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Hemochromatosis (*HFE*) gene mutations and risk of gastric cancer in the European Prospective Investigation into Cancer and Nutrition (EPIC) study

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Hereditary hemochromatosis (HH) is a strong risk factor for hepatocellular cancer, and mutations in the *HFE* gene associated with HH and iron overload may be related to other tumors, but no studies have been reported for gastric cancer (GC). A nested case–control study was conducted within the European Prospective Investigation into Cancer and Nutrition (EPIC), including 365 incident gastric adenocarcinoma and 1284 controls matched by center, sex, age and date of blood collection. Genotype analysis was performed for two functional polymorphisms (C282Y/rs1800562 and H63D/rs1799945) and seven tagSNPs of the *HFE* genomic region. Association with all gastric adenocarcinoma, and according to anatomical localization and histological subtype, was assessed by means of the odds ratio (OR) and 95% confidence interval (CI) estimated by unconditional logistic regression adjusted for the matching variables. We observed a significant association for H63D with OR (per rare allele) of 1.32 (CI = 1.03–1.69). In subgroup analyses, the association was stronger for non-cardia anatomical subsite (OR = 1.60, CI = 1.16–2.21) and intestinal histological subtype (OR = 1.82, CI = 1.27–2.62). Among intestinal cases, two tagSNPs (rs1572982 and rs6918586) also showed a significant association that disappeared after adjustment for H63D. No association with tumors located in the cardia or with diffuse subtype was found for any of the nine SNPs analyzed. Our results suggest that H63D variant in *HFE* gene seems to be associated with GC risk of the non-cardia region and intestinal type, possibly due to its association with iron overload although a role for other mechanisms cannot be entirely ruled out.

Introduction

Previous studies have reported that non-cardia gastric cancer (GC) risk was significantly associated with increasing intakes of total, red and processed meat (1). This could be due to a high exposure to *N*-nitroso-compounds (NOC), such as nitrosamines and nitrosamides. Meat may have a high content of such compounds, but it is also a source of precursors of NOC (nitrates, nitrites and proteins) and of heme iron that could act as nitrosating agent; thus, the observed association could be due to endogenous nitrosation (2). Following these results, we have also reported that GC risk was associated with increasing dietary intake of heme iron, mainly among subjects with low plasmatic levels of vitamin C (3).

Iron has long been suggested to play a role in carcinogenesis, based mainly on animal model studies, and to a smaller extent, observational studies in humans (4). The main putative mechanism is believed to be iron-induced oxidative stress (5). Redox cycling of iron is closely related with the production of reactive oxygen species able to induce lipid peroxidation and oxidative damage to DNA. Furthermore, reactive oxygen species produced by iron have been shown to specifically target some tumor suppressor genes (5). As mentioned above, heme

Abbreviations: CI, confidence interval; CRC, colorectal cancer; EPIC, European Prospective Investigation into Cancer and Nutrition; GC, gastric cancer; HCC, hepatocellular carcinoma; HH, hereditary hemochromatosis; MAF, minor allele frequency; NOC, N-nitroso-compounds; OR, odds ratio; SNP, single nucleotide polymorphisms.

iron also plays an important role in endogenous nitrosation; the group nitrosyl iron of heme acts as a nitrosating agent and in the presence of amines or amides this could lead to the formation of NOC, including known human carcinogens (6).

If iron plays a role in carcinogenesis, individuals with elevation of total body iron stores or iron overload could be at higher risk of developing cancer. Hereditary hemochromatosis (HH) is the most severe clinical expression of iron overload, leading to dysfunction of liver, pancreas, heart and other organs (7). The commonest clinical form is HH type 1, an autosomal recessive disease caused by mutations in the *HFE* gene (8). Most individuals affected by HH are homozygous for the polymorphism C282Y in *HFE*. The variant form is relatively common in European populations, with allelic frequencies 7–10% in Great Britain, 4–8% in Central and Northern Europe and 3% or below in Spain and Italy. Another common polymorphism is H63D, with allelic frequency 10–20% in European populations. The prevalence estimates in US population (non-Hispanic whites) are 6% for C282Y and 15% for H63D (9). H63D is also considered a mutation associated with HH, but its penetrance is much lower than for C282Y. Compound heterozygotes for C282Y and H63D or homozygous variant H63D rarely develop clinical disease, but they have moderate degree of overload, with high serum ferritin and transferrin saturation (8).

