Title

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Increased frequency of *GNPAT* p.D519G in compound *HFE* p.C282Y/p.H63D heterozygotes with elevated serum ferritin levels

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Abstract

Glyceronephosphate O-acyltransferase (*GNPAT*) p.D519G (rs11558492) was identified as a genetic modifier correlated with more severe iron overload in hemochromatosis through whole-exome sequencing of *HFE* p.C282Y homozygotes with extreme iron phenotypes. We studied the prevalence of p.D519G in *HFE* p.C282Y/p.H63D compound heterozygotes, a genotype associated with iron overload in some patients. Cases were Australian participants with elevated serum ferritin (SF) levels ≥300μg/L (males) and ≥200μg/L (females); subjects whose SF levels were below these cut-offs were designated as controls. Samples were genotyped for *GNPAT* p.D519G. We compared the allele frequency of the present subjects, with/without elevated SF, to p.D519G

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Declaration of competing interest

The authors declare no competing interests.

frequency in public datasets. *GNPAT* p.D519G was more prevalent in our cohort of p.C282Y/p.H63D compound heterozygotes with elevated SF (37%) than European public datasets: 1000G 21%, gnomAD 20% and ESP 21%. We conclude that *GNPAT* p.D519G is associated with elevated SF in Australian *HFE* p.C282Y/p.H63D compound heterozygotes.

Keywords

GNPAT; *HFE*; Hemochromatosis; Genetic modifiers; Iron overload

1. Introduction

Dysregulation of systemic iron homeostasis leads to either an increase or a decrease in body iron levels [1]. Hereditary hemochromatosis (HH) is an iron overload disorder caused by mutations in either *HFE* [2], hemojuvelin (*HJV*) [3], transferrin receptor 2 (*TFR2*) [4], ferroportin (*FPN1*) [5] or hepcidin (*HAMP*) [6]. *HFE*-associated HH is the most common form, and in the original description by Feder et al., 83% of patients with a hemochromatosis phenotype were homozygous for the *HFE* p.C282Y allele [2]. Heterozygosity for p.C282Y is generally not associated with iron overload except occasionally in combination with another *HFE* mutation, p.H63D (compound heterozygosity; p.C282Y/p.H63D). The p.H63D allele alone is not associated with iron overload except very rarely in persons with p.H63D homozygosity. Although ~1:200 people of Caucasian origin are *HFE* p.C282Y homozygotes [7], a minority of homozygotes develop significant iron overload related disease [8]. This variability in phenotype is attributed in part to the presence of putative genetic modifiers of iron homeostasis [8].

Recently, a polymorphism in the *GNPAT* gene that encodes glyceronephosphate O-acyltransferase (*GNPAT* p.D519G [rs11558492]) was identified as a potential modifier of iron overload in a whole-exome sequencing study of p.C282Y homozygotes with extreme iron overload phenotypes [9]. Other studies have either substantiated [10,11] or contradicted [12,13] the importance of *GNPAT* p.D519G as a genetic modifier of iron phenotypes in *HFE* p.C282Y homozygotes. In two studies, *GNPAT* p.D519G was associated with increased intestinal iron absorption of an orally-administered iron test dose in women without *HFE* mutations [14,15], suggesting an independent effect of *GNPAT* p.D519G on the regulation of iron absorption. *GNPAT* p.D519G is also a risk factor for familial (but not sporadic) porphyria cutanea tarda [16].

Although some patients with hemochromatosis phenotypes are *HFE* p.C282Y/p.H63D compound heterozygotes, the prevalence of iron overload in this group is lower than in p.C282Y homozygotes and the degree of iron overload tends to be milder than in the most severely-affected p.C282Y homozygotes [17]. The reason for the variability in phenotypic expression among p.C282Y/p.H63D compound heterozygotes is unknown. The prevalence of *GNPAT* p.D519G has been evaluated in French and Austrian *HFE* p.C282Y/p.H63D compound heterozygotes with elevated serum ferritin (SF) [18,19]. In the French study [18], the compound heterozygotes with higher iron stores had an increased *GNPAT* p.D519G allele frequency compared to certain general populations, suggesting a relationship between

GNPAT and iron homeostasis. The study of Austrian patients [19] found no significant difference in the prevalence of *GNPAT* p.D519G in *HFE* p.C282Y/p.H63D compound heterozygotes and *HFE* p.C282Y homozygotes ($p = 0.06$).

