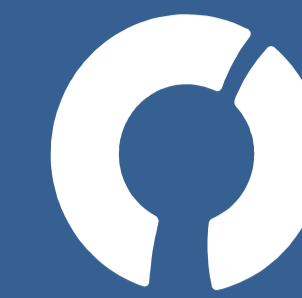


VARIANTS OF HEPcidIN GENE PROMOTER MODULATE IRON STATUS IN H63D INDIVIDUALS

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Introduction

Hepcidin is a 25 amino-acid peptide hormone known as the main regulator of iron homeostasis. It is able to decrease the absorption of dietary iron in the duodenum and the release of recycled iron from macrophages as well as the export of the stored iron from hepatocytes¹.

Hepcidin deficiency causes iron overload and, in contrast, its overproduction is associated with iron deficiency/anemia².

Several mutations located in the hepcidin gene (HAMP) have already been associated to the development of iron overload or hereditary hemochromatosis (HH). Additionally, some HAMP promoter variants were described, however their functional consequences remain unclear. One of them is the polymorphism c.-582 A>G that was recently described as worsening iron overload phenotype in beta-thalassemia major patients³ but that has no effect on the iron status in the healthy population⁴. Functional assays performed have revealed that hepcidin expression was slightly reduced under the promoter variant G when transactivated by the upstream stimulatory factors 1 and 2 (USF1/USF2) in HepG2 cells⁵.

The aims of this study were to determine: *i)* the frequency of the c.-582 A>G HAMP polymorphism in patients presenting iron overload and the common HFE mutations (H63D and/or C282Y); *ii)* if it is a modulator of iron overload in these patients and, *iii)* which are the upstream stimuli that are impaired by the polymorphism presence by performing functional *in vitro* studies in HAMP promoter.

Methods

→ 266 individuals with ferritin levels higher than 400ng/mL were screened for the 2.7-kb HAMP promoter variants

- (i) 191 individuals homozygous or heterozygous for the H63D allele
- (ii) 75 individuals carrying one or more C282Y alleles (HH/CY, HH/YY or HD/CY)

→ The 1.5-kb HAMP promoter sequence was cloned into the pGL2-enhancer vector and site-directed mutagenesis performed to obtain the polymorphic construct.

→ Huh-7 hepatoma cells were seeded in 35-mm plates, and the pGL2-enhancer constructions co-transfected along with pGL4.70 vector. Three hours post-transfection cells were submitted to different stimuli: (i) 20μM holo-transferrin, (ii) 4-6μM ferric citrate, (iii) 20ng/mL interleukin-6, (iv) 200μM cobalt chloride and (v) GDF15 at physiological and pathological concentrations (500 and 150000pg/mL, respectively). Finally cells were harvested and luminescence assays performed.

Discussion

In our study sample, the c.-582 A>G HAMP promoter polymorphism seems to be in linkage disequilibrium with the c.-1010 C>T HAMP polymorphism.

In individuals that present ferritin levels higher than 400ng/mL along with H63D or C282Y mutations in HFE gene, the AG/CT and GG/TT genotypes are significantly more frequent than in the control caucasian population (NCBI database). This is also revealed by the significant differences obtained for the allele frequencies in the group presenting one or two H63D alleles (HD/CC and DD/CC individuals). However, this is not observed in the group presenting at least one C282Y allele (HH/CY, HH/YY and HD/CY individuals).

In silico studies show that both polymorphisms can disrupt highly predictable transcription factor binding sites, such as USF2 and TATA. We tried to find which stimuli are impaired by these variants, however after performing luminescence assays we have found that neither holo-Tf, ferric citrate, IL-6, hypoxia nor GDF-15 seem to be the stimuli that become unable to trigger the HAMP promoter activity.