From observational studies in humans, there is little doubt that HH is a strong risk factor for hepatocellular carcinoma (HCC). Two recent meta-analyses (10,11) reported a strong association between variant C282Y (homozygous or allelic) and risk of HCC, but the evidence is limited for the association with non-hepatic localizations. For colorectal cancer (CRC), three studies (12–14) have shown a positive association for homozygous C282Y or carriers of at least one mutation in C282Y or H63D, whereas others did not find any association with CRC (15,16) or colorectal adenomas (17). Women with C282Y were found to have an increased risk of breast (12) and epithelial ovarian cancer (18), but no associations were reported for C282Y or H63D variants with prostate or breast cancer in males (12,19), pancreatic cancer (20) or endometrial cancer (18). The presence of H63D mutation was associated with an increased risk of acute lymphoblastic leukemia, but not with other types of acute leukemia (21).

To our knowledge, no studies have been published on the association between gastric cancer and hemochromatosis. The purpose of this work was to assess the potential effect of polymorphism in the *HFE* gene on the risk of gastric adenocarcinoma, according to anatomical localization and histological subtype, in a prospective study in European populations.

Materials and methods

Study design and participants

The study subjects were participants from the European Prospective Investigation into Cancer and Nutrition (EPIC), following a nested case–control design. Methods and rationale of the EPIC study have been reported elsewhere (22). Briefly, the EPIC cohort includes 521 457 participants recruited between 1992 and 2000 in 23 centers from 10 European countries (Denmark, France, Germany, Greece, Italy, the Netherlands, Norway, Spain, Sweden and the United Kingdom). At enrollment, each subject provided information about usual diet, lifestyle factors and anthropometric measurements; blood samples were also collected for most participants. All participants provided a written informed consent at recruitment, and the study was approved by the ethical committees at the International Agency for Research on Cancer (IARC) and in each of the EPIC centers.

Identification of cancer cases was based on population cancer registries in most countries, except in France, Germany and Greece where it was mainly achieved by active contact with study subjects and review of health insurance and pathology reports. Cases for our study were subjects without any previous cancer, newly diagnosed with histologically confirmed primary gastric adenocarcinoma during the follow-up, which started at the date of recruitment and was performed through 2003 to 2006 depending on the study center. During this period, 571 gastric cancers were diagnosed within the cohort, defined by code C16 of the 10th Revision of the International Classification of Diseases (ICD-10). An independent panel of pathologists reviewed original slides and/or cuts from paraffin blocks as well as pathology reports provided by each EPIC center to confirm and validate the diagnosis, tumor site and morphology (23). A total of 10 cases were excluded because they had no malignant tumors, 31 because they had no primary GC and 46 because they had a prevalent tumor (the subject had already been diagnosed with another cancer). Furthermore, 6 cases with tumors located in the gastric stump and 65 with morphology other than adenocarcinoma were excluded, leaving a total of 413 incident primary gastric adenocarcinomas for analysis.

Within the EPIC cohort, a nested case–control study (EurGast) was designed to assess the genetic susceptibility and dietary biomarkers in relation with GC risk. For each case, up to four control subjects were randomly selected among cohort members alive and free of cancer at the time of diagnosis of the case, matched by center, sex, age at baseline (±2.5 years) and date of blood collection (±45 days). Following these criteria, 413 GC cases and 1565 matched controls with blood samples available were included. After excluding subjects without DNA available or whose DNA could not be amplified (48 cases and 281 controls), our final data set for genotype analysis included 365 cases and 1284 controls (Table I).

Single nucleotide polymorphisms selection

We aimed to analyze the two common functional variants in *HFE* associated with HH: the G to A at nucleotide 845 in exon 4, resulting in a cysteine to tyrosine substitution at amino acid 282 (C282Y, rs1800562), and a second variant in exon 2 (rs1799945), a C to G at nucleotide 187, resulting in a histidine to aspartic acid substitution at amino acid 63 (H63D). Furthermore, we compiled a list of single nucleotide polymorphisms (SNPs) from 10 kb upstream to at least 3 kb downstream of the *HFE* gene from HapMap data for Caucasians (phase II CEU population, releases 23a or 24 based on dbSNP v126 and NCBI genome build 36). Haplotype blocks were defined using Haploview v4.0, and tagging SNPs (tagSNPs) were selected using the Tagger algorithm as reported previously (24). Criteria for tagSNP selection were minor allele frequency (MAF) ≥5% in Caucasians, $r^2 \geq 0.8$ between each pair of tagged and tagSNPs (pair-wise tagging), and SNPs tagging haplotypes with a frequency ≥5%. Following these criteria, seven tagSNPs were selected (rs4529296, rs1572982, rs707889, rs1045537, rs17596719, rs6918586 and rs1543680), among which one (rs1543680) is located in the *HIST1H4C* gene (Table II).