To examine the question whether *GNPAT* p.D519G is associated with the likelihood of increased iron stores in *HFE* p.C282Y/p.H63D compound heterozygotes, we compared the prevalence of the p.D519G allele in Australian p.C282Y/p.H63D compound heterozygotes in the QIMR Berghofer Medical Research Institute Haemochromatosis database [20], with or without elevated SF levels. We also compared *GNPAT* p.D519G allele frequency of the present p.C282Y/p.H63D compound heterozygotes with those of large population cohorts.

2. Participants and methods

This study was approved by the QIMR Berghofer Human Research Ethics Committee. Informed and signed consent was obtained from all patients. Subjects were identified via referral for hemochromatosis, a family history of hemochromatosis, or liver disease evaluation. *HFE* genotype (compound heterozygous p.C282Y/p.H63D), gender, age at the time of testing, SF, and transferrin saturation (TSAT) at the time of testing, were extracted from the database [20]. Subjects with SF levels ≥300μg/L (men) and ≥200μg/L (women) were classified as having elevated SF [21]. Subjects with SF below these levels were classified as controls.

The identified samples were genotyped for *GNPAT* p.D519G (rs11558492) using the TaqMan SNP genotyping assay (SYTO Green, Life Technologies). For comparison, population allele frequencies of *GNPAT* p.D519G were extracted from the following datasets: 1000G (http://www.internationalgenome.org/), gnomAD (http://gnomad.broadinstitute.org/) and ESP6500 (http://evs.gs.washington.edu/EVS/) and limited to the European populations. These databases contain variant frequencies derived from the genomic sequencing of large populations with different ethnic backgrounds and can be used to assess the frequency and potential pathogenicity of variants in various populations. The frequencies were then compared to the *GNPAT* p.D519G allele frequency of the present cohort of p.C282Y/p.H63D compound heterozygotes. Categorical variables were analysed between groups using Fisher's exact test. For these and subsequent analyses described below, $p \leq 0.05$ indicated statistical significance.

The univariate proportional odds model was utilized to study the correlation between the outcome, allele count, and the covariate, case/control status [21]. The proportion of subjects having 0, 1 and 2 alleles were represented as p0, p1 and p2. The score test for the proportional odds assumption was performed to examine whether the assumption was violated or not in the given data. The estimated odds ratio and the 95% confidence limit were reported for the covariate case. SAS version 9.4 was utilized for performing logistic regression analyses.

In additional analyses, the natural logarithm transformation (ln) was applied to SF to induce the normality of the distribution. Descriptive statistics (mean, median, standard deviation, minimum, and maximum) were summarised by gender (male/female) and positivity (yes/no)

for the *GNPAT* p.D519G allele for continuous variables that included age, ln(SF), and TSAT. Pearson correlation coefficients were obtained between SF and TSAT, and between ln(SF) and TSAT. Linear regression analyses [22] were performed to model the outcomes SF and ln(SF) separately from sets of covariates that included age, gender (male/female), positivity (yes/no) for *GNPAT* p.D519G allele, and with and without TSAT. However, only the analyses with TSAT are shown, as the analyses without TSAT yielded similar results.

3. Results

3.1. Characterisation of the patient cohort into control and elevated serum ferritin groups

We identified 72 p.C282Y/p.H63D compound heterozygotes in the QIMR Berghofer Medical Research Institute Haemochromatosis Database with DNA available for analysis. Of these, 9 had missing SF data and were excluded from the analyses. *GNPAT* p.D519G was assessed in the remaining 63 subjects, which were divided into two groups: elevated SF (≥300μg/L males, ≥200μg/L females, n=27) and controls (< 300 males, <200 females, n = 36). The cohort was analysed for the following characteristics: age, gender, and *GNPAT* p.D519G genotype (Table 1), where homozygotes were denoted GG/hom, heterozygotes with GA/het and wildtype with AA/WT.