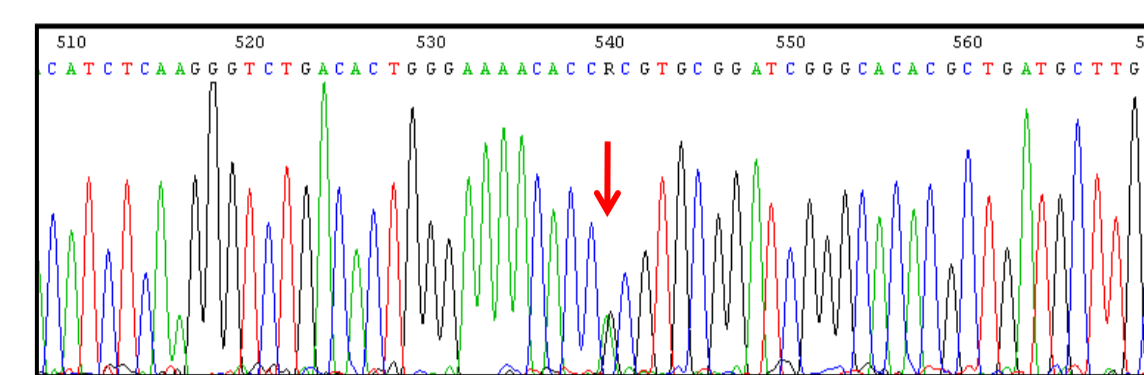
In conclusion, c.-582 A>G and c.-1010 C>T polymorphisms seem to be a risk factor to iron overload development in individuals that by their H63D or C282Y background are already prone to develop this phenotype.

References

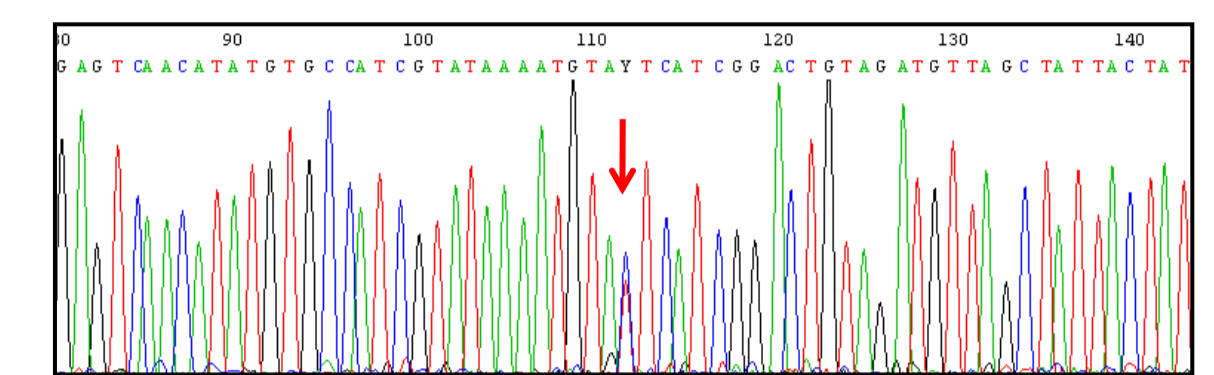
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HAMP promoter variants screening



c.-582 A>G



c.-1010 C>T

→ Two polymorphisms were found in the proximal region of the HAMP promoter, the **c.-582 A>G** and **c.-1010 C>T**

→ These polymorphisms appear to be in linkage disequilibrium in our sample

		Caucasians (CEU) from NCBI database	HH/CY, HH/YY and HD/CY individuals (n.75)		HD/CC, DD/CC individuals (n.191)	
c.-582 A>G / c.-1010C>T		Frequency (%)	Frequency (%) [n]	p*	Frequency (%) [n]	p*
Allele	A/C	83.6	78.7 [118]	0.18572	68.8 [263]	0.00006
	G/T	16.4	21.3 [32]		31.2 [119]	
	Total	100.0	100.0 [150]		100.0 [382]	
Genotype	AA/CC	69.0	62.7 [47]	0.01464	47.1 [90]	<0.00001
	AG/CT	29.3	32.0 [24]		43.5 [83]	
	GG/TT	1.7	5.3 [4]		9.4 [18]	
	Total	100.0	100.0 [75]		100.0 [191]	

