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A *CBS* haplotype and a polymorphism at the *MSR* gene are associated with cardiovascular disease in a Spanish case–control study

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Abstract

Objectives: The aim of this study was to evaluate the association of polymorphisms present in genes related to homocysteine (Hcy) metabolism with coronary artery disease (CAD).

Design and methods: We examined 8 polymorphisms in the cystathionine β-synthase (*CBS*), glutamate carboxypeptidase II (*GCPII*), methionine synthase (*MS*), methionine synthase reductase (*MSR*) and methylenetetrahydrofolate reductase (*MTHFR*) genes in 140 CAD patients and 113 controls, by means of Chi-square, logistic regression, ANOVA and the Mann–Whitney *U* test.

Results: The c.66 G allele of *MSR* conferred an odds-ratio for CAD of 1.76 (95% CI 1.12–2.77), while a *CBS* haplotype [c.699C–c.844wt–c.1080C] was found over-represented in CAD [OR of 2.16 (1.29–3.63)].

Conclusions: Our results not only highlight the involvement of the *MSR* and *CBS* genes in the etiology of cardiovascular disease, but also emphasize the strength of haplotype analyses in association studies.

Keywords: CBS; SNP; Haplotype; CAD; Assocaition

Introduction

Numerous publications over the last 30 years have shown that a high level of plasma total homocysteine (tHcy) is an independent risk factor for cardiovascular diseases [1–5]. A meta-analysis published by Boushey et al. [6] revealed that hyperhomocysteinemia is responsible for 10% of coronary artery disease (CAD) cases in the general population.

The level of tHcy is affected by many non-genetic factors such as folic acid, vitamins B_2, B_6, and B_{12}, age, sex, physical activity and smoking [7], as well as by still unidentified genetic factors. Even though the measurement of tHcy plasma levels could help predict the risk of cardiovascular disease, both in the general population and in ischemic patients, the complexity of the laboratory methods currently available and the high intra- and inter-assay variability makes it difficult to routinely include this analysis in general screenings for cardiovascular disease. Alternatively, the identification of polymorphisms involved in the onset of these diseases could be useful for this purpose. Severe deficiencies in genes involved in both re-methylation and transsulphuration of homocysteine are known to cause homocystinuria (MIM #236200), an autosomal recessive condition characterized by homocysteine plasma levels between 10 and 30 times the upper reference value and often associated, among other clinical symptoms, with premature cardiovascular disease. While several studies have analyzed the relationship between polymorphisms present in these genes and the risk of CAD or hyperhomocysteinemia, their results have proven inconsistent, possibly due to differences in ethnicity and sample stratification, among other reasons.

In this study, we investigated the possible effects of several genetic factors on the risk of cardiovascular disease in a Spanish

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population. We had previously studied the effect of the *MTHFR* c.677C>T polymorphism in a Spanish cohort [8]. Here, we analyzed eight polymorphisms of five genes related to homocysteine metabolism, as well as the haplotypes present in two of these genes, *CBS* and *MTHFR*, both alone and in combination with other classic cardiovascular risk factors such as hypertension, diabetes or smoking.

Materials and methods

Subjects

A previously described cohort of 76 male patients and 95 controls [8] was enlarged, following the same inclusion criteria, to 140 patients and 113 healthy controls of the same sex, age and geographic origin. Disease was defined as having had at least one episode of myocardial infarction, angina pectoris, or coronary artery bypass graft surgery before the age of 55. Patients were recruited at Hospital de Bellvitge (Barcelona) during 2001–2004. Control individuals were selected from the staff of Hospital Sant Joan de Déu (Barcelona) and from routine health check-ups at outpatient facilities of the Barcelona area. Exclusion criteria for both patients and controls were: overt chronic disease including kidney and liver dysfunction, alcohol abuse, medication with anticonvulsants, and acute illness or surgery in the previous 3 months. Individuals with at least one of two surnames suspected to be foreign were also excluded.

This study was approved by the Ethics Committee of Hospital de Bellvitge and informed written consent was obtained from all the participants.

Data collection

Data on the participating individuals were collected during the course of medical examinations (anthropometric data such as weight, height, and blood pressure) and via standardized questionnaires (medical history, pharmacologic treatments, vitamin supplements taken, or lifestyle). The "classical" risk factors used in this study were defined according to Meco et al. [9]. The Body Mass Index (BMI) was calculated as: weight (kg)/height^2 (m^2).