Genotyping, DNA extraction and quality control

Genomic DNA was extracted from a 0.5 ml aliquot of buffy coat, following procedures described previously (25). DNA concentrations were measured by PicoGreen dsDNA quantitation assay (Molecular Probes, The Netherlands), and 0.75–1 µg of DNA at ~50 ng/µl was pipetted to 96-well plates for genotyping. Genotyping was carried out using the Illumina BeadStation Platform and GoldenGate technology (Illumina, San Diego, CA), at the laboratory of the Spanish National Genotyping Center (CEGEN, Barcelona, Spain).

Genotyping of the nine SNPs selected for this study was included within a 1536 SNPs panel of a candidate genes analysis (24). In the main study, SNPs were excluded when they failed in >20% of samples ($n = 4$), were monomorphic ($n = 22$) or deviated from Hardy–Weinberg equilibrium (HWE) among controls ($n = 9$). An additional 214 SNPs were excluded either because they could not be amplified or because their genotyping signal or cluster separation was not good enough. None of the excluded SNPs was in the *HFE* region. In addition to the internal genotyping controls included by CEGEN, 5% of the samples ($n = 100$) were genotyped in duplicate with overall agreement of 99.2%. It must be kept in mind, however, that genotyping of *HFE* gene was based on the *a priori* hypothesis of the potential relationship of iron overload and GC. Thus, although the selected *HFE* SNPs were included within the panel of a larger study (24), the analysis of *HFE* was considered totally independent of the other 248 genes/genomic regions genotyped.

Other factors: iron intake

The usual diet over the previous year was measured at recruitment by country-specific validated questionnaires (22). Using this information, dietary iron intake was computed using country-specific food composition tables (3).

Statistical analysis

For each polymorphism, HWE and pair-wise linkage disequilibrium were tested separately for cases and controls. Association between each SNP and GC risk was assessed by the odds ratio (OR) and 95% confidence interval (CI) estimated by unconditional logistic regression, adjusted for the matching variables sex, age (5 year categories), center and date of blood collection (quarters of year). Given the matched design of the study, we checked that this approach provided approximately the same results obtained by means of conditional logistic regression. Other covariates potentially related with GC risk are expected to be independent of genetic variation and were not included in the model. The potential effect modification by iron intake was considered in stratified analyses, and interaction with the SNP of interest was assessed by means of a likelihood ratio test. The possibility of population stratification was considered within the context of the main study. The observed distribution of

Table I. Main characteristics of cases and controls

		Cases (365)	Controls (1284)	*P* value[a]
		n (%)	*n* (%)	
Sex	Male	214 (58.6)	759 (59.1)	—
	Female	151 (41.4)	525 (40.9)	
Country	France	2 (0.5)	3 (0.2)	—
	Italy	56 (15.3)	206 (16.0)	
	Spain	41 (11.2)	134 (10.4)	
	United Kingdom	41 (11.2)	135 (10.5)	
	The Netherlands	26 (7.1)	99 (7.7)	
	Greece	24 (6.6)	88 (6.9)	
	Germany	48 (13.2)	186 (14.5)	
	Sweden	64 (17.5)	220 (17.1)	
	Denmark	61 (16.7)	205 (16.0)	
	Norway	2 (0.5)	8 (0.6)	
Education	None	21 (5.8)	82 (6.4)	0.49
	Primary school	157 (43.0)	507 (39.5)	
	Technical/professional	91 (24.9)	305 (23.8)	
	Secondary school	40 (11.0)	140 (10.9)	
	University	48 (13.2)	218 (17.0)	
	Missing	8 (2.2)	32 (2.5)	
Smoking status	Never smoker	128 (35.1)	542 (42.2)	0.001
	Former smoker	115 (31.5)	427 (33.3)	
	Current smoker	118 (32.3)	292 (22.7)	
	Missing	4 (1.1)	23 (1.8)	
		Mean (SD)	Mean (SD)	
Age at recruitment (years)		58.4 (7.9)	58.4 (7.7)	—
Dietary intake (per day)				
Energy (kcal)		2142 (631.5)	2137 (696.9)	0.85
Iron (mg)		13.2 (4.3)	13.3 (4.7)	0.59
Vegetables (g)		183.2 (140.5)	185.7 (133.4)	0.66
Fruit (g)		215.7 (182.0)	235.6 (182.7)	0.04
Red meat (g)		50.8 (35.6)	48.6 (37.8)	0.28
Processed meat (g)		39.4 (38.5)	35.5 (33.3)	0.01
Alcohol (g)		16.9 (23.4)	15.0 (22.8)	0.17

