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A common SNP near *BMP2* is associated with severity of the iron burden in *HFE* p.C282Y homozygous patients: A follow-up study

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ABSTRACT

Background and Objectives: It is now generally admitted that penetrance of the common *HFE* p.C282Y/p.C282Y genotype is incomplete, and identification of modifier genes is the concern of a growing number of research projects. We recently identified a significant association between pretherapeutic serum ferritin level and the common rs235756 single nucleotide polymorphism (SNP) of the *BMP2* gene region. Our results further suggested an interactive effect between the *BMP2* rs235756 SNP and the rs16827043 SNP in *HJV*, with a small additive effect of the rs4901474 SNP in *BMP4*.
Design and Methods: The present study has been designed as a replication study in an independent cohort of 450 *HFE* p.C282Y homozygous patients from a nearby French region (Brittany). Information on individual alcohol consumption and amount of iron removed by phlebotomy being available for a substantial part of this cohort, additional analyses were conducted.
Results: Only the use of the Amount of Iron Removed by phlebotomy (AIR) as marker of iron burden has provided positive results. Indeed, a significant association was detected between rs235756 and AIR adjusted for sex and age, with a mean AIR increasing with the number of *BMP2* T alleles in the genotype groups. The effect of rs235657 was not strong enough to detect effects of gene combinations. Still, the trend in two-locus genotype risks involving *BMP2* and *HJV* for AIR was concordant with the specific interactive effect described in the initial study.
Interpretation and Conclusions: Although we failed to replicate results of the initial study, we argue that, altogether, our results help to consider genes involved in the regulation of hepcidin synthesis as potential modifiers of the p.C282Y/pC282Y genotype expression and especially *BMP2*.

Introduction

The historical and predominant form of hemochromatosis (HC) is an adult-onset autosomal recessive condition usually associated with the *HFE* p.C282Y/p.C282Y genotype (OMIM # 235200). In Northern European populations, this genotype is carried by approximately 1 person in 200 [1]. However, its phenotypic expression is heterogeneous and clearly depends on a balance between accentuating and reducing factors. The influence of gender, age and lifestyle components, such as alcohol abuse, has been well documented [2-4]. Involvement of modifier genes or epigenetic mechanisms is less obvious, but is supported by studies in *Hfe* knockout mice, as well as by the greater concordance between biochemical and clinical iron overload phenotypes within families than between families [1,5,6].

In an effort to identify genes that modulate expression of the *HFE* p.C282Y/p.C282Y genotype, we recently investigated 79 tag single nucleotide polymorphisms (SNPs) in 592 patients. We emphasized genes involved in the four non-*HFE* hemochromatosis forms (*HJV*, *HAMP*, *TFR2*, *SLC40A1*) and genes involved in regulation of hepcidin synthesis (*BMP2*, *BMP4*, *SMAD1*, *SMAD4*, *SMAD5* and *IL6*), using pretherapeutic serum ferritin as marker of the level of iron burden. Only one SNP, rs235756, reached statistical significance ($p = 4.42 \times 10^{-5}$). This SNP is located ~6 kb downstream of the *BMP2* gene. In separate analyses focused on *BMP2* and other genes of the BMP pathway, we were also able to detect an interactive effect on serum ferritin of rs235756 in *BMP2* and an SNP in *HJV* (rs16827043), with a small additive effect of an SNP in *BMP4* (rs4901474) [7].

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In the first attempt to replicate these positive findings, we analyzed an independent cohort of 450 *HFE* p.C282Y/p.C282Y patients. This cohort fulfilled the inclusion criteria of the initial study and genotype–phenotype associations were assessed using the pretherapeutic serum ferritin level as quantitative phenotypic trait. In parallel, we selected patients for whom the amount of iron removed by phlebotomy or the daily alcohol consumption or both were available. Choosing a better marker for iron burden and also considering a well-recognized confounding factor, and despite a smaller sample size, we expected more precise estimates of the reported modifier gene effects.

Materials

Study patients

The cohort of patients originated from Brittany (the Western part of France). The inclusion criteria were identical to those of the initial study [7]; they included availability of sex, age at diagnosis and serum ferritin level before any venesection therapy. All the patients presented with a transferrin saturation level greater than or equal to 45%.