Biochemical analyses

Venous blood samples were collected after a 12-hour fasting period, aliquoted and frozen at −40 °C. Biochemical analyses were performed in the laboratory of Hospital Sant Joan de Déu (Barcelona). Plasma total homocysteine was measured by HPLC with fluorescent detection of the 7-fluorobenzo-2oxa-1,3-diazole-4-sulfonate (SBDF) derivatives [10]. Serum folate and vitamin B_{12} were determined by a competitive protein binding chemiluminescent assay (IMMULITE; Diagnostic Products Corporation). Other biochemical parameters were measured using standard methods (Olympus AU510, Merck-Igoda S.A., Merck Darmstadt, Germany) as described previously [8].

Genetic analysis

Genomic DNA was obtained from peripheral blood white cells using the *Wizard Genomic DNA purification Kit* (Promega, Madison, WI). The 8 polymorphisms analyzed are listed in Table 1, together with the corresponding restriction enzymes and references. The *CBS* polymorphism c.844ins68 described by Sebastio et al. [11] was analyzed using the primers described in Urreizti et al. [12]. The rest of the polymorphisms were analyzed using the primers described in the references listed in Table 1.

We used PHASE (v.2.0.2) [13,14] to reconstruct the haplotypes of each individual.

Statistical analyses

The analysis of the minimal statistical power was performed post hoc using the Genetic Power Calculator software [15]. For a sample size of 140 patients and 113 controls, assuming a lifetime risk of 2 and MAF of 0.12–0.47, the statistical power ranges from 45% to 79%. For the same assumptions but an MAF of 0.22 and a risk of 2.16, the statistical power is 81.2%.

Categorical risk factors, both genetic (genotypes, haplotypes and alleles) and non-genetic (hypertension, obesity, diabetes and smoking) were analyzed using the χ^2 test. Continuous variables were checked for normality using the Kolmogorov–Smirnov test. Those following a normal distribution (age, BMI, tHcy, folate and vitamins B_6 and B_{12}) were analyzed using variance analysis models (ANOVA). Alternatively, the variance of factors that did not fit a normal distribution (creatinine and glucose) was analyzed using the Mann–Whitney *U* test. Relative risks (RR) and their 95% confidence intervals (CI) were obtained using logistic regression. The significance level was initially established at $p<0.05$. For the multiple comparison correction, we considered all tests performed and assumed false discovery rates (FDR, [16]) of 5% using the *Q*-value R library, which corresponds to a significance threshold of $p<0.003$. Statistical analyses were performed using the statistical software package SPSS (v. 11.5.1, Chicago, IL) for Windows.

Results

The biochemical and anthropometric parameters studied in the patients and controls are listed in Table 2. No significant

Table 1
Polymorphisms analyzed

Gene	rs number	cDNA	Protein	Restriction site	Reference
CBS	rs234706	699C>T	Y233Y	*Rsa*I	[33]
CBS	–	844ins68			[11]
CBS	rs17850743	1080C>T	A360A	*Sfi*I	[33]
GCPII	–	1561C>T	H475Y	*Acc*I	[34]
MTHFR	rs1801133	677C>T	A222V	*Hinf*I	[35]
MTHFR	–	1298A>C	E429A	*Mbo*II	[36]
MSR	rs1801394	66A>G	I22M	*Nde*I	[22]
MS	rs1805087	2756A>G	D919G	*Hae*III	[37]

Table 2
Anthropometric and biochemical parameters of the cohort

Factor	*N*	Patients	Controls	*p*	OR (95% CI)[a]
	(253)	(140)	(113)		
Hypertension (%)	226	36.9	15.9	0.001[b]	2.41 (1.04–5.15)
Obese (%)	236	30.4	14.3	0.004[b]	2.10 (0.94–4.69)
Smoker (%)	237	86.2	58.6	<0.001[b]	4.37 (2.05–9.31)
Diabetic (%)	242	16.3	5.6	0.01[b]	6.75 (1.34–33.97)
Age (years) (*M*±SD)	249	48.8 (7.09)	49.58 (6.30)	0.363[c]	–
BMI (*M*±DS)	236	28.11 (3.43)	26.71 (3.06)	0.001[d]	1.09 (0.97–1.21)
tHcy (μmol/L) (*M*±SD)	133	12.65 (4.35)	11.55 (2.30)	0.072[c]	–
Folate (nmol/L) (*M*±SD)	237	14.90 (5.76)	16.19 (5.81)	0.089[c]	–
Vitamin B_{12} (pmol/L) (*M*±SD)	131	306.60 (74.68)	316.70 (90.90)	0.487[c]	–
Vitamin B_6 (nmol/L (*M*±SD)	133	12.28 (4.94)	11.55 (4.69)	0.387[c]	–
Creatinine (μmol/L) (*M*±SD)	247	96.18 (15.47)	86.12 (14.36)	<0.001[d]	1.05 (1.03–1.07)
Glucose (mmol/L) (*M*±SD)	248	6.23 (2.04)	5.48 (0.79)	<0.001[d]	1.33 (0.99–1.76)