[a]*P* values are calculated excluding subjects with missing values; data not shown for variables on which cases and controls were matched.

P values of the 1287 SNPs analyzed (24) was well fitted by a uniform distribution, indicating that genomic control was not needed and the potential ethnic heterogeneity was corrected by adjustment for center.

The main analysis to explore the potential relationship between each SNP and GC risk was based on the log-additive (per allele) model, meant to be the most sensitive to detect an association. In subsequent analyses, other genetic models such as dominant, recessive and codominant were further explored. These analyses were carried out in the whole data set and for each tumor localization and histological type. To account for multiple testing related to assessing several SNPs, a gene-based permutation test was performed. After 10 000 permutations, the distribution of minimum *P* values of each of the nine SNPs analyzed was fitted to a beta distribution, with parameter gamma estimated by the maximum likelihood method, and used to calculate a minimum adjusted *P* value.

Haplotype analysis of the genotyped SNPs of the *HFE* genomic region was carried out using the EM = Expectation - Maximization algorithm as implemented in the haplo.stats R package. Haplotype frequencies were inferred, and association of each haplotype with frequency ≥5% was assessed by its OR and 95% CI compared with the most frequent haplotype among controls, taken as the referent group.

Results

The mean age of both cases and controls was 58.4 years, and they also had very similar distribution by sex and country, as expected from the matched design (Table I). Compared with controls, cases had a significantly higher proportion of current smokers (32 versus 23%), ate significantly less fruit than controls (216 and 236 g/day in average, respectively) and more processed meat (39 versus 36 g/day). Out of the 365 cases, 107 (29%) had tumors located in the cardia and 181 (50%) in distal parts of the stomach (non-cardia); 6 cases had a mixed localization including both gastric regions and the tumor site was unknown for the remaining 71. Regarding the histology, our case series had about the same prevalence (35%) of the 2 main subtypes of Lauren's classification (126 intestinal for 128 diffuse type), whereas 8 had a mixed type and 103 could not be classified.

The nine genotyped SNPs were in HWE among controls, and the allelic frequencies were in good agreement with the expected prevalence in Caucasian populations (Table II). The variant alleles were relatively common for the seven tagSNPs (MAF ranging from 10 to 37%), as well as for the variant form of H63D (allele G at nucleotide 187), with a frequency of 12.7%. Regarding the polymorphism with strongest association with HH (C282Y), the variant A had frequency of 4.2%.

In the association analysis based on the log-additive model (Table III), only H63D (rs1799945) was significantly associated with the risk of GC, with OR of 1.32 (1.03–1.69). This association was even more marked when the analysis was restricted to cases located in the distant stomach (non-cardia) and those of intestinal type, with ORs 1.60 (1.16–2.21) and 1.82 (1.27–2.62), respectively. Regarding GC of intestinal type, in addition to H63D, two tagSNP showed a significant increased risk: rs1572982 (OR = 1.40, CI = 1.08–1.83) and r66918586 (OR = 1.39, CI = 1.06–1.82). There were no significant associations of any SNP (including H63D) for cases located in the cardia or cases of diffuse type. Moreover, no significant associations were found for the cases with unknown localization or histological subtype of the tumor (results not shown).

