 0.01). Also, the in-vitro results confirm previous observations in different population groups and add new information concerning the functionality of rs3811647. We therefore suggest that basal differences in circulating Tf levels between individuals may be ascribed to SNP rs3811647, which is located in intron 11 of Tf gene (Chr3q22.1).

The tissue-specificity of Tf expression is accomplished by the recruitment of different combinations of transcription factors. In hepatocytes, binding sites of transcription factors that are well-known to regulate Tf expression have been described. Proximal region I (PRI) and proximal region II (PRII) within the Tf promoter positively regulate its expression in the liver whereas distal regions repress Tf expression. However, our study shows that in addition to the positive regulation of Tf by proximal promoter elements, there are intronic elements such as rs3811647 that could act as enhancers of Tf transcription. This is intriguing because no known function especially in relation to relative risk would have been predicted for intronic sequences, considering that disease associations have hitherto been limited to coding-region mutations only. We found that presence of the A allele of rs3811647 might constitute a binding site for the GR.

Glucocorticoids influence the expression of a number of genes involved in iron metabolism including ferritin, ferroportin, DMT-1 and iron regulatory protein-1. GR might therefore regulate glucocorticoid-dependent differences in Tf allele expression; further studies will ascertain this. Although it is more frequent to find regulatory regions upstream of the start site of transcription, in some cases transcription factors are able to drive gene expression from within coding regions. It is therefore not entirely surprising that we found regulatory regions within the Tf intron.

In our previous study, the women that presented the A allele also had lower transferrin saturation, which may indicate a reduction in iron transport to tissues. Since low ferritin levels have been associated with this
SNP, it could be related to low iron status. Another important observation was made in a placebo-controlled nutritional intervention study an with iron-fortified food in iron-deficient women. Dietary iron-fortification markedly increased the iron status in all women. However, carriers of the minor A allele showed Tf levels higher than the rest during the 16-week intervention period. All of these observations suggest that this SNP may affect iron metabolism.

In conclusion, we found that the A allele of the SNP rs3811647 enhances Tf expression compared with the G allele, and that this might explain the association between this SNP and the high serum Tf levels observed in different population groups.

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References