Obesity: BMI >30; smoker: includes present and former smokers; diabetes includes glucose intolerance.
[a] Corrected for hypertension, BMI, smoking, glucose and creatinine.
[b] χ^2 test.
[c] ANOVA.
[d] Mann–Whitney *U* test.

differences were observed between the two groups for age, tHcy, folate, or for B_6 and B_{12} vitamin levels. On the other hand, the patient group exhibited significantly higher levels of creatinine and glucose ($p<0.001$) and included a significantly higher proportion of hypertensive or smoker subjects ($p<0.001$) as well as obese or diabetic individuals ($p<0.01$).

All 140 patients and 113 controls were genotyped for three different polymorphisms of the *CBS* gene (c.699C>T, c.844ins68 and c.1080C>T), 2 of *MTHFR* (c.677C>T and c.1298A>C), as well as polymorphisms c.1561C>T, c.66A>G and c.2756A>G of *GCPII*, *MSR* and *MS*, respectively. All 8 polymorphisms were in Hardy–Weinberg equilibrium.

The frequency distribution of genotypes for all of these polymorphisms in patients and controls was analyzed by means of a χ^2 test, and no significant association was found between any given genotype and disease (data not shown). Only genotype c.66GG of *MSR* appeared slightly over-represented in patients ($p=0.068$). Allelic distributions were then compared in the two groups using the same method. As seen in Table 3, the c.66G allele was over-represented in patients ($p=0.026$). Once adjusted for smoking, hypertension, BMI, glucose and creatinine, the odds-ratio of this allele versus the c.66A allele was 1.76 (1.12–2.77). Additionally, the c.1080C allele of *CBS* was slightly over-represented in patients, whereas the c.844ins68 allele of this gene appeared under-represented. However, neither of these deviations was statistically significant ($p=0.083$ and 0.09, respectively).

Finally, haplotypes of the *CBS* gene were reconstructed from the genotypes at the three intragenic polymorphisms and those of *MTHFR* from its two intragenic SNPs. We subsequently tested their association with the disease by means of a χ^2 test. The overall *CBS* haplotype distribution appeared to be significantly associated with the disease ($p=0.0025$; Table 4) and, in particular, the [c.699C–c.844wt–c.1080C] (C–wt–C) haplotype was over-represented in the patient group. At the haplogenotype level, the results were similar: a dominance model comparing the two groups "presence of at least one copy of C–wt–C" and "bearing no copy of it" showed that the first group was over-represented in patients ($p=0.001$). Once adjusted for smoking, hypertension, BMI, glucose and creatinine, the relative risk of haplotype C–wt–C versus the other haplotypes was 2.16 (1.29–3.63). No association was found between the *MTHFR* haplotypes and cardiovascular diseases.

Table 3
Allelic frequencies of the 8 polymorphisms in patients and controls

	N	Patients		Controls		*p*
		+[a] (freq)	−[b] (freq)	+[a] (freq)	−[b] (freq)	
699C>T *CBS*	504	201 (0.72)	77 (0.28)	149 (0.66)	77 (0.34)	0.122
844Ins68 *CBS*	506	259 (0.93)	21 (0.08)	199 (0.88)	27 (0.12)	0.090
1080C>T *CBS*	504	195 (0.70)	83 (0.30)	142 (0.63)	84 (0.37)	0.083
1561C>T *GCP II*	504	265 (0.95)	15 (0.05)	210 (0.94)	14 (0.06)	0.669
677C>T *MTHFR*	506	161 (0.58)	119 (0.43)	143 (0.63)	83 (0.37)	0.187
1298A>C *MTHFR*	504	201 (0.72)	79 (0.28)	150 (0.67)	74 (0.33)	0.242
66A>G *MSR*	498	120 (0.43)	158 (0.57)	117 (0.53)	103 (0.47)	0.026
2756A>G *MS*	506	237 (0.85)	43 (0.15)	193 (0.85)	33 (0.15)	0.813

[a] + corresponds to the major allele in this control population.
[b] − corresponds to the minor allele.

Table 4
Association between *CBS* haplotypes and CAD

CBS haplotype[a]	Patients (freq)	Controls (freq)	RR (95% CI)
C–wt–C	101 (0.36)	50 (0.22)	2.16 (1.29–3.63)
C–wt–T	81 (0.30)	74 (0.33)	0.89 (0.55–1.46)
C–ins–C	21 (0.08)	25 (0.11)	0.62 (0.29–1.35)
T–wt–C	75 (0.27)	67 (0.30)	0.77 (0.45–1.25)
T–wt–T	2 (0.01)	8 (0.04)	0.18 (0.03–1.12)

Overall χ^2 $p=0.0025$.
[a] c.699C or T-c.844 wt or ins68–c.1080C or T.