A more detailed analysis exploring other genetic models was carried out for the three SNPs with significant association according to the log-additive model (Table IV). In the whole data set, H63D showed only an almost significant association with GC risk for the dominant model (OR = 1.33, *P* = 0.056), but significant associations were found for the codominant and dominant models in the non-cardia cases and in all models (codominant, dominant and recessive) for intestinal type. However, the strongest association was seen for the dominant

Table II. Description of SNPs genotyped in the *HFE* genomic region and frequencies in cases and controls

SNP	Gene	SNP location[a]	Aminoacid change	Alleles[b]	MAF CEU (%)[c]	Cases/controls genotyped	MAF (controls) (%)	HWE test *P* value (controls)
rs4529296	HFE	Flanking 5′ UTR	—	C/G	40.0	363/1277	37.2	0.81
rs1799945	HFE	Coding	H63D	C/G	12.9	323/1157	12.7	0.29
rs1800562	HFE	Coding	C282Y	G/A	4.2	365/1283	4.2	0.48
rs1572982	HFE	Intron	—	G/A	42.0	365/1282	45.6	0.12
rs707889	HFE	Flanking 3′ UTR	—	C/T	20.0	365/1277	19.3	0.09
rs1045537	HFE	Flanking 3′ UTR	—	G/C	12.7	365/1283	10.5	0.23
rs17596719	HFE	Flanking 3′ UTR	—	G/A	12.5	365/1282	12.4	0.70
rs6918586	HFE	Flanking 3′ UTR	—	T/C	35.8	365/1280	39.6	0.82
rs1543680	HIST1H4C	Flanking 5′ UTR	—	G/A	20.0	362/1282	18.9	0.14

[a]SNP location relative to the gene.
[b]The more common allele in Caucasians reported first.
[c]HapMap CEU, from dbSNP build 131.

Table III. Association between SNPs in HFE region and gastric adenocarcinoma, overall and according to localization and histological type. OR and 95% CI for log-additive model (per allele effect) estimated by unconditional logistic regression, adjusted for sex, age, center and date of blood collection

SNP	All gastric adenocarcinoma			Cardia (107 cases)		Non-cardia (181 cases)		Intestinal type (126 cases)		Diffuse type (128 cases)	
	Cases/ controls	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value	OR (95% CI)	*P* value
rs4529296	363/1277	1.09 (0.91–1.29)	0.35	1.04 (0.77–1.41)	0.79	1.13 (0.90–1.43)	0.30	1.22 (0.93–1.61)	0.15	0.99 (0.75–1.31)	0.97
rs1799945[a]	323/1157	1.32 (1.03–1.69)	0.031	1.14 (0.71–1.82)	0.59	1.60 (1.16–2.21)	0.005	1.82 (1.27–2.62)	0.002	1.36 (0.92–1.99)	0.13
rs1800562[b]	365/1283	1.12 (0.75–1.67)	0.59	0.70 (0.33–1.51)	0.35	1.37 (0.81–2.32)	0.25	0.95 (0.48–1.88)	0.88	1.12 (0.59–2.10)	0.74
rs1572982	365/1282	1.09 (0.93–1.29)	0.29	1.06 (0.80–1.41)	0.70	1.07 (0.86–1.34)	0.54	1.40 (1.08–1.83)	0.012	1.11 (0.85–1.44)	0.45
rs707889	365/1277	0.96 (0.77–1.18)	0.67	0.96 (0.67–1.37)	0.81	0.97 (0.73–1.29)	0.83	0.88 (0.62–1.24)	0.46	0.85 (0.60–1.20)	0.35
rs1045537	365/1283	0.96 (0.74–1.26)	0.79	0.86 (0.53–1.38)	0.51	0.95 (0.65–1.39)	0.79	0.83 (0.52–1.32)	0.43	1.24 (0.83–1.86)	0.30
rs17596719	365/1282	0.95 (0.73–1.24)	0.71	1.24 (0.79–1.92)	0.35	0.82 (0.57–1.19)	0.29	1.08 (0.73–1.61)	0.70	0.80 (0.52–1.24)	0.31
rs6918586	365/1280	1.11 (0.94–1.32)	0.23	1.06 (0.79–1.43)	0.68	1.14 (0.91–1.44)	0.26	1.39 (1.06–1.82)	0.017	1.17 (0.89–1.54)	0.27
rs1543680	362/1282	0.93 (0.75–1.15)	0.48	0.95 (0.66–1.37)	0.80	0.96 (0.72–1.28)	0.76	0.81 (0.57–1.15)	0.23	0.82 (0.58–1.17)	0.28

[a]H63D.
[b]C282Y.

model, with OR = 1.73 (P = 0.004) and OR = 1.93 (P = 0.004) for the non-cardia and intestinal cases, respectively. A similar pattern was observed for the two tagSNP associated with increased risk of intestinal GC (rs1572982 and r6918586).

