The haptoglobin 2-2 genotype is associated with inflammation and carotid artery intima-media thickness

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Abstract

The haptoglobin 2-2 genotype is associated with atherosclerosis in type 2 diabetes mellitus. We examined the associations of the haptoglobin 2-2 genotype with C-reactive protein (high-sensitivity C-reactive protein) and carotid artery intima-media thickness, adjusting for age, gender, ethnicity, type 2 diabetes mellitus, smoking status, body mass index, blood pressure, glycated haemoglobin, non-high-density lipoprotein cholesterol and medications via logistic multivariate regression in 200 subjects (160 type 2 diabetes mellitus versus 40 healthy individuals). The prevalence of the haptoglobin 2-2 genotype was 58% (115/200), higher in the Indians than in Chinese (72% versus 45%, $p = 0.001$). Multivariate analysis showed that the haptoglobin 2-2 genotype was associated with high-sensitivity C-reactive protein [mean: 3.5 ± 3.9 versus 2.2 ± 2.6 mg/L (non-haptoglobin 2-2), $p < 0.001$], haptoglobin concentration [mean: $116.9 \pm 54.4.0$ versus 147.2 ± 54.5 mg/dL (non-haptoglobin 2-2), $p < 0.001$] and average carotid artery intima-media thickness (multiplied by 10) [6.15 ± 1.22 versus 5.98 ± 1.20 mm (non-haptoglobin 2-2), $p = 0.013$]. This pilot study shows an association of the haptoglobin 2-2 genotype with low-grade inflammation, haptoglobin concentration and carotid artery intima-media thickness in multi-ethnic Singapore.

Keywords

Haptoglobin polymorphism, carotid atherosclerosis, C-reactive protein

Background

Haptoglobin (Hp) is an acute-phase antioxidant that binds circulating free haemoglobin (Hb).[1] In humans, Hp is characterised by a genetic-polymorphism resulting in three genotypes (Hp 1-1, Hp 2-1 and Hp 2-2) which results from expression of two different alleles (Hp1 and Hp2) of the Hp gene.[1] The protein product of the Hp2 allele is an inferior antioxidant compared to that of Hp1 allele.[1]

The Hp 2-2 genotype is associated with higher cardiovascular risk attributed to decreased affinity to Hb, diminished clearance of Hp 2-2–Hb complexes, impaired anti-inflammatory pathways, low-density lipoprotein (LDL) oxidation and dysfunctional reverse cholesterol transport of high-density lipoprotein (HDL).[1] In patients with type 2 diabetes mellitus (T2DM), there is further down-regulation of CD163 in Hp 2-2 individuals, and the Hp 2-2 protein is less efficient at blocking haem transfer from glycosylated Hb in T2DM.[2] Hyperglycaemia and Hp status thus synergise and exacerbate cardiovascular disease (CVD) risk further in T2DM.

Carotid artery intima-media thickness (CIMT) is a robust surrogate marker for atherosclerosis with good correlation seen with prevalent CVD.[3] High-sensitivity C-reactive protein (hsCRP), an inflammatory marker, is a strong univariate predictor of cardiovascular events with higher concentrations in Indians compared to other ethnicities in Singapore.[4] Longitudinal studies have found that Hp 2-2 genotype is associated with a twofold to

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fivefold increased risk of incident CVD in T2DM.[5–8] However, these have not been reported in Asian populations. We compared the associations of Hp 2-2 genotype with hsCRP, Hp concentrations and CIMT in the multi-ethnic population of Singapore.

Methods

In this cross-sectional pilot study, we recruited 200 individuals (160 T2DM vs 40 healthy individuals) at a tertiary hospital. The exclusion criteria included acute illness 2 weeks prior, serum creatinine >200 μmol/L, cardiovascular events (i.e. myocardial infarction, coronary revascularisation procedure), ischaemic cerebrovascular disease (i.e. ischaemic stroke, carotid artery disease) or peripheral arterial disease (i.e. amputation due to vascular disease or intermittent claudication). This study was approved by the institutional review board [Domain Specific Review Board (DSRB) ref nos: 2013/01235 and 2014/00236] and conducted according to the ethical principles embodied in the Declaration of Helsinki.