In total, 249 men and 201 women were included. Table 1 presents the characteristics of this sample together with those of the study of reference for comparison. Sex ratio and mean age at diagnosis by sex are consistent in the two studies. Mean serum ferritin levels tended to be lower with a reduced variance in the present sample.

All patients signed an informed consent form.

Daily alcohol consumption and amount of iron removed (AIR) by phlebotomy

Alcohol use was documented by individual interviews using a structured questionnaire [3]. Daily alcohol consumption was assessed distinguishing the amounts of beer, wine and liquor consumed, and was then recoded following standards considered by the World Health Organization (http://www.who.int/en): abstinent (no consumption declared), moderate or occasional drinkers (not more than 2 units for women and 3 units for men) and excessive drinkers (2 units or more for women and 3 units or more for men). One unit corresponds to 12 g of ethanol. Alcohol consumption was available for 408 patients: 244 were abstinent, 106 were moderate or occasional drinkers and 58 were excessive drinkers.

AIR was assessed from the volume of blood extracted during depletion therapy, assuming that 500 ml of blood contains 250 mg of elemental iron. AIR was available for 390 subjects (177 women and 213 men), mean values by sex are given in Table 4.

SNP genotyping

SNP genotyping was performed by coupling multiplex PCR and primer-extension analyses on a denaturing high-performance liquid chromatography (D-HPLC; Transgenomic, Omaha, NE, USA) system. Briefly, multiplex PCR led to the amplification of a 238-bp product from the *BMP2* locus, a 218-bp product from the *BMP4* locus, and a 228-bp product from the *HJV* locus. PCR products were treated to remove unincorporated primers and dNTPs using the ExoSAP-IT™ enzymatic mix from GE Healthcare. The PCR purified products were subsequently used in two primer-extension reactions (the *BMP2* SNP was subjected to an independent analysis, whereas the *BMP4* and *HJV* SNPs were analyzed together). The PCR primer sequences and conditions for all enzymatic reactions are available upon request.

The three SNPs (rs235756, rs4901474 and rs16827043) were successfully genotyped for all 450 subjects and were in Hardy–Weinberg equilibrium. Sample minor allele frequencies–0.39 for rs235756, 0.44 for rs4901474 and 0.10 for rs16827043–did not significantly differ from those of the initial study.

Statistical analyses

Serum ferritin levels and AIR were both normalized using a $\log_e$ transformation and adjusted for age (with consideration of age groups of 10 years) and sex before testing for association. For the log-transformed serum ferritin levels, the final multiple-regression model was similar to the one of the initial study. It included a parameter for each of the six independent age groups, a parameter for sex and an interaction parameter age × sex for each age group. For the log-transformed AIR, the final multiple-regression model included a parameter for each of the six independent age groups and a parameter for sex. Individual effects of alcohol consumption on log-transformed serum ferritin and AIR were both highly significant (p-value < 0.0001). When including alcohol consumption in the model, the interaction between age and sex was no longer significant for serum ferritin level. Thus, for both the log-transformed ferritin level and AIR, the multiple-regression models adjusted for alcohol consumption contain a parameter for each age group and for sex and three parameters for the alcohol consumption variables. The correlation between log-transformed serum ferritin level and log-transformed AIR was high, with a coefficient of determination for simple linear regression $r^2 = 0.72$. The relation between these two variables is best described by a second degree polynomial (see Fig. 1).

To test the replication of the allelic association between rs235756 SNP in *BMP2* and either adjusted ferritin or AIR, we used a linear regression with a one-sided t-test.

The exploratory models involving *HJV* and *BMP4* SNPs and proposed in the study of reference–*BMP2*+*HJV*+*BMP2*x*HJV* and *BMP2*+*BMP4*+*HJV*+*BMP2*x*HJV* where *BMP2* stands for rs235756 SNP, *HJV* for rs16827043 and *BMP4* for rs4901474–were tested for adjusted ferritin and AIR using a multiple-regression approach. The genetic analyses were performed using R [8].