According to Haploview, H63D (rs1799945) is located in the recombination point of two blocks, one at 5′ of the gene tagged by the tagSNP rs4529296 and another tagged by the remaining 7 SNPs (including C282Y). Because rs4529296 was not associated with GC in previous analyses, we performed a haplotype analysis including H63D (rs1799945) plus the seven SNPs in the linkage disequilibrium block at 3′ of the *HFE* gene (Table V). Only six haplotypes had frequency >5%; the commonest (34.2%) was formed by the wild-type allele of each SNP and was taken as the referent in the analysis. Only one haplotype with frequency 14% was significantly associated with increased GG risk (OR = 1.34, CI = 1.04–1.73). This haplotype was the only one containing the variant allele of rs1799945 (H63D), but it also contained the variant alleles of the two tagSNPs found to be associated with GC risk of intestinal type (rs1572982 and r6918586). Therefore, in order to try to disentangle the independent effects of each SNP, we performed a multivariate analysis, where the effect of every SNP was adjusted for each other; this approach was applied to cases of intestinal type. In the multivariate analysis, the log-additive OR for H63D was 1.61 (1.05–2.48), slightly lower than the univariate estimate but still significantly associated with GC risk, whereas the ORs for rs1572982 and rs6918586 became non-significant.

Finally, we explored whether the effect of H63D was modified by iron intake by means of stratified analysis, where strata were defined according to the median of iron intake among controls (12.75 mg/day). The OR (log-additive model) was 1.42 (0.99–2.03) for those with higher iron intake compared with 1.17 (0.81–1.70) for those with lower iron intake. However, these two ORs were not statistically different (P value for interaction 0.83) and one cannot really tell whether the effect of H63D is greater among those with high iron intake than among those with lower iron intake. On the other hand, as iron from meat (mostly heme iron) is more readily absorbed than iron from plant foods, we also assessed the effect of H63D according to the level of heme iron intake (below or above the median of controls of 1.15 mg/day). The ORs (log-additive model) were 1.63 (1.11–2.39) and 1.14 (0.80–1.61) for those with lower and higher heme iron intake, respectively. Again these two ORs were not significantly different (P value for interaction 0.18), and it cannot be stated whether the effect of H63D is modified by heme iron intake.

Discussion

We have observed that the variant G at nucleotide 187 in exon 2 of *HFE* (H63D, rs1799945) is associated with increased GC risk in European populations. This association seems to be restricted to cases located in the non-cardia anatomical subsite (distant stomach) and those of intestinal histological subtype. No association was observed for the cardia cases or those of diffuse subtype. This is in agreement with our previous results on the association of GC risk with meat and heme iron intake: red and processed meat were associated with increased risk in non-cardia but not in cardia GC (1). The risk associated with heme iron intake was also higher in non-cardia compared with cardia cases although no differences were observed for the histological types (3).

To our knowledge, an association between *HFE* mutations and GC risk has not been reported so far. There is strong evidence that

Table IV. Association between selected SNPs of HFE gene and gastric adenocarcinoma (overall, non-cardia cases and intestinal type). ORs and 95% CIs estimated by unconditional logistic regression, adjusted for sex, age, center and date of blood collection

