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# 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 genetic factors influence.**

***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 variant. 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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Key words: *Transferrin gene. SNP rs3811647. Serum transferrin. Iron metabolism. Gene expression.*

## EL SNP INTRÓNICO rs3811647 DEL GEN DE LA TRANSFERRINA HUMANO, MODULA SU EXPRESIÓN EN CÉLULAS HEPÁTICAS

## Resumen

***Introducción:*** **La transferrina (Tf) ejerce una función crucial en el mantenimiento de la homeostasis sistémica del hierro. La expresión del gen de la transferrina es controlada a nivel transcripcional, aunque la posible influencia de factores genéticos todavía es desconocida.**

***Objetivo:*** **Estudiar el papel del rs3811647 en la expresión de la transferrina mediante un ensayo *in-vitro* en células de hepatoma.**

***Diseño y métodos:*** **Células Hep3B fueron co-transfectadas con vectores que contenían las variantes A (VarA-Tf-luc) y G (VarG-Tf-luc) del rs3811647, utilizandose la luciferasa como marcador de la expresión del gen Tf.**

***Resultados:*** **Los ensayos con la luciferasa mostraron un mayor aumento de la expresión del gen Tf en presencia de la variante A comparada con la G ($p < 0,05$). El análisis in silico del SNP rs3811647 mostró que la presencia del alelo A puede constituir un sitio de unión del receptor de glucocorticoides (GR).**

***Conclusión:*** **El alelo A del SNP rs3811647 incrementa la expresión del gen Tf de modo que podría modular la variación interindividual en los niveles de transferrina sérica observados en diferentes poblaciones.**

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Palabras clave: *Gen de la transferrina. SNP rs3811647. Transferina sérica. Metabolismo del hierro. Expresión génica.*

## 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 TfR2/HFE mediated pathway.[3]

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The expression of the *Tf* gene is controlled by transcriptional mechanisms and is tissue-specific.[4] Many environmental factors are known to affect plasma Tf levels: in iron deficiency, the rate of Tf synthesis in the liver increases significantly,[5] whereas inflammatory or immunologic stimuli may decrease the levels of circulating Tf.[6] Recent studies observed increased Tf levels under hypoxia, a response that may facilitate iron supply for erythropoiesis.[7] Nevertheless, little is known about the genetic factors that influence Tf levels in humans, although its expression pattern appears to show sexual dimorphism.[8] Our research group recently published that only a few SNPs could explain a large percentage of the heritable variation of serum transferrin levels; one of these loci is SNP rs3811647, located in intron 11 of the human transferrin gene (*Tf*),[9] which as in agreement with other data from the bibliography.[10] Based on these studies, we hypothesised 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, CATGCTAGCGGCTTGCACACAGGATTTTT; antisense, CATCTCGAGAATCAGTGGAAGTGGCAAGG; *NheI* and *XhoI* 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 for 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: GGGAGTTTACAGACAGATCgTCTAGGATTATACATCTAGGAAGGG. 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 $10^4$ 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 gal activity in the samples.

### *In silico analysis*

For the prediction of putative transcription factor binding sites, a sequence of 25 bases to 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

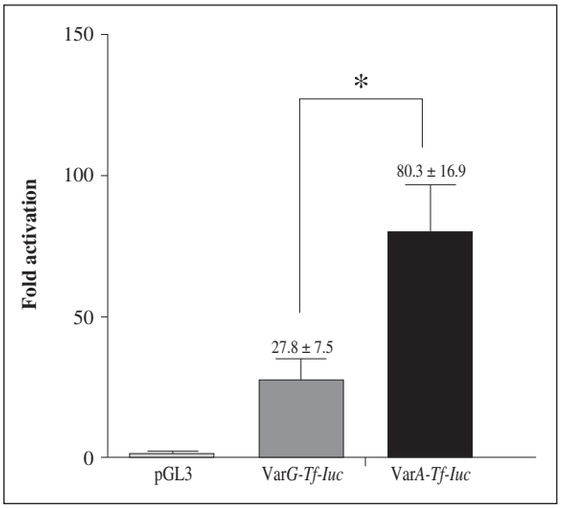


*Fig. 1.—SNP rs3811647 luciferase assay. Hep3B cells were transfected with VarA-Tf-luc or VarG-Tf-luc constructs; pGL3 (Promoter) vector backbone was used as negative control. Data were normalized to the expression levels of β-galactosidase internal control, and represent the means of two independent experiments (± SEM), *p < 0.05.*

