HEPCIDIN GENE PROMOTER C.-1010T AND C.-582G VARIANTS ARE MODULATORS OF IRON OVERLOAD DEVELOPMENT IN INDIVIDUALS CARRYING THE H63D MUTATION IN THE *HFE* GENE

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Introduction

Hepcidin is a 25 amino-acid peptide hormone known to be a crucial regulator of iron homeostasis. It is able to decrease the absorption of dietary iron in the duodenum and the release of recycled iron from macrophage, as well as the export of the stored iron from hepatocytes[1].

It is known that the deficiency in hepcidin levels leads to the development of iron overload and that, in contrast, its overproduction causes iron deficiency/anemia[2].

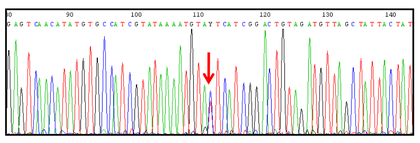
Several mutations located in the hepcidin gene (HAMP) have already been associated to the development of iron overload or hereditary hemochromatosis (HH). Additionally, some HAMP promoter variants were described, however their functional consequences remain unclear. One of them is the polymorphism c.-582 A>G, recently described to be in association with an increased iron overload phenotype in beta-thalassemia major patients[3], but that has no effect on the iron status in the healthy population[4]. Also, functional assays have revealed that hepcidin expression becomes slightly reduced under the promoter G variant when transactivated by the upstream stimulatory factors 1 and 2 (USF1/USF2) in HepG2 cells[5].

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

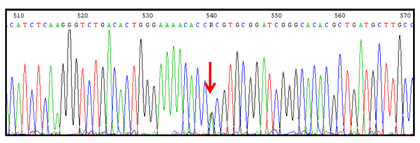
HAMP promoter variants screening

→ 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 desequilibrium in our sample



c.-1010 C>T



c.-582 A>G

		Caucasians (CEU) from NCBI database	HH/CY, HH/YY and HD/CY individuals (n.75)		HD/CC, DD/CC individuals SF>400ng/mL) [n.191]		HD/CC, DD/CC Individuals (SF<400ng/mL) [n.98]	
c.-1010C>T /c.-582 A>G		Frequency (%)	Frequency (%) [n]	*p**	Frequency (%) [n]	*p**	Frequency (%) [n]	*p**
Allele	**C/A**	83.6	78.7 [118]	0.1857	68.8 [263]	**0.00006**	78.1 [153]	0.1374
	T/G	16.4	21.3 [32]		31.2 [119]		21.9 [43]	
	Total	100.0	100.0 [150]		100.0 [382]		100.0 [196]	
Genotype	**CC/AA**	69.0	62.7 [47]		47.1 [90]		62.2 [61]	
	CT/AG	29.3	32.0 [24]	**0.0146**	43.5 [83]	**<0.00001**	31.6 [31]	**0.0022**
	TT/GG	1.7	5.3 [4]		9.4 [18]		6.1[6]	
	Total	100.0	100.0 [75]		100.0 [191]		100.0 [98]	

•*p*-values were obtained by the Chi-square test
•SF - Serum Ferritin

→ The **TG haplotype** frequency (31.2%) observed in the group of individuals presenting one or two H63D alleles (HD/CC and DD/CC individuals) with high SF is much **higher** than the one observed in the CEU population (16.4%), while **no significant difference** was observed in the individuals carrying at least one C282Y allele (21.3%) or in HD/CC and DD/CC individuals with normal SF

→ We have found differences in the genotype distribution in all the groups of analysed individuals. However it showed to be **highly significant** (p<0,00001) between the **genotype distribution in HD/CC, DD/CC individuals** with high SF when compared to the control population. Genotype appear to be biased by a high frequency of TT/GG and CT/AG individuals (9.4 and 43.5%, respectively) and a lower frequency in CC/AA individuals (47.1%)