SNP	Genotype (model)	All gastric adenocarcinoma			Non-cardia cases			Intestinal type		
		Cases/controls	OR 95% (CI)	*P* value[a]	Cases/controls	OR 95% (CI)	*P* value[a]	Cases/controls	OR 95% (CI)	*P* value[a]
rs1799945 (H63D)	CC	230/885	1.00	0.090	107/885	1.00		73/885	1.00	
	CG	82/249	1.27 (0.94–1.71)		51/249	1.71 (1.17–2.50)	0.015	34/249	1.79 (1.14–2.83)	0.007
	GG	11/23	1.97 (0.92–4.22)		5/23	2.01 (0.71–5.69)		6/23	3.45 (1.29–9.27)	
	CG/GG versus CC	Dominant	1.33 (1.00–1.76)	0.056	Dominant	1.73 (1.20–2.51)	0.004	Dominant	1.93 (1.25–2.98)	0.004
	GG versus CC/CG	Recessive	1.85 (0.87–3.94)	0.12	Recessive	1.71 (0.61–4.81)	0.33	Recessive	2.90 (1.10–7.70)	0.048
rs1572982	GG	96/393	1.00	0.26	48/393	1.00		25/393	1.00	
	GA	188/608	1.26 (0.95–1.68)		92/608	1.21 (0.82–1.77)	0.62	66/608	1.74 (1.06–2.85)	0.025
	AA	81/281	1.18 (0.84–1.65)		41/281	1.14 (0.72–1.80)		35/281	2.01 (1.16–3.49)	
	AA/GA versus GG	Dominant	1.24 (0.95–1.61)	0.11	Dominant	1.19 (0.83–1.70)	0.35	Dominant	1.83 (1.15–2.91)	0.008
	AA versus GG/GA	Recessive	1.02 (0.76–1.35)	0.92	Recessive	1.01 (0.68–1.49)	0.97	Recessive	1.39 (0.91–2.14)	0.14
rs6918586	TT	118/469	1.00	0.33	53/469	1.00		33/469	1.00	
	TC	186/608	1.21 (0.93–1.58)		100/608	1.43 (0.99–2.06)	0.15	67/608	1.61 (1.03–2.52)	0.041
	CC	61/203	1.19 (0.83–1.70)		28/203	1.19 (0.72–1.97)		26/203	1.88 (1.07–3.28)	
	CC/TC versus TT	Dominant	1.21 (0.94–1.55)	0.14	Dominant	1.37 (0.97–1.95)	0.073	Dominant	1.68 (1.10–2.57)	0.014
	CC versus TT/TC	Recessive	1.06 (0.77–1.46)	0.72	Recessive	0.96 (0.61–1.50)	0.84	Recessive	1.40 (0.87–2.25)	0.18

[a]The first *P* value for each SNP corresponds to the significance of the codominant model.

Table V. Haplotype analysis of SNPs of HFE gene and risk of gastric adenocarcinoma

Haplotype[a]	Frequency[b] (%)	OR[c]	(95% CI)	*P* value[c]
CGGCGGTG	34.7	1.00	(Reference)	—
CGGTGGTA	14.5	1.00	(0.77–1.30)	0.99
GGA**C**GG**C**G	14.3	1.34	(1.04–1.73)	0.024
CGA**C**GA**C**G	12.3	1.01	(0.76–1.33)	0.96
CGA**C**CG**C**G	10.5	1.05	(0.79–1.40)	0.74
CGA**C**GGTG	6.2	1.05	(0.74–1.49)	0.77

[a]Each haplotype is formed by the alleles corresponding to each of eight SNPs, including H63D (rs1799945) and seven tagSNPs of a linkage disequilibrium block in the *HFE* genomic region, ordered according to the localization in the gene: rs1799945, rs1800562, rs1572982, rs707889, rs1045537, rs17596719, rs6918586 and rs1543680. The first haplotype corresponds to the wild-type allele for each SNP. Marked with bold type, the alleles corresponding to the three SNPs are significantly associated with the univariate analysis: rs1799945, rs1572982 and rs6918586.
[b]Only haplotypes with frequency 5% or above are presented; among haplotypes not shown, no significant associations were observed.
[c]ORs and *P* values compared with the most frequent haplotype estimated by unconditional logistic regression, adjusted for sex, age and center.

C282Y is associated with hepatocellular carcinoma (10,11), but a causal relationship between *HFE* mutations and other tumors is still debated. Moreover, the specific polymorphisms involved in such associations may differ. For instance, among the three studies that reported association of *HFE* mutations with CRC, one study observed such association with both C282Y and H63D (13), another found the association for compound C282Y/H63D heterozygotes (14) and the third observed the increased risk only for homozygous C282Y (12). Regarding other tumor sites, acute lymphoblastic leukemia was found to be associated with H63D (21), whereas epithelial ovarian cancer was associated with C282Y only (18). We found that GC risk is associated with H63D but not with C282Y; because our hypothesis was that increases in body iron status may promote gastric carcinogenesis, the reasons for this finding are unclear. Some *HFE* variants associated with HH have relatively high frequencies in some populations, probably due to selection because of their protective effect from iron deficiency. Some have proposed that, given this positive selection, if C282Y is not frequent in a population its role is assumed by H63D (26). In our study, the frequency of variant alleles for H63D and C282Y was 12.7 and 4.2%, respectively, in agreement with the expected (9). However, C282Y has variable frequency across European countries, with prevalence of 7–10% in Great Britain, 4–8% in Central and Northern Europe and 3% or less in Southern Europe. In our study, leaving out France and Norway given the small number of cases, the allelic frequency of this variant was 2.6% in Southern Europe (Greece, Italy and Spain), 4.1% in Northern Europe (Denmark and Sweden) and 6% in Central Europe (Germany, The Netherlands and the United Kindom).

