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GNPAT variant (D519G) is not associated with an elevated serum ferritin or iron removed by phlebotomy in patients referred for C282Y-linked hemochromatosis

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ABSTRACT

Background. Previous studies in high and low expressors has demonstrated that a variant in the *GNPAT* gene (D519G, Rs11558492, chromosome 1, exon 11) has been associated with severe iron overload in C282Y homozygotes for hemochromatosis. In this study, a *GNPAT* variant was assessed prospectively in patients referred for *HFE* testing over a range of serum ferritin levels. **Material and methods.** Consecutive patients sent for *HFE* testing were studied for the *GNPAT* variant using a TaqMan kit assay (Life Technologies, Burlington, ON). Serum ferritin and iron removed by phlebotomy was compared in C282Y homozygotes with and without the *GNPAT* variant. The frequency of the *GNPAT* variant in referred patients was compared to a control population of voluntary blood donors without *HFE* mutations. **Results.** There were 533 patients that had *GNPAT* analysis. The allele frequency for the *GNPAT* variant in C282Y homozygotes (n = 75) was 0.226 and in wild type control patients (n = 458) was 0.213 (p = .07). Forty-eight percent (of the C282Y homozygotes were heterozygous (n = 28) or homozygous (n = 8) for the *GNPAT* variant. The mean (log)ferritin and iron removed did not significantly differ between C282Y homozygous with *GNPAT* homozygotes, *GNPAT* heterozygotes, and without the *GNPAT* variant (p = 0.84). **Conclusions.** C282Y homozygotes referred for *HFE* testing commonly have a *GNPAT* variant. This *GNPAT* variant does not appear be a co-modifying gene affecting expression of *HFE* related hemochromatosis in this population. The *GNPAT* variant does not predict the severity of iron overload.

Key words. Iron overload. Haemochromatosis. Ferritin. Peroxisome. Iron.

INTRODUCTION

Since a genetic test (*HFE*) has been available for the diagnosis of hemochromatosis since 1996, many studies have reported a wide range of clinical expression in C282Y homzoygotes. The cause of this variability has not been clearly established and environmental and genetic factors have been considered.[1] The presence of co-modiftying genes remains a possibility and this has been studied using candidate gene analysis.[2] genome wide association studies (GWAS),[3] and exome sequencing.[4] A recent study using exome sequencing and sequence kernel association testing suggested that a variant of the *GNPAT* gene may be associated with more severe iron overload.[4] Gene silencing of the *GNPAT* gene led to a reduction in intracellular hepcidin. If this *GNPAT* mutation could predict severity of iron overload it could be a useful diagnostic test to predict follow up care and prognosis. In this study, *GNPAT* mutations were studied in a prospective population of male and female C282Y homozygotes with a broad spectrum of iron overload as assessed by serum ferritin and iron removed by phlebotomy.

MATERIAL AND METHODS

This study was approved by the Human Ethics Committee of Western University.

Consecutive patients sent for *HFE* testing were studied for the *GNPAT* variant Serum ferritin at the time of presentation in untreated C282Y homozygotes was compared with and without the *GNPAT* variant. The frequency of the *GNPAT* variant in referred C282Y homozygotes was compared to a control population of voluntary blood donors without *HFE* mutations. Not all of the blood donors