Table 1
Sample characteristics.

	Global sample		Milet et al. sample	
	Female	Male	Female	Male
Sample size	201	249	262	330
Age at diagnosis[a] (years)	48.1 ± 13.9	45.6 ± 12.5	46.1 ± 14.3	44.0 ± 13.1
Serum ferritin[b] (mg/l)	326 (182–587)	820 (490–1585)	400.5 (186–699)	1040 (585–2356)
	Subsample			
Sample size	177	213		
AIR[1] (g)	1.95 (1.0–3.6)	4.73 (3.1–8.0)		

Milet et al. [7] sample characteristics are given for comparison.
[a] Mean value ± std.
[b] Median value (25th–75th percentile).
[1] Amount of the Iron Removed by phlebotomy.

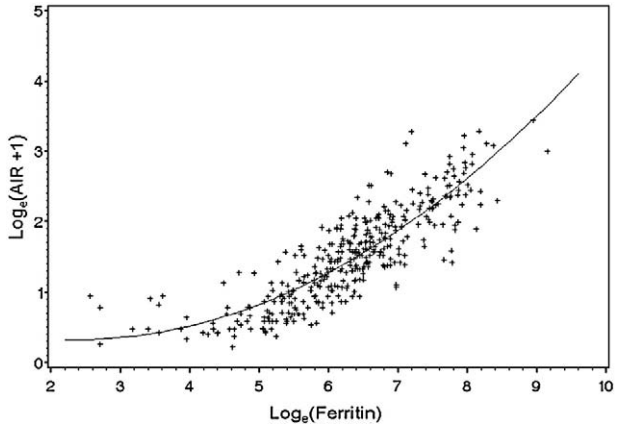

Fig. 1. Correlation between serum ferritin level and AIR in 390 individuals. The polynomial curve is given by the equation: $\mathrm{Log_e}(\mathrm{AIR}+1) = 0.73 - 0.34\mathrm{Log_e}(\mathrm{Ferritin}) + 0.07\mathrm{Log_e}(\mathrm{Ferritin})^2$.

To test whether the effect of rs235756 SNP on serum ferritin level significantly differed between the present 450 C282Y homozygote sample and the 592 C282Y homozygote sample of the study of reference, log-transformed serum ferritin data of the two samples, adjusted for sex and age, were pooled. A global linear model was fitted to the data: $Y_{ij} = \alpha + \beta S_{ij} + \gamma_1 G^1_{ij} + \gamma_2 G^2_{ij} + \kappa_1 S_{ij}\ x\ G_{ij}1 + \kappa_2 S_{ij} \times G^2_{ij}$, where $Y_{ij}$is the transformed and adjusted serum ferritin level of individual i from sample j, where $j = 1$ if i is from the previously studied sample and 0 otherwise, S_i is the sample of origin of individual i with $S_{ij} = 1$ if i is from the previously studied sample and 0 otherwise, and $G^1{}_{ij}$ *and* $G^2{}_{ij}$ are the variables coding the rs235756 genotype information. This model includes a sample effect (measured by parameter β), a genotype effect of rs235756 (measured by parameters $\gamma 1$ and $\gamma 2$) and a genotype × sample interaction effect (measured by parameters $\kappa 1$ and $\kappa 2$). To test the homogeneity of the rs235756 effect in the two samples, we evaluated the significance of the two $\kappa 1$ and $\kappa 2$ parameters, with a 2-*df* *F*-test. If $\kappa 1$ and $\kappa 2$ are not significant, this means that the model in which the effect of the rs235756 on serum ferritin level is the same in the two samples is a better description of the data.

Results

Serum ferritin as marker of the iron burden

As can be seen from Table 2, the association between rs235756 and serum ferritin level did not reach significance in the present cohort of *HFE* p.C282Y/p.C282Y patients ($n = 450$; one-sided p-value = 0.25). Taking the alcohol consumption into account in the regression did not change the result (one-sided p-value = 0.125). However, as viewed in the initial study, the mean ferritin level adjusted for age and sex was an increasing function of the number of T alleles in the individuals:

Table 2
Mean serum ferritin level as a function of rs235756 genotypes (*BMP2*) in the present sample and in Milet et al. for comparison.