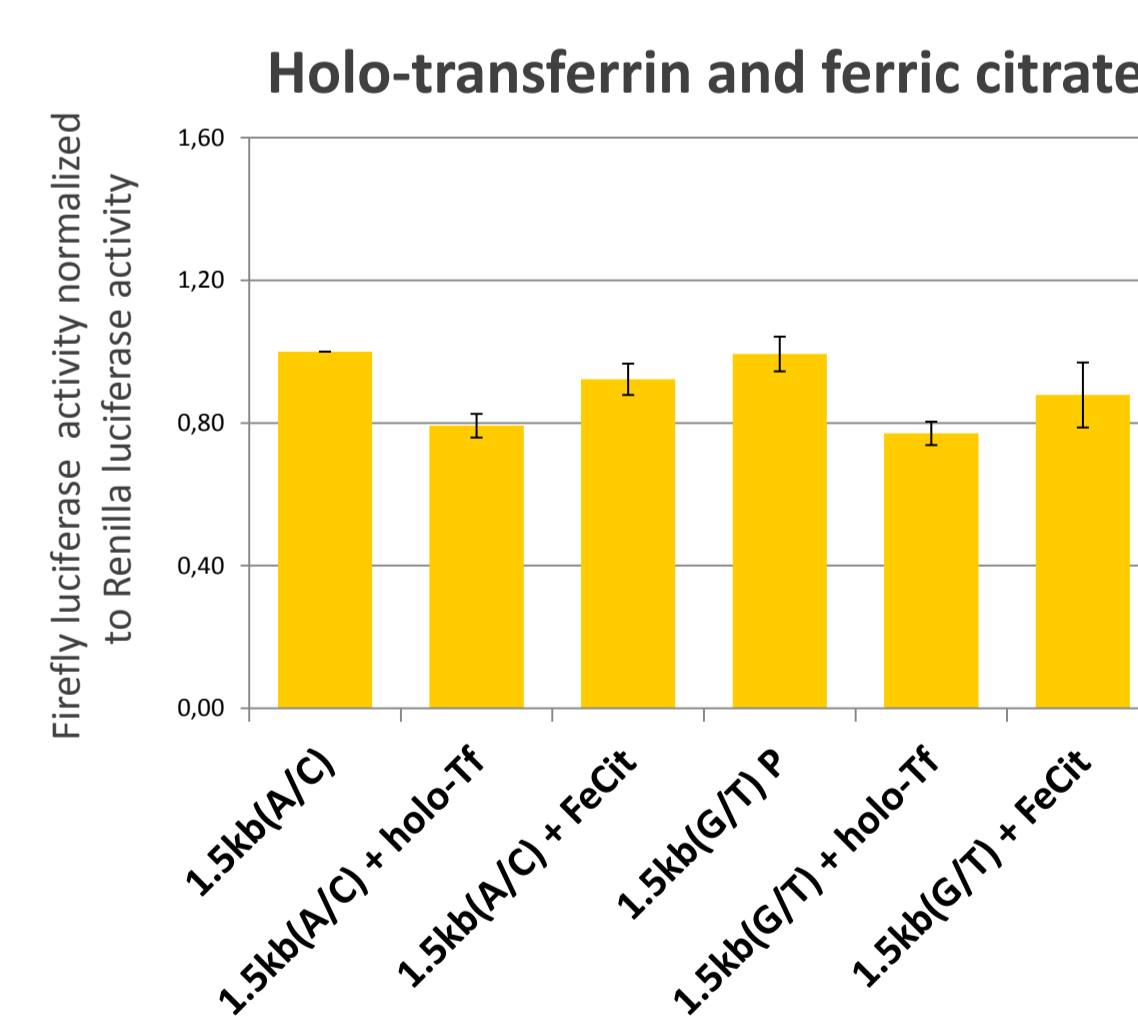
* p-values were obtained by the Chi-square test

→ The G/T haplotype frequency (31.2%) observed in the group of individuals presenting one or two H63D alleles (HD/CC and DD/CC individuals) is much higher than the one observed in the CEU population (16.4%), while no significant differences are observed in the individuals carrying at least one C282Y allele (21.3%)

→ The genotype distribution in the sample of individuals carrying at least one C282Y mutation is significantly different ($p=0.01464$) from the one obtained for the control population, since the frequency of GG/TT genotype is 3.1-fold higher in this study group