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had serum ferritin available. Peripheral blood was taken from patients and extracted to an aqueous solution or salt precipitate. Prior to testing, salt precipitate samples were rehydrated in 300 μL TE Buffer (Teknova). Rehydrated and aqueous samples were tested for the *HFE* and *GNPAT* variant using a TaqMan genotyping assay (Life Technologies Burlington, ON), prepared using a clinically validated *HFE* run protocol and the Roche Lightcycler 480. Each run was analyzed using the endpoint genotyping setting of the Lightcycler 480 software (release 1.5.1.61). Sanger sequencing of *GNPAT* exon 11 was conducted on three samples, homozygous, heterozygous, and wildtype, that served as D519G standards throughout the study. Sequence was run on the 3730 DNA analyzer (Applied Biosystems) using custom primers (Primer 3, SNPCheck) and the results were analyzed using Mutation surveyor (version 4.0.7). The allele frequency of *GNPAT* mutations in the C282Y homozygotes and a control population without *HFE* mutations were compared using the Fisher exact test. Ferritin was converted to Log (Ferritin) for comparative analysis because of the lack of a Gaussian distribution. Log (Serum ferritin) was compared across groups by ANOVA. Iron removed by phlebotomy in grams of iron was the number of 500 mL phlebotomies to bring the initial serum ferritin down to 50 μg/L x 0.25. The effects of a *GNPAT* mutation on log (ferritin) and iron removed (g) were studied in a multiple regression model with age, gender and C282Y homozygosity as the other variables.

RESULTS

There were 533 patients that had *GNPAT* analysis. There were 75 C282Y homozygotes. Median age was 57 with a range from 25-77 years. The allele frequency for the *GNPAT* variant in C282Y homozygotes (n = 75) was 0.226 and in wild type control patients (n = 458) was 0.213 (p = 0.07, Fisher exact test). Forty eight percent of the C282Y homozygotes were heterozygous (n = 28) or homozygous (n = 8). The mean ferritin ± standard deviation did not significantly differ between C282Y homozygous who were *GNPAT* homozygotes (n = 8, 1450 μg/L ± 1529), *GNPAT* heterozygotes (n = 28, 1012 μg/L ± 1,626) and without the *GNPAT* variant (n = 39, 1,464 µg/L ± 1,169). Mean (log) serum ferritin did not differ between the 3 groups. Serum ferritin in *GNPAT* homozygotes, heterozygotes and wild type in 75 C282Y homozygotes is shown in figure 1. Not all patients tested for *GNPAT* had serum ferritin available. Iron removed by phlebotomy was similar between C282Y homozygotes with and without the *GNPAT* mutation (Figure 2) (p = 0.33). The multiple regression models demonstrated that male gender (p = 0.04) and C282Y homozygosity (p = 0.01) had significant effects on an elevation in log serum ferritin (Table 1). Male

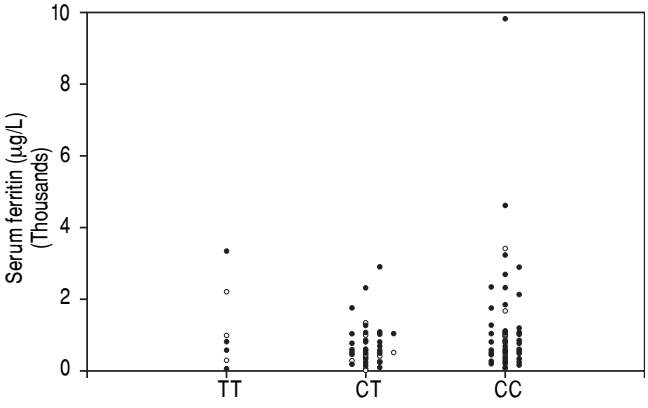

Figure 1. *Serum ferritin in* GNPAT *homozygotes (TT), heterozygotes (CT) and wild type (CC)(●: male. ○: female 1). Log10(ferritin) was compared in these 3 groups by ANOVA with no significant differences between groups (p = 0.56).*

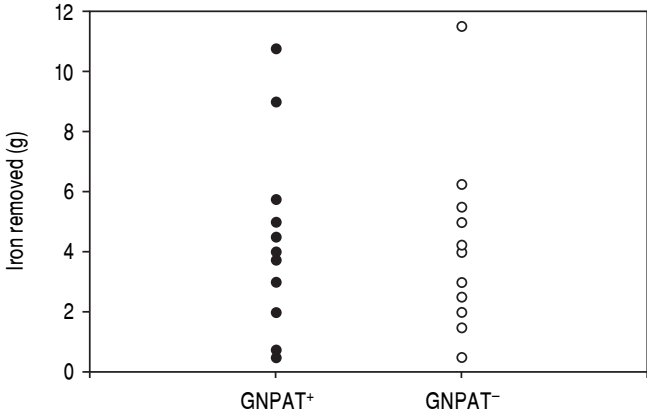

Figure 2. *Iron removed by phlebotomy in grams of iron is compared in C282Y homozygotes with and without the* GNPAT *mutation (p = 0.33).*

Table 1. Least squares multiple regression of independent variables which effect log serum ferritin.