	Sample size	Mean value			*p*-value
		TT	TC	CC	
Serum ferritin level					
Milet et al. sample	592	655 mg/l	516 mg/l	349 mg/l	
Present sample					
No adjustment	450	510 mg/l	475 mg/l	437 mg/l	0.25
Adjusted for alcohol	408	625 mg/l	587 mg/l	573 mg/l	0.12

All data are adjusted for sex and age.
Data adjusted for alcohol consumption are also presented for the present sample. One-sided *p*-values of the allelic association test are provided.

Table 3
Mean amount of iron removed adjusted for sex and age as a function of rs235756 genotypes (*BMP2*).

	Sample size	Mean value			*p*-value
		TT	TC	CC	
Amount of iron removed					
No adjustment	390	3.20 g	2.82 g	2.55 g	0.018
Adjusted for alcohol	368	3.82 g	3.33 g	3.05 g	0.015

Adjustment for alcohol consumption is also considered. One-sided *p*-values of the allelic association test are provided.

510 ng/ml among individuals with TT genotypes, 475 ng/ml among individuals with TC genotypes and 437 ng/ml among individuals with CC genotypes. The same tendency was observed when alcohol consumption was taken into account (625 ng/ml among individuals with TT genotypes, 587 ng/ml among individuals with TC genotypes and 573 ng/ml among individuals with CC genotypes).

When analyzing the pooled transformed serum ferritin data of the two samples, the *p*-value of the 2-*df* *F*-test testing the significance of the two genotypes × sample of origin interaction parameters $\kappa 1$ and $\kappa 2$ was p value = 0.08. This means that the model in which the effect of the rs235756 genotypes is different in the two samples is not a better description of the data than the simpler model in which the effect of the rs235756 genotypes on serum ferritin is the same in the two samples.

The model including individual effects of *BMP2* and *HJV* with an interactive effect of the two SNPs was rejected in the present cohort of iron overloaded patients. The *p*-value of the test comparing this model to the null model with no SNP effect was 0.52 when considering transformed serum ferritin level adjusted for age and sex and 0.59 when alcohol consumption was further added to the model. Adding the effect of *BMP4* to the model does not change the outcome of the test (p-value = 0.62 and p-value = 0.67 with alcohol consumption in the model).

Amount of iron removed by phlebotomy as marker of the iron burden

The association between rs235756 and AIR was significant in the 390 patients phenotyped for that trait. Allele T was associated with a higher AIR (p-value = 0.018) and this association was robust to modelling of the daily alcohol consumption (p-value = 0.015), as can be seen from Table 3.

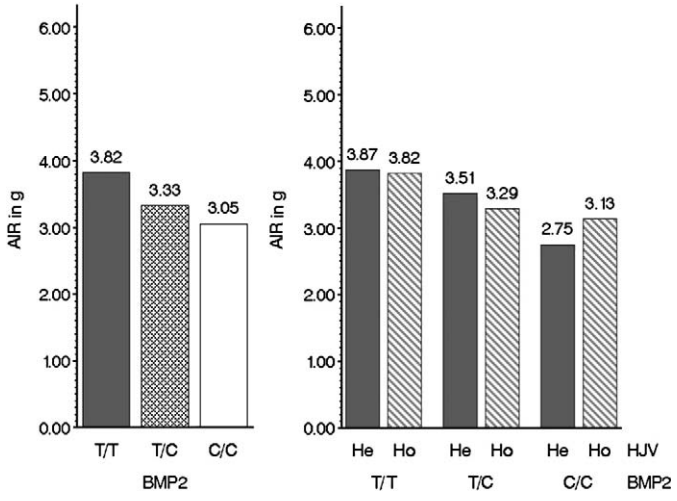

Fig. 2. Mean amount of iron removed adjusted for sex, age and alcohol consumption as a function of (A) rs235756 genotypes (*BMP2*) and (B) both rs235756 (*BMP2*) and rs16827043 (*HJV*) genotypes. For rs16827043, genotypes are grouped in "He" for AG and GG genotypes and "Ho" for AA genotypes.