The mean age at the time of testing was 34 years (males 32, females 34) in the control group and 48 years (males 47, females 51) in subjects with elevated SF levels. Regarding genotypes, 6% (males 0%, females 8%) of the control subjects and 11% (males 15%, females 0%) of the cases were homozygous, and 39% of the control group (males 36%, females 40%) and 52% of the case subjects (males 50%, females 57%) were heterozygous for *GNPAT* p.D519G. Among cases, mean SF was 622μg/L (median 599μg/L, IQR 388–861μg/L), while in the control group, it was 88μg/L (median 59μg/L, IQR 42 – 125μg/L). Mean TSAT levels in subjects with elevated SF was 51% versus 36% in the control group.

3.2. Descriptive statistics for serum ferritin, age, gender, transferrin saturation, and positivity for p.D519G

Analyses of ln(SF) included descriptive statistics of the age in years (mean = 40, SD = 15.9) for 62 persons with available data. The median (minimum and maximum) values of SF were 155μg/L (13.0, 1150.0). Corresponding values for natural log-transformed SF was 5.0 (2.6, 7.0). The median (minimum and maximum) TSAT value in 60 participants was 35.5% (16.0, 103.0) (Table 1). Pearson correlation coefficients were computed between SF and TSAT (0.42) and between ln(SF) and TSAT (0.47), p = 0.0008 and p = 0.0002, respectively, indicating a positive correlation between SF and TSAT.

3.3. Comparison of the GNPAT p.D519G allele frequency in the elevated serum ferritin group with public datasets

GNPAT p.D519G allele frequency was greater in the elevated SF group (37%) than the control group (25%), but this difference was not statistically significant (p = 0.1718). The allele frequency of *GNPAT* p.D519G was significantly greater in our cohort of p.C282Y/p.H63D compound heterozygotes with elevated SF than in most of the publicly available datasets that we analysed (Table 2). The p.D519G allele frequency (37%) was significantly

higher in the elevated SF group than the following populations; 1000G: Europe 21% (p = 0.0106), including subsets: Utah residents with Northern and Western European ancestry (CEU) 17% (p = 0.0026) and Finnish in Finland (FIN) 11% (p < 0.0001), gnomAD: non-Finnish European 20% (p = 0.0059), Finnish European 8% (p < 0.0001) and ESP: European American 21% (p = 0.0061). The p.D519G allele frequency (37%) was not significantly higher in the elevated SF group than in 1000G: GBR, 1000G: IBS and 1000G: TSI subpopulations, 25% (p = 0.0843), 29% (p = 0.3234) and 24% (p = 0.0582) respectively.

3.4. The odds ratio of allele count and case/control status

The relationship between the allele count and case/control status was not significantly different in males than in females (Joint Test, p=0.31). There was no significant correlation between the allele count and gender or case/control status (Type 3 analysis of Effects, p = 0.63, and p = 0.13 respectively). The test of the proportional odds assumption was not significant (p = 1.00), which indicates that the proportional odds assumption is reasonable. For a case (elevated SF), the odds of having more allele counts was 2.13 (95% CI, 0.80, 5.66) times higher than that for a control.

3.5. Linear regression model of serum ferritin with age, gender, and positivity for p.D519G

When TSAT was included in the set of covariates, both age and gender still had a significant correlation with SF when the positivity for p.D519G and TSAT were adjusted. The TSAT had a significant correlation with SF (p = 0.015) when age, gender, and the positivity for p.D519G were adjusted. However, there was no significant correlation either between SF and the positivity for p.D519G in this model (p =0.0847). The expected average of SF in men was 276.9μg/L higher than the expected average of SF in women when age, positivity for p.D519G, and TSAT were adjusted (Table 3). Other regression models without TSAT as a covariant yielded similar result (data not shown).

3.6. Linear regression model of the natural log-transformed serum ferritin with age, gender, and positivity for p.D519G

When the natural log-transformed SF was the outcome and the set of covariates included TSAT, then age, gender, TSAT, and the positivity for p.D519G had a significant correlation with the transformed SF (p = 0.002). The expected average of transformed SF in men was 1.02 ln(ug/L) higher than the expected average of transformed SF in women when the other covariates were adjusted (Table 4). Other regression models without TSAT as a covariant yielded similar results (data not shown).