Discussion

In this case–control study of Spanish cardiovascular disease patients, a significant association was detected between the *MSR* gene polymorphism c.66A>G and the disease. At the *CBS* locus, none of the three polymorphisms tested was individually associated. However, one particular haplotype, C–wt–C, was.

The *MSR* gene c.66A>G polymorphism was described in 1999 by Wilson et al. [17]. This nucleotide change determines the substitution of an isoleucine at position 22 of the enzyme by a methionine. The effect of this modification is a diminished affinity of the MSR enzyme for MS [18,19]. Several studies have described associations of the 22M variant of MSR and diseases such as Down syndrome [20,21] or neural tube defects [22]. However, its relation to cardiovascular disease was less extensively studied. In the present report, the *MSR* c.66A>G was the only one of eight polymorphisms to be significantly associated with the disease, with a relative risk of 1.76 (1.12–2.77) for the G allele, compared with the A allele. Regarding the *CBS* gene, we analyzed two silent changes (c.699C>T and c.1080C>T), together with the c.844ins68 variant. None of them seemed to be a clear risk factor on its own. However, when analyzed together, they appeared to be associated with the disease so that the C–wt–C haplotype was over-represented in patients. The presence of this haplotype confers a 2.16 (1.29–3.63) fold increase in the risk of suffering a CAD, after adjusting for smoking, hypertension, BMI, glucose and creatinine.

The c.844ins68 variant was originally described as a disease-causing mutation [11], but was later shown to be a polymorphism [23]. Its frequency in the general population varies widely, ranging from 0 in Eastern Asia to 0.4 in some Sub-Saharan areas [24]. In the present study, its frequency in Spain was found to be 0.12, in agreement with previous reports [24]. This insertion has been extensively studied in relation both to cardiovascular disease and to plasma homocysteine levels, mainly with negative results [3,25]. Only a few studies reported positive results with the disease [26], or the homocysteine values [27,28]. Regarding the c.699C>T and the c.1080C>T polymorphisms, Kruger et al. [29] found their haplotypic combination to be significantly associated both with CAD and with the response to folate treatment. Another study by Aras et al. [30] found an association between these polymorphisms and the post-methionine-loading (PML) homocysteine. However, these results were not reproduced in the study of Lievers et al. [31]. This disparity may be attributed to the different characteristics or size of the studied populations, to environmental factors, to sample stratification, or a dissimilar definition of the pathology.

In the present study, the three *CBS* polymorphisms were analyzed together for the first time. The particular haplotype that we found associated with the disease (C–wt–C) proved different from that identified by Kruger et al. (C–T; the ins68 was not analyzed). Since none of these polymorphisms is supposed to be functional, we hypothesize that the effect results from another polymorphism in linkage disequilibrium with those studied; e.g., a variant affecting a regulatory motif of the promoter. Under this hypothesis, we envisage this variant being carried by different haplotypes in various populations.

In our previous study [8], we reported the high heritability of tHcy, only partially explained by the c.677 T allele of *MTHFR*. In the present study, we could not identify any additional genetic factors to account for the variability of this biochemical parameter, among the other polymorphisms analyzed (data not shown). This may be due both to lack of sufficient power of the study and to the existence of additional genetic variants. In this respect, Souto et al. [32] performed a genome-wide linkage analysis to search for loci involved in tHcy determination and found a peak at 11p23, where the gene for nicotinamide *N*-methyltransferase is located.

An obvious limitation of this study is the reduced sample size and, as a consequence, a limited power to detect small effects. In the case of the *MSR* c.66 G allele, the relative risk of 1.76 is rather small and the p value of 0.026 would be above a threshold for significance corrected for multiple testing. Thus, we take these data with caution and only as hypothesis-raising results. On the other hand, we found that the *CBS* (C–wt–C) haplotype conferred a relative risk of 2.16 ($p=0.0025$), large enough to be detected in a sample like ours and significant enough to stand the FDR significance threshold. Additionally, the significance value of the corresponding haplogenotype analysis ($p=0.001$) stands both the FDR threshold and a restrictive Bonferroni correction (taking into account the total number of 37 tests performed).

In conclusion, our results suggest the involvement of the *MSR* and *CBS* genes in the etiology of cardiovascular disease and emphasize the strength of haplotype analyses in association studies.

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