Table 4
Mean age at diagnosis as a function of rs235756 genotype and sex.

rs235756 genotype	All (adjusted for sex)
TT	47.9
TC	46.8
CC	44.4
p-value of the mean age comparison test	0.17

Fig. 2 shows the mean AIR adjusted for age, sex and alcohol consumption as a function of rs235756 genotypes. Interestingly, AIR increased with the number of T alleles in the genotype (3.82 among TT genotypes, 3.33 among TC genotypes and 3.05 among CC genotypes).

The model that includes individual effects of *BMP2* and *HJV* with an interactive effect of the two SNPs was not a better descriptor of AIR distribution than the simpler model including only *BMP2* (*p*-value of the model comparison 0.18). Nevertheless, the variation of the mean AIR with the two-SNP genotypic distributions is concordant with that described in the initial study (see Fig. 2). The two-locus combinations at greater risk (TT at rs235756 and AG or GG at rs16827043) and lower risk (AA at rs235756 and AG or GG at rs16827043) involve the same HJV genotypes (AG or GG). The more complex model with an additive effect of *BMP4* was also rejected.

Finally, note that rs235756 had no impact on age at diagnosis in the whole sample adjusted for sex (ANOVA *p*-value for mean age adjusted for sex is 0.17, results presented in Table 4), a result concordant with that of the initial study.

Discussion

Failure to replicate the association between serum ferritin level and the rs235756 common variant should not be interpreted as necessarily refuting the initial finding. Rather, it has to be considered by looking for differences between samples and implementation of the studies.

Given that we were able to confirm the relationship between the *BMP2* allele T and higher serum ferritin level, it is possible that we did not reach statistical significance because of insufficient power. This could be due to an inadequate sample size (450 versus 592 patients), but also to the inherent heterogeneity related to disease. Despite a conservative approach (a follow-up study where patients from a nearby region were selected based on identical inclusion criteria), we observed differences in distribution of serum ferritin between the two samples. This suggests that confounding factors and involuntary inclusion of subgroups of patients led to imprecise estimates. Owing to this concern, we must admit that exaggeration of significance levels in the initial study and devaluation of the associations sought in the follow-up study are both imaginable. Thus, as reported in other genetic studies of complex traits [9-12], sample size and population stratification are possible reasons for the lack of replication.

The amount of iron removed by phlebotomy is an excellent marker of iron burden. However, it cannot be obtained before several weeks or months and needs to be evaluated under a regular practice [13]. This is an important limitation when constituting a large cohort of patients. Although subject to increases not related to iron overload [13,14], pretherapeutic serum ferritin is therefore more widely used. Here, we were able to study a total of 390 patients who had been followed up by in the same healthcare center. This allowed us to use AIR as a phenotypic trait in novel multiple-regression models. A significant association was detected between rs235756 and AIR adjusted for sex and age, with a mean AIR increasing with the number of *BMP2* T alleles in the genotype groups. The effect of rs235657 was not strong enough to detect effects of gene combinations. Still, the trend in two-locus genotype risks involving *BMP2* and *HJV* for AIR was concordant with the specific interactive effect described in the initial study.

Another point merits attention in the design of our follow-up study. We were able to evaluate the daily alcohol consumption of a substantial proportion of the selected cohort. Given that alcohol abuse has clearly been posited as a confounding factor of iron overload in *HFE* p.C282Y/p.C282Y patients [3,15], the adjustment of AIR for alcohol intake reinforces the validity of our observations.

Overall, we believe that the results of the present follow-up study confirm that variations in the *BMP2* gene region have an effect on iron burden among *HFE* p.C282Y homozygous patients, with possible combination effects with other genes involved in hepcidin synthesis. The usefulness of incorporating evidence from multiple levels must be a concern for future investigations. Without overlooking the need for other follow-up studies, and the particular interest of analyzing patients of different origins, efforts should be made to determine whether the Tag-SNP rs235657 is functional. Given its localization and the frequency of the allele at greater risk (T), it seems more reasonable to argue for linkage disequilibrium with one or several variants in the *BMP2* gene.

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