4. Discussion and conclusions

Studies of humans with GNPAT mutations other than p.D519G revealed reduced transferrin receptor recycling [23]. Other GNPAT mutations/deletions occur in people with peroxisomal disease, a class of disorders characterized by increased hepatic iron [24]. p.D519G was associated with significantly higher serum iron levels and higher TSAT after an oral iron challenge in healthy Taiwanese women, a population in which HFE p.C282Y does not occur, suggesting that p.D519G is involved in the regulation of hepcidin independently of p.C282Y

[14]. Similarly, in European subjects without hemochromatosis-associated *HFE* genotypes, serum iron levels and transferrin saturation (TS) after an oral iron challenge were significantly higher in p.D519G-positive than in p.D519G-negative subjects [15]. In the human liver cell line HepG2/C3A (ATCC HB-8065), GNPAT knockdown decreased the baseline activity of the BMP-SMAD pathway, although the function of the pathway was normal [9]. In mice with Gnpat-knockout ($Gnpat^{-/-}$). Gnpat/Hfe double-knockout ($Gnpat^{-/-}Hfe^{-/-}$ or DKO) mice, and hepatocyte-specific Gnpat-knockout mice (Gnpatfl/fl;Alb-Cre), hepcidin expression was normal and no effect on either systemic iron metabolism or iron-overload phenotypes under normal or high dietary iron conditions was observed [25].

Our results demonstrate that *GNPAT* p.D519G is associated with elevated SF levels in Australian *HFE* p.C282Y/p.H63D compound heterozygotes and that the *GNPAT* p.D519G allele frequency is greater in *HFE* p.C282Y/p.H63D compound heterozygotes with elevated SF than in several large European population cohorts. Whether this is due to differences in the genetic makeup of the Australian population from which the study subjects were derived or that the high p.D519G allele frequency observed is being driven by the effect of the *GNPAT* p.D519G variant on iron metabolism would require a larger study of cases and controls. The *GNPAT* p.D519G allele frequency appeared higher in the group with elevated SF (37%) than among the control group (25%), but this difference was not statistically significant ($p = 0.1718$).

We found no significant correlation between the allele count and the case/control status between males and females (Joint Test, $p = 0.31$). The odds of having more alleles was 2.13 times higher in a case versus a control. Using linear regression analyses to model the outcomes SF and ln(SF) separately from sets of covariates, we found that age and gender had a significant correlation with SF ($p < 0.0001$) when TSAT and *GNPAT* p.D519G were adjusted in the model. Covariates including age, gender, and TSAT were significantly associated with ln(SF) when positivity for p.D519G was adjusted ($p = 0.002$).

The correlation coefficient (r) of TSAT and SF at diagnosis in the present HFE p.C282Y/p.H63D compound heterozygotes was 0.42. This coefficient was statistically significant but not especially high. Transferrin saturation is a surrogate measure of the rate at which available iron is released from macrophages via ferroportin, whereas serum ferritin is a surrogate measure of body iron stores [26,27]. In patients with hemochromatosis, the correlation coefficients of TSAT and log SF at diagnosis with iron stores removed by phlebotomy to achieve iron depletion in 54 p.C282Y homozygotes were 0.54 and 0.74, respectively, and in eight p.C282Y/p.H63D compound heterozygotes were 0.95 and 0.85, respectively [28].