Demographic data and clinical parameters were collected, and standardised questionnaires were administered. The complete list of medications was reviewed from the online prescription database and also confirmed with patients. Blood pressure (BP) was measured from left arm in the supine position and the average of two readings were taken. Hp concentrations and hsCRP were measured by turbidimetry. Glycated haemoglobin (HbA1c) was measured by immunoturbidimetric assay using the Beckman Coulter Synchron LX®20 (Beckman Coulter Inc., Brea, CA, USA) clinical chemistry analyser. Lipids were measured after a 10-h overnight fast using standard coupled enzymatic methods. LDL cholesterol was calculated by the Friedewald equation.

Genomic DNA was extracted from peripheral blood using QIAamp DNA kit (Qiagen, Hilden, Germany). Hp genotyping was performed using TaqMan-based real-time polymerase chain reaction (PCR) according to Soejima and Koda.[9] Three sets of primers and probes (Applied Biosystems, Foster City, USA) were used to target the HPdel breakpoint, the breakpoint of the duplication region specific for HP2, and 5′ region of exon 1 as an internal control (HP5′). Analysis of all ambiguous samples was repeated and 5% of the samples were amplified twice for precision checks. The amplification plots were used to detect HPdel and homozygous HP1. Subjects who were homozygous for HP2 and those harbouring HP1 and HP2 were discriminated by ΔΔCt method. The HP2/HP5′ ratio of each sample ($2^{-\Delta\Delta Ct}$) was calculated by comparing HP2-specific sequence relative to HP5′ and a previously genotyped sample as reference. The TaqMan assay was validated with a PCR-based method according to Koch et al.[10] Two separate primer pairs were used to generate specific amplicons indicative of alleles Hp1 (1757 bp) and

Hp2 (349 and 3481 bp). Genotypes were determined by distinct sizes of HP2/HP1 and HP2/HP2 PCR products.

Carotid ultrasonography was performed using a 5.0- to 13.0-MHz multi-frequency high-resolution linear transducer probe (GE Logiq P5) by two operators trained before study initiation. Pilot examination on 23 volunteers showed acceptable limits of inter- and intra-user agreement with a coefficient of variance of ±0.1 by Bland–Altman analysis. Auto-IMT software was used for CIMT measurements in order to optimise reproducibility. These measurements were made following the recommendations of the Mannheim CIMT consensus.[11] The operators were blinded to the disease state and the Hp genotypes.

Statistical methods

We checked for Hardy–Weinberg equilibrium (HWE) in the control and the diabetes population using chi-square test. Univariate analysis was performed to examine Hp genotypes against demographics, clinical variables, medications, metabolic variables, Hp concentrations, hsCRP and CIMT. The associations between Hp genotypes and Hp, hsCRP and average CIMT were subsequently analysed using logistic multivariate regression model after adjusting for other variables. All analysis was done using STATA version 13.

Results

The allele frequencies were as follows: Hp2: 150/174 (86%) in Indians, 16/20 (80%) in Malays versus 133/206 (65%) in Chinese; Hp1: 23/174 (13%) in Indians, 4/20 (20%) in Malays and 69/206 (34%) in Chinese; and Hp0: 1/174 (0.6%) in Indians and 4/206 (1.9%) in Chinese. The prevalence of Hp 2-2 genotype was 115/200 (58%) in this sample with the prevalence in Indians 63/87 (72%), Malays 6/10 (60%) and Chinese 46/103 (45%). The prevalence of Hp 2-1 genotype was 66/200 (33%), with prevalence in Chinese 39/103 (45%), Malays 4/10 (40%) compared to Indians 23/87 (27%). The Hp 1-1 genotype was only seen in the Chinese 14/103 (14%) in our sample population. Two Chinese subjects had the 1-del genotype, two Chinese and one Indian had the 2-del genotype. The healthy group was in HWE ($p = 0.06$), while the T2DM group was not in HWE ($p = 0.02$).

In the univariate analysis, we found that the Hp 2-2 genotype was more prevalent in non-Chinese and was associated with higher hsCRP and lower Hp levels when compared to non-Hp 2-2 genotype ($p < 0.05$). We also found that the CIMT significantly correlated to age, systolic BP, lipid profile, glucose and HbA1c ($p < 0.05$), while hsCRP significantly correlated to body mass index (BMI), blood pressure, HbA1c and glucose ($p < 0.05$) and Hp concentrations correlated to BMI, HbA1c, and glucose ($p < 0.05$) (data not shown). Univariate regression analysis

Table 1. Univariate and multivariate logistic regression analysis looking at associations between the Hp genotypes and CIMT, hsCRP and Hp concentrations.