Independent variables	Coefficient	t	p
Age	12.89	1.185	0.24
Male gender	622.44	2.052	0.04
GNPAT+	-396.78	-1.459	0.15
C282Y homozygote	702.35	2.483	0.01

Table 2. Percentage distribution of GNPAT alleles in C282Y homozygotes and wildtype.

	N	TT(wild type)	CT(heterozygote)	CC (homozygote)	Allele Freq C
C282Y homozygote	75	39 (52%)	28 (37%)	8 (11%)	0.226
Wild type	458	290 (63%)	149 (33 %)	19 (4%)	0.213

There was no difference in GNPAT allele frequency between C282Y homozygotes and wild type patients (p = 0.07, Fisher exact test).

Table 3. Frequency of D519G in patients with ferritin ≥ 1,000 μg/L and < 1,000 μg/L.

Ferritin	Levstik (Canada)	Ryan (Ireland)	Besson (France)	McLaren (U.S.)
≥ 1,000 μg/L	20.5% (n = 39)	19.3% (n = 57)	31.5% (81)	38.6% (22)
< 1,000 μg/L	27.9% (n = 68)	25.4% (n = 263)	19.9% (91)	0% (13)

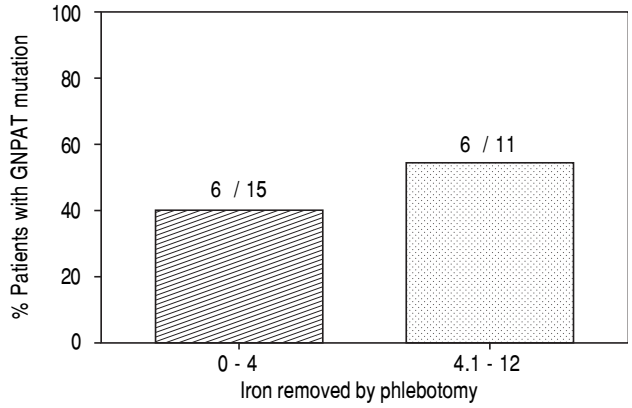

Figure 3. *The proportion of patients with a* GNPAT *mutation (homozygote or heterozygote) in relation to the iron removed by phlebotomy.*

gender was related to iron removed (p = 0.05) There was no effect of the *GNPAT* mutation on serum ferritin or iron removed in the regression models. There were no significant differences in mean serum in a grouping of patients with a serum ferritin ≥ 1,000 μg/L and < 1,000 ≥ g/L (p = 0.49) (Table 3). The relationship between iron removed by phlebotomy and the presence of a *GNPAT* mutation is shown in figure 3.

DISCUSSION

In this study, we have demonstrated that the D519G frequency did not differ significantly in a broad spectrum of C282Y homozygotes and a reference wild type population. This result agrees with the findings of Ryan, *et al.,*[5] but differs from the findings of Besson, *et al.*[6] and McLaren, *et al.*[4] who found D519G enrichment in high expressing C282Y homozygotes.[11] Our findings are also consistent with the observations of Bardou-Jacquet, *et al.,* who re-evaluated the role of *GNPAT* in a large GWAS study of C282Y homozygotes.[7] In a study of 83 healthy volunteers in Taiwan, an oral iron tolerance test showed an increase in serum iron and transferrin saturation in 19 participants with *GNPAT* variants. Fasting serum iron was also higher in subjects with *GNPAT* variants than in wild type subjects.[8] The cause of these iron abnormalities is not clear but could be mediated by serum hepcidin since gene silencing of the *GNPAT* gene in cultured hepatocytes reduced intracellular hepcidin.[4] Serum hepcidin could be of interest in these patients but hepcidin can fluctuate widely even in iron overloaded patients[9] and the diagnostic role of serum hepcidin has not been clearly established.[10]