One possible explanation of our findings is that H63D is associated with chronic subclinical increases in body iron stores, which in turn promotes increased oxidative stress and induces DNA damage (5). Increased body iron status may also promote endogenous nitrosation resulting in the formation of NOC (6). We found an increased risk for H63D among subjects with high iron intake although there was no significant interaction between both factors. Moreover, intake is not a good indicator of body iron stores. Other mechanisms may also contribute to the association between *HFE* gene mutations and GC risk observed in our population. The peptide hormone gastrin, originally identified as a stimulant of gastric acid secretion, has been demonstrated to act as a growth factor in the gastrointestinal mucosa (27). Therefore, elevated plasma gastrin concentrations can be considered an indicator of GC risk, particularly in the presence of *Helicobacter pylori* although there is a complex interplay between *H.pylori*-induced gastritis, gastrin levels and GC risk. *H.pylori* infection in the antral portion of the stomach usually induces chronic gastritis

without atrophy, with pronounced hyperchlorhydria but normal or slight increase in gastrin levels, often leading to peptic ulcer but no increased cancer risk, whereas the corpus-dependent atrophic gastritis is associated with low acid secretion and hypergastrinemia, and may result in increased GC risk (28,29). Interestingly, it has been demonstrated that circulating concentrations of both amidated and non-amidated forms of gastrin were significantly greater in patients with hemochromatosis compared with a group of normal controls with a similar mean age and sex ratio (30). The potential effect of H63D mediated by gastrin is also consistent with the finding of a significant association of H63D among non-cardia GC; moreover, this mechanism could be shared with *H.pylori*. However, in recent analysis we have shown that eventually all non-cardia GC cases have been previously infected, suggesting that *H.pylori* infection is a necessary cause of sporadic non-cardia GC (31). Therefore, it would be very difficult to examine whether there is an interaction of H63D with *H.pylori* infection on cancer risk.

One limitation of our study is that we have not considered polymorphisms in other genes involved in iron metabolism and homeostasis (8,32). However, these polymorphisms are less common than H63D or C282Y and their clinical significance is uncertain. On the other hand, although H63D has a functional effect on increasing iron stores, it is not clear to what extent it is directly involved in GC risk, or whether it is a marker of other variant. For instance, C282Y had been found to be associated with childhood acute lymphoblastic leukemia. Because *HFE* is located within the extended HLA complex, several variants have been analyzed from the histone gene *HIST1H1C* to *HIST1H1T*, and an intergenic SNP (rs807212) was identified as tagging most common haplotypes of this region. This SNP has been shown to be strongly associated with lymphoblastic leukemia, and accounted for the original C282Y association, which became weaker and no significant after adjustment for rs807212 (33). Finally, it should be considered that the reported association may be observed by chance owing to the multiple comparisons performed in this analysis. To take this into account, we carried out a permutation test for the log-additive model, adjusted for the matching variables. The estimated minimum adjusted P values from the permutation test were 0.27 for the whole data set, but remained significant for the GC of intestinal type ($P = 0.02$) or marginally significant ($P = 0.05$) for the non-cardia cases.

In conclusion, in our prospective study, the mutation H63D in *HFE* gene was found to be associated with increased risk of GC in European populations. This finding is consistent with previous results in the same population showing a relationship between GC and meat and iron intake. The association seems to be restricted to tumors located in the distal region of the stomach (non-cardia cases) and tumors of intestinal type. This effect could be due to a potential role of chronic iron overload associated with H63D, but other mechanisms could also be involved. These results should be replicated in order to confirm a role of *HFE* mutations in GC risk, and extensive analysis of determinants of body iron homeostasis is needed to gain insight on the potential role of iron in gastric carcinogenesis.

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