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 with 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 ($p = 0.01$).[9] Also, the *in-vitro* results confirm previous observations in different population groups[10-13] 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.[14] 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,[15] 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.[16,17] 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.[18] 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.[9] Since low ferritin levels have been associated with this

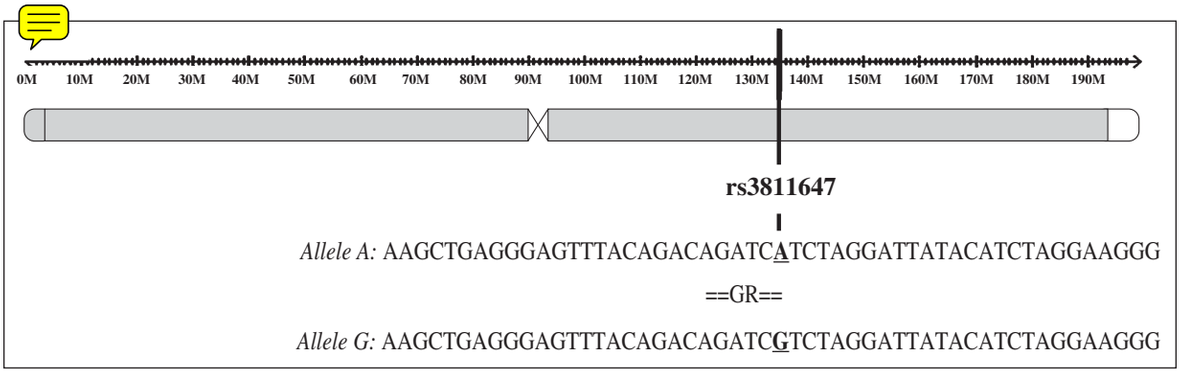


*Fig. 2.—Genomic arrangement (upper panel) and sequences proximal to SNP rs3811647 (bold letter, lower panel). Sequence analysis with prediction algorithms showed that the A allele in rs3811647 might create a binding site for the glucocorticoid receptor (GR).*

SNP, it could be related to low iron status.[10] Another important observation was made in a placebo-controlled nutritional intervention study with iron-fortified food in iron-deficient women.[19] 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.[20] 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