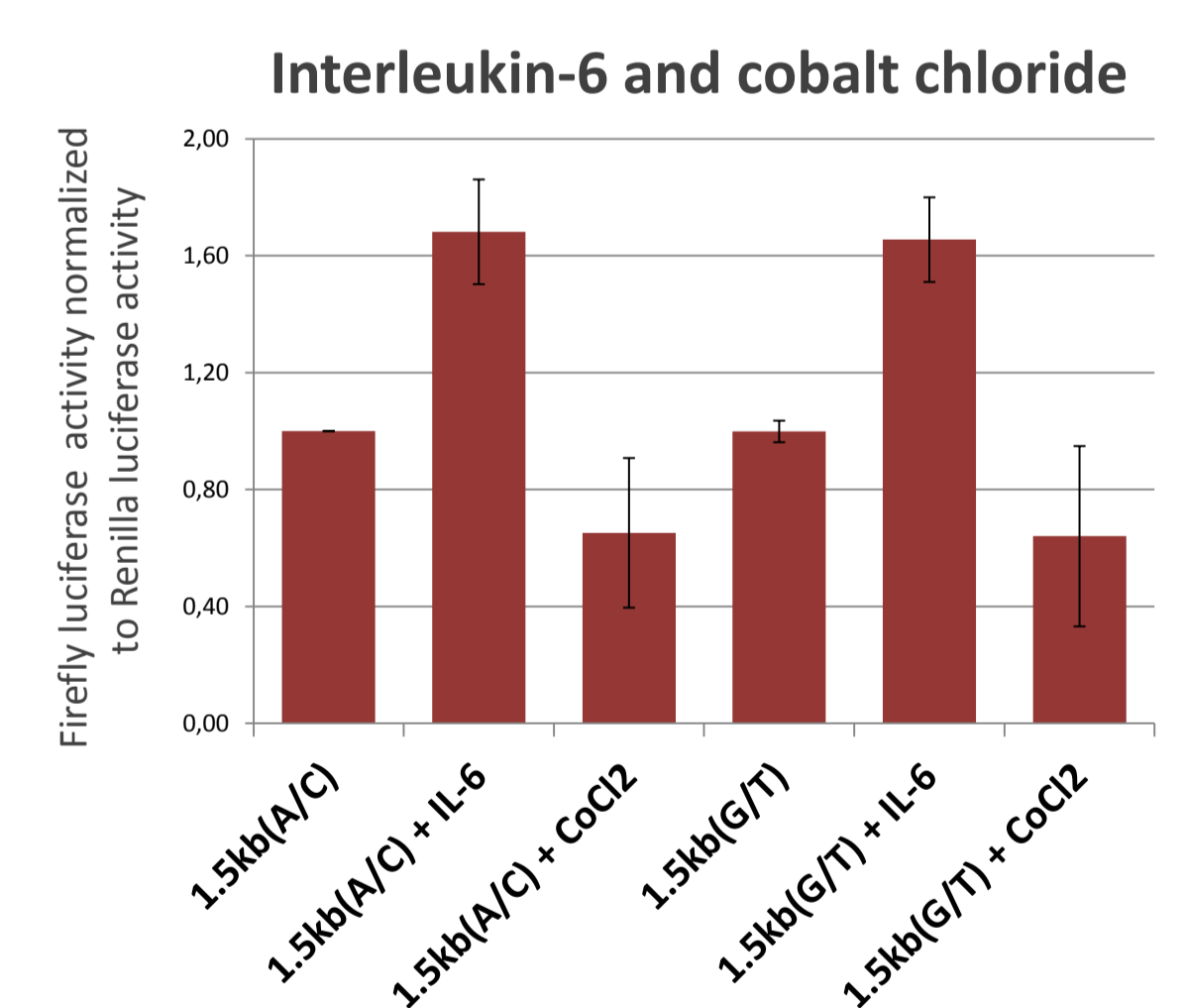
→ Also, we observe an highly significant difference ($p<0,00001$) between the genotype distribution in HD/CC, DD/CC individuals when compared to the control population. Genotype appear to be biased by a high frequency of GG/TT and AG/CT individuals (9.4 and 43.5%, respectively) and a lower frequency in AA/CC individuals (47.1%)