→ Interestingly, we observed that TG haplotype distribution (31.2%) in the **HD/CC and DD/CC individuals with high SF** is significantly higher than the one observed in HD/CC and DD/CC individuals with normal SF (**p= 0.0196**)

Methods

→ 364 individuals with mutations in the HFE gene were screened for the 2.7-kb HAMP promoter variants

(i) 75 individuals carrying one or more C282Y alleles (HH/CY, HH/YY or HD/CY) with serum ferritin (SF) higher than 400 ng/mL

(ii) 191 individuals homozygous or heterozygous for the H63D allele with SF higher than 400 ng/mL

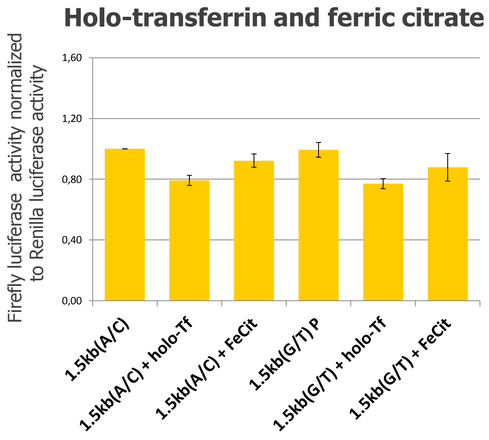
(iii) 98 individuals homozygous or heterozygous for the H63D allele with SF lower than 400 ng/mL

→ 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

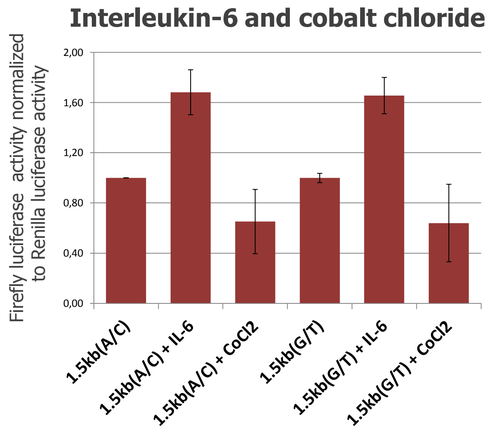
Luminescence assays

Holo-transferrin and ferric citrate



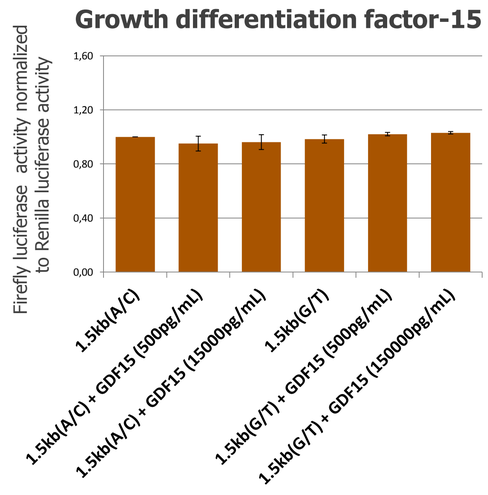
→ **IL-6** is able to **increase** the promoter activity, while **cobalt chloride**, mimicking hypoxia, **represses it**

Interleukin-6 and cobalt chloride



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

Growth differentiation factor-15



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

No differences in the luciferase activity were observed in the polymorphic promoter when compared to the normal under the different analysed stimuli

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 H63D or C282Y mutations in HFE gene, the CT/AG and TT/GG genotypes are significantly more frequent than in the control caucasian population (NCBI database). However, when analysing the allele frequencies we only found significant differences in the group of individuals that have SF higher than 400ng/mL along with one or two H63D alleles (HD/CC and DD/CC individuals). We also observe that the allele frequency is also significantly different when comparing individuals having the same HD/CC or DD/CC background, but with different SF levels (higher polymorphism frequency found in individuals with SF levels higher than 400ng/mL).

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.-1010 C>T and c.-582 A>G 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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