The patient selection differs in the current study from the original observations of McLaren, *et al.* In their study, only men were included and extreme expression (high and low) was assessed using body iron stores as assessed by liver iron concentration and/or quantitative phlebotomy. The advantages of not using serum ferritin as the marker of iron overload is that patients with false positive elevations in ferritin from alcohol use or inflammation would be excluded. We have been able to analyze a sub-group of patients with iron removed by phlebotomy and have not shown an effect of the *GNPAT* mutation. The study of low expressors by McLaren, *et al*.[4] found no *GNPAT* mutations whereas our current study has demonstrated *GNPAT* mutations across the spectrum of serum ferritin and is consistent with other population studies of *GNPAT* allele frequency. There is a trend showing an increase in *GNPAT* mutation as iron removed increases but the presence of the mutation across all groups limits the value of *GNPAT* as a diagnostic test to predict severity of iron overload.

The role of *GNPAT* mutations in iron metabolism remains elusive[8] but this study clearly demonstrates that the test will not be useful to predict severity of iron overload in C282Y homozygotes.

REFERENCES

1. Pietrangelo A. Genetics, Genetic Testing and Management of Hemochromatosis: 15 years since hepcidin. *Gastroenterology* 2015; 1240-51.
2. Barton JC, Lafreniere SA, Leiendecker-Foster C, Li H, Acton RT, Press RD, Eckfeldt JH. *HFE*, SLC40A1, HAMP, HJV, TFR2, and FTL mutations detected by denaturing high-performance liquid chromatography after iron phenotyping and *HFE* C282Y and H63D genotyping in 785 HEIRS Study participants. *Am J Hematol* 2009; 710-14.
3. de TM, Roth MP, Jouanolle AM, Coppin H, le GG, Piperno A, Ferec C, et al. Genome-wide association study identifies TF as a significant modifier gene of iron metabolism in *HFE* hemochromatosis. *J Hepatol* 2015; 62: 664-72.
4. McLaren C, Emond M, Subramanian N, Phatak P, Barton JC, Adams PC, Goh JB, et al. Exome sequencing in *HFE* C282Y homozygous men with extreme phenotypes identifies a *GNPAT* variant associated with severe Iron overload. *Hepatology* 2015; 62: 429-39.
5. Ryan J, Russell J, Ryan J, Crowe J, Stewart S. *GNPAT* variant is not associated with severe iron ovelroad in Irish C282Y homozygotes. *Hepatology* 2016: 63; 2055-6.
6. Besson C, Martinez M. Further support for the association of *GNPAT* variant rs11558492 with severe iron overload in hemochromatosis. *Hepatology* 2016: 63; 2054-5.
7. Bardou-Jacquet E, De Tayrac M, Mosser J, Deugnier Y. *GNPAT* Variant associated with severe iron overload in *HFE* hemochromatosis. *Hepatology* 2015; 62: 1917-8.
8. Hsiao S, Lee CT, Pei S. *GNPAT* variant is associated with iron phenotyp in healthy Taiwanese women: A population without the *HFE* C282Y mutation. *Hepatology* 2016: 63; 2057-8.
9. Ravasi G, Pelucchi S, Trombini P, Mariani R, Tomosugi N, Modignani GL, Pozzi M, et al. Hepcidin expression in iron overload diseases is variably modulated by circulating factors. *PLoS One* 2012; 7: e36425.
10. Vermeulen E, Vermeersch P. Hepcidin as a biomarker for the diagnosis of iron metabolism disorders: a review. *Acta Clin Belg* 2012; 67: 190-7.
11. Handa P, Kowdley KV. Glyceronephosphate O-acyltransferase as a hemochromatosis modifier gene: Another iron in the fire? *Hepatology* 2015; 62: 337-9.

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