Luminescence assays



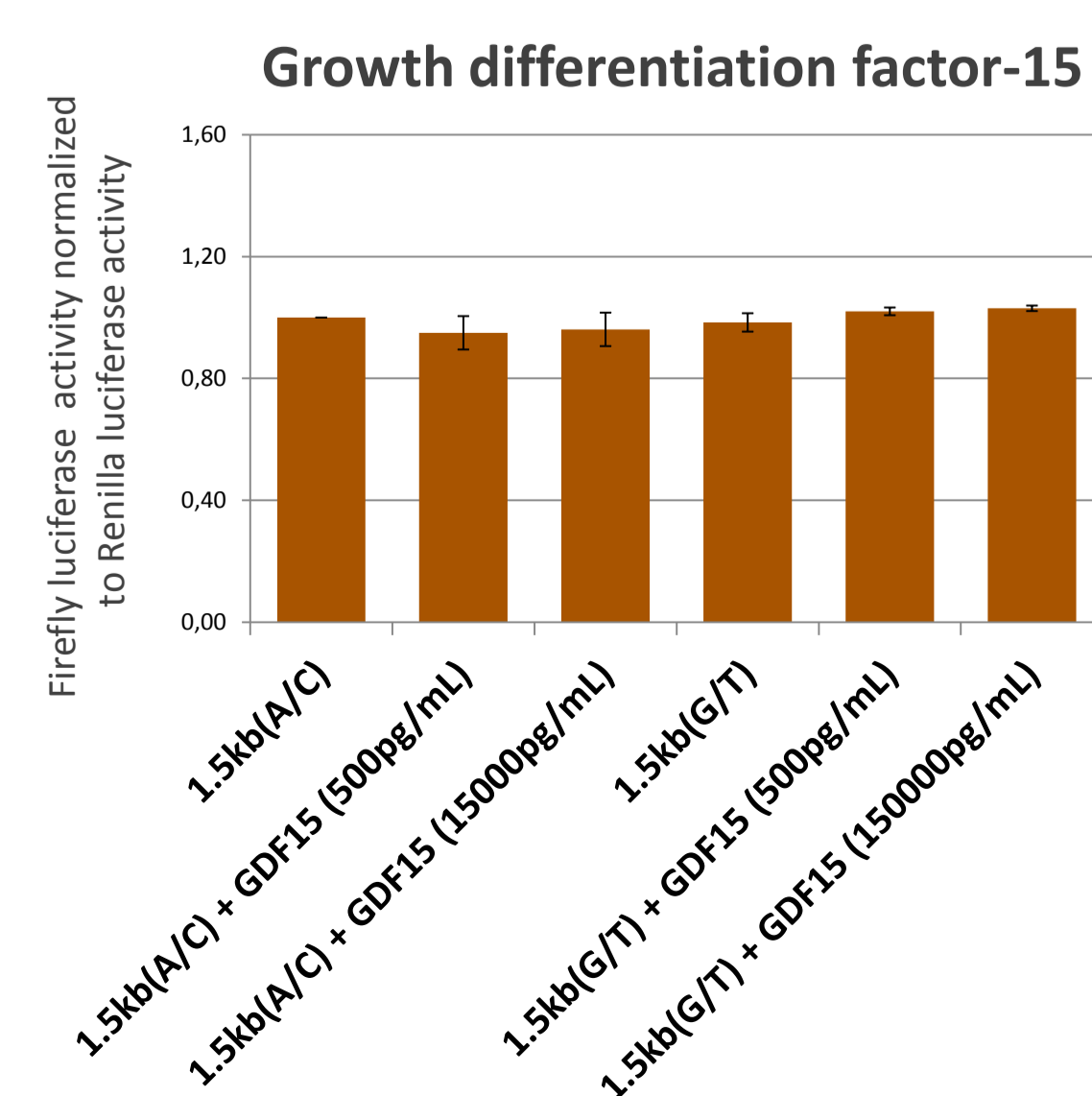
→ Both holo-transferrin and ferric citrate stimuli partially inhibit the 1.5kb-HAMP promoter activity

→ No differences in the luciferase activity were observed in the polymorphic promoter when compared to the normal



→ IL-6 is able to increase the promoter activity, while cobalt chloride, mimicking hypoxia, represses it.

→ Also, no differences in the luciferase activity were observed in the polymorphic promoter when compared to the normal



→ GDF-15 stimulus seems not to affect the HAMP promoter activity neither at physiological nor pathological (beta-thalassemia) concentrations

→ Again, no differences in the luciferase activity were observed in the polymorphic promoter when compared to the normal