In a study from Southern France which included 77 male p.C282Y/p.H63D compound heterozygotes (SF > 500 μg/L), *GNPAT* p.D519G allele frequency was significantly higher than in individuals from Northwest Europe (CEU) used for the HapMap project ($p < 0.0001$) and in 4300 European Americans from the National Heart, Lung, and Blood Institute Exome Sequencing Project ($p < 0.0001$) (http://evs.gs.washington.edu/EVS/) [18]. This is similar to our results, as we also found the *GNPAT* p.D519G allele frequency to be higher in our cohort of p.C282Y/p.H63D compound heterozygotes with elevated SF, than in the CEU

cohort, 37% versus 17% ($p = 0.0026$), respectively. In Austrian hemochromatosis patients, they looked at the prevalence of GNPAT p.D519G in HFE p.C282Y/p.H63D compound heterozygotes and HFE p.C282Y homozygotes but found no significant difference ($p = 0.06$) [19]. The implications of a possibly higher prevalence of *GNPAT* p.D519G among *HFE* p.C282Y/p.H63D compound heterozygotes than among *HFE* p.C282Y homozygotes is still unknown. The results of our current study are consistent with the previous findings in French male p.C282Y/p.H63D compound heterozygotes with elevated SF [18] and extend these results in Australian male and female p.C282Y/p.H63D compound heterozygotes with elevated SF, in whom the frequency of *GNPAT* p.D519G was higher in participants with elevated SF than in several public databases. This provides further evidence that *GNPAT* p.D519G is more prevalent in p.C282Y/p.H63D compound heterozygotes with elevated SF than in the general population. Thus, the variability in SF among p.C282Y/p.H63D compound heterozygotes may be explained in part by co-inheritance of *GNPAT* p.D519G.

A limitation of our study is that the number of *HFE* p.C282Y/p.H63D compound heterozygotes with elevated SF and positivity for GNPAT p.D519G was small. This may have limited our ability to demonstrate significant differences between iron phenotypes of subjects positive and negative for p.D519G, if they exist. It is plausible but unproven that a lack of elevated SF in some of the present subjects with p.D519G suggests that iron homeostasis in p.C282Y/p.H63D compound heterozygotes with or without p.D519G is also affected by genetic factors such as sex or inheritance of other modifier alleles for which we did not test, or acquired factors such as dietary iron content, medications, medical conditions, blood donations, or age. Nonetheless, the present observations may inform expanded studies of iron phenotypes in p.C282Y/p.H63D compound heterozygotes in Australia or elsewhere.

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Abbreviations:

CEU	Utah residents with Northern and Western European ancestry
CI	confidence interval
FIN	Finnish in Finland
FPN	ferroportin
Freq	frequency
GBR	British in England and Scotland

GNPAT	Glyceronephosphate O-acyltransferase
HAMP	hepcidin
HH	hereditary hemochromatosis
HJV	1hemojuvelin
het	heterozygote
HFE	homeostatic iron regulator protein
hom	homozygote
IBS	Iberian populations in Spain
IQR	interquartile range
ln	natural logarithm
SF	serum ferritin
SEM	standard error of the mean
TFR2	transferrin receptor 2
TSAT	transferrin saturation
TSI	Toscani in Italy
WT	wildtype

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Table 1

Characteristics of the C282Y/H63D compound heterozygote patients.

Patient characteristics	**Controls (Males SF < 300) (Females SF < 200) *n* = 36**			**Elevated SF (Males SF ≥ 300) (Females SF ≥ 200) *n* = 27**		
	All	**Males**	**Females**	**All**	**Males**	**Females**
Age	Count (frequency)			Count (frequency)		
< 40	23 (0.64)	7 (0.64)	16 (0.64)	9 (0.33)	8 (0.4)	1 (0.14)
40–50	6 (0.17)	0 (0)	6 (0.24)	5 (0.19)	3 (0.15)	2 (0.29)
> 50	6 (0.17)	3 (0.27)	3 (0.12)	13 (0.48)	9 (0.45)	4 (0.57)
Unknown	1 (0.03)	1 (0.09)	0 (0)	0 (0)	0 (0)	0 (0)
Average	34	32	34	48	47	51
Genotype	Count (frequency)			Count (frequency)		
Homozygous (AA)/WT	20 (0.56)	7 (0.64)	13 (0.52)	10 (0.37)	7 (0.35)	3 (0.43)
Heterozygous (GA)/het	14 (0.39)	4 (0.36)	10 (0.4)	14 (0.52)	10 (0.5)	4 (0.57)
Homozygous (GG)/hom	2 (0.06)	0 (0)	2 (0.08)	3 (0.11)	3 (0.15)	0 (0)
	Mean (SEM)	Median	IQR	Mean (SEM)	Median	IQR
Serum ferritin (ug/L)	88.39 (11.48)	59	41.5–125.25	622.37 (56.38)	599	387.5–861
Transferrin saturation (%)	36.37 (2.49)	34	26–40.5	51.12 (5.15)	47	29–65
	N	Median	Mean	Standard deviation	Minimum	Maximum
Age (year)	62	39.0	39.8	15.9	10.0	73.0
SF	63	155	317.2	331.1	13.0	1150.0
ln(SF)	63	5.0	5.1	1.3	2.6	7.0
TSAT (%)	60	35.5	42.5	21.2	16.0	103.0