1. World Health Organization. Assessing the iron status of populations. Geneva, 2007.
2. Gkouvatsos K, Papanikolaou G, Pantopoulos K. Regulation of iron transport and the role of transferrin. *Biochim Biophys Acta* 2012; 1820 (3): 188-202.
3. Gao J, Chen J, Kramer M, Tsukamoto H, Zhang AS, Enns CA. Interaction of the hereditary hemochromatosis protein HFE with transferrin receptor 2 is required for transferrin-induced hepcidin expression. *Cell Metab* 2009; 9 (3): 217-27.
4. Zakin MM, Baron B, Guillou F. Regulation of the tissue-specific expression of transferrin gene. *Dev Neurosci* 2002; 24 (2-3): 222-6.
5. Idzerda RL, Huebers H, Finch CA, McKnight GS. Rat transferrin gene expression: tissue-specific regulation by iron deficiency. *Proc Natl Acad Sci USA* 1986; 83 (11): 3723-7.
6. Djeha A, Perez-Arellano JL, Hayes SL, Oria R, Simpson RJ, Raja KB, et al. Cytokine-mediated regulation of transferrin synthesis in mouse macrophages and human T lymphocytes. *Blood* 1995; 85 (4): 1036-42.
7. Rolfs A, Kvietikova I, Gassmann M, Wenger RH. Oxygen-regulated transferrin expression is mediated by hypoxia-inducible factor-1. *J Biol Chem* 1997; 272 (32): 20055-62.
8. Whitfield JB, Cullen LM, Jazwinska EC, Powell LW, Heath AC, Zhu G et al. Effects of HFE C282Y and H63D polymorphisms and polygenic background on iron stores in a large community sample of twins. *Am J Hum Genet* 2000; 66 (4): 1246-58.
9. Blanco-Rojo R, Baeza-Richer C, Lopez-Parra AM, Perez-Granados AM, Brichs A, Bertoncini S et al. Four variants in transferrin and HFE genes as potential markers of iron deficiency anaemia risk: an association study in menstruating women. *Nutr Metab (Lond)* 2011; 8: 69.
10. Benyamin B, McRae AF, Zhu G, Gordon S, Henders AK, Palotie A et al. Variants in TF and HFE explain approximately 40% of genetic variation in serum-transferrin levels. *Am J Hum Genet* 2009; 84 (1): 60-5.
11. Benyamin B, Ferreira MA, Willemsen G, Gordon S, Middelberg RP, McEvoy BP, et al. Common variants in TMPRSS6 are associated with iron status and erythrocyte volume. *Nat Genet* 2009; 41 (11): 1173-5.
12. McLaren CE, Garner CP, Constantine CC, McLachlan S, Vulpe CD, Snively BM, et al. Genome-wide association study identifies genetic Loci associated with iron deficiency. *PLoS One* 2011; 6 (3): e17390.
13. Constantine CC, Anderson GJ, Vulpe CD, McLaren CE, Bahlo M, Yeap HL et al. A novel association between a SNP in CYBRD1 and serum ferritin levels in a cohort study of HFE hereditary haemochromatosis. *Br J Haematol* 2009; 147 (1): 140-9.
14. Sawaya BE, Aunis D, Schaeffer E. Distinct positive and negative regulatory elements control neuronal and hepatic transcription of the human transferrin gene. *J Neurosci Res* 1996; 43 (3): 261-72.
15. Tabor HK, Risch NJ, Myers RM. Candidate-gene approaches for studying complex genetic traits: practical considerations. *Nat Rev Genet* 2002; 3 (5): 391-7.
16. He F, Ma L, Wang H, Shen Z, Li M. Glucocorticoid causes iron accumulation in liver by up-regulating expression of iron regulatory protein 1 gene through GR and STAT5. *Cell biochemistry and biophysics* 2011; 61 (1): 65-71.
17. Wang L, Wang H, Li L, Li W, Dong X, Li M, Lv L. Corticosterone induces dysregulation of iron metabolism in hippocampal neurons *in vitro. Biol Trace Elem Res* 2010; 137 (1): 88-95.
18. Schrem H, Klempnauer J, Borlak J. Liver-enriched transcription factors in liver function and development. Part I: the hepatocyte nuclear factor network and liver-specific gene expression. *Pharmacol Rev* 2002; 54 (1): 129-58.
19. Blanco-Rojo R, Perez-Granados AM, Toxqui L, González-Vizcayno C, Delgado MA, Vaquero MP. Efficacy of a microencapsulated iron pyrophosphate-fortified fruit juice: a randomised, double-blind, placebo-controlled study in Spanish iron-deficient women. *Br J Nutr* 2011; 105 (11): 1652-9.
20. Blanco-Rojo R, Pérez-Granados AM, López-Parra AM, Baeza C, Toxqui L, Arroyo-Pardo E et al. Influence of SNP rs3811647 on Fe metabolism and response to an Fe supplemented food in menstruating women. *Proceedings of the Nutrition Society* 2011; 70 (OCE4): E212.