SF: serum ferritin, No.: number, Freq: frequency, Hom: homozygous, Het: heterozygous, WT: wildtype, SEM: standard error of the mean, IQR: interquartile range.

Table 2

Frequencies of the *GNPAT* variant in the 1000 Genomes Project, Genome Aggregation Database, and Exome Sequencing Project data sets.
Gene (SNP number) *GNPAT (rs11558492)*; Genetic position chr1:231408091A > G.
Nucleotide/amino acid change C.1556A > G/p.Asp519Gly.

Database	Population	Allele count A	Allele count G (freq)	Control[a]	Elevated SF[b]
1000G	Europe	792	214 (0.21)	0.4591	0.0106 *
	CEU	164	34 (0.17)	0.1642	0.0026 **
	FIN	177	21 (0.11)	0.0054 **	<0.0001 ****
	GBR	137	45 (0.25)	>0.9999	0.0843
	IBS	151	63 (0.29)	0.5462	0.3234
	TSI	163	51 (0.24)	0.8739	0.0582
gnomAD	Non-Finnish European	102,715	26,407 (0.20)	0.3794	0.0059 **
	Finnish European	23,051	2071 (0.08)	<0.0001 ****	<0.0001 ****
ESP6500	European American	6830	1770 (0.21)	0.3794	0.0061 **
C282Y/H63D compound heterozygote patients		54[a]	18 (0.25)[a]	0.1718	
		34[b]	20 (0.37)[b]		

CEU: Utah residents with Northern and Western European ancestry, FIN: Finnish in Finland, GBR: British in England and Scotland, IBS: Iberian populations in Spain, TSI: Toscani in Italy, Control: control group (males<300μg/L, females>200μg/L), Elevated SF: Elevated SF group (males ≥300μg/L, females ≥200μg/L).

*Denotes significance ($P < 0.05$).

**($P < 0.01$).

****($p < 0.0001$).

[a]Denotes control group.

[b]Denotes elevated SF group.

Table 3

The linear regression model of serum ferritin with covariates including Age, Gender, Positivity for p.D519G, and transferrin saturation.

E[SF] = −338.011 + 7.450 × Age + 276.861 × Male + 112.895 × Positivity for p.D519G + 4.0 × transferrin saturation.

Parameter	Estimate	Standard error	Pr > |t|	95% Confidence limits	
Intercept	−338.011	104.966	0.0022	−548.456	−127.567
Age	7.450	2.018	0.0005	3.404	11.496
Gender 1.Male	276.861	67.345	0.0001	141.841	411.880
p.D519G 1.Positive	112.895	64.275	0.0847	−15.968	241.758
TSAT	3.998	1.587	0.0148	0.816	7.181

Table 4

The linear regression model of ln(SF) with covariates including Age, Gender, Positivity for p.D519G, and transferrin saturation.

E[Ln(SF)]=2.368+0.030×Age+1.016×Male +0.560×Positivity for P.D519G + 0.018 × transferrin saturation.

Parameter	Estimate	Standard error	Pr > |t|	95% Confidence limits	
Intercept	2.368	0.370	<0.0001	1.625	3.111
Age	0.029	0.007	0.0001	0.015	0.044
Gender 1.Male	1.016	0.238	<0.0001	0.540	1.493
p.D519G 1.Positive	0.560	0.227	0.0167	0.106	1.015
TSAT	0.018	0.006	0.0022	0.007	0.029