Variables	Hp genotype		Univariate	Multivariate			
	Non-Hp 2-2 (n = 85)	Hp 2-2 (n = 115)	p value	p value	OR	95% CI	
Age, years; mean (SD)	50.82 (13.22)	48.77 (12.04)	0.253	0.002	0.92	0.88	0.97
Gender, n							
Female	45 (52.94)	67 (58.26)	0.454	–	–	–	–
Male	40 (47.06)	48 (41.74)		0.165	0.54	0.23	1.29
Ethnicity, n							
Chinese	57 (67.06)	46 (40.00)	<0.001	–	–	–	–
Non-Chinese	28 (32.94)	69 (60.00)		<0.001	4.47	2.04	9.77
Indian	24 (28.24)	63 (54.78)					
Malay	4 (4.71)	6 (5.22)					
Smoker, n							
Non-smokers	73 (85.88)	101 (87.83)	0.686	–	–	–	–
Ever smoked	12 (14.12)	14 (12.17)		0.620	1.37	0.39	4.79
T2DM, n							
No	18 (21.18)	22 (19.13)	0.721	–	–	–	–
Yes	67 (78.82)	93 (80.87)		0.395	2.31	0.34	15.95
BMI (kg/m²), mean (SD)	26.52 (6.02)	27.38 (5.60)	0.295	0.420	0.97	0.89	1.05
Systolic BP (mmHg), mean (SD)	131.29 (21.02)	132.11 (21.43)	0.786	0.965	1.00	0.97	1.03
Diastolic BP (mmHg), mean (SD)	73.74 (10.88)	74.47 (10.24)	0.623	0.653	0.99	0.94	1.04
Non-HDL (mmol/L), mean (SD)	3.34 (1.01)	3.28 (0.81)	0.688	0.075	0.67	0.43	1.04
HbA1c (%), mean (SD)	7.93 (2.02)	7.96 (2.01)	0.917	0.416	1.14	0.83	1.58
Fasting serum glucose (mmol/L), mean (SD)	8.20 (3.77)	8.54 (3.91)	0.547	0.475	0.95	0.82	1.09
Average CIMT (multiplied by 10) (mm), mean (SD)	5.98 (1.20)	6.15 (1.22)	0.308	0.013	1.73	1.12	2.66
hsCRP (mg/L), mean (SD)	2.15 (2.56)	3.53 (3.89)	0.005	<0.001	1.62	1.29	2.03
Haptoglobin (mg/dL), mean (SD)	147.40 (56.82)	116.90 (54.42)	<0.001	<0.001	0.98	0.97	0.98
Subjects on statins, n (%)	54 (63.53)	70 (60.87)	0.623	0.679	1.26	0.43	3.68
Subjects on aspirin, n (%)	10 (11.76)	13 (11.30)	0.896	0.690	1.31	0.34	5.00
Subjects on ACE/ARB, n (%)	42 (49.41)	53 (46.01)	0.585	0.862	1.08	0.43	2.70
Subjects on insulin, n (%)	38 (44.71)	40 (34.78)	0.136	0.036	0.32	0.11	0.93
Subjects on metformin, n (%)	56 (65.88)	84 (73.04)	0.331	0.644	1.39	0.34	5.60
Subjects on sulphonylureas, n (%)	27 (31.76)	43 (37.39)	0.444	0.733	1.18	0.46	3.06

Hp: haptoglobin; CIMT: carotid artery intima-media thickness; hsCRP: high-sensitivity C-reactive protein; OR: odds ratio; CI: confidence interval; SD: standard deviation; T2DM: type 2 diabetes mellitus; BMI: body mass index; BP: blood pressure; HbA1c: glycated haemoglobin; HDL: high-density lipoprotein; ACE/ARB: angiotensin-converting enzyme/angiotensin receptor blocker.

using two-sample t-test with equal variance showed that hsCRP concentrations were negatively associated with aspirin use; Hp levels were negatively associated with insulin and aspirin use and CIMT was negatively associated with statin, aspirin, angiotensin-converting enzyme/angiotensin receptor blocker (ACE/ARB), insulin, metformin and sulphonylurea use (data not shown). In the logistic regression analysis, after adjusting for age, gender, ethnicity, smoking status, T2DM, medications, BMI, systolic and diastolic BPs, non-HDL cholesterol, HbA1c and glucose, we found that Hp 2-2 genotype significantly correlated with a higher CIMT ($p = 0.013$), lower Hp levels ($p < 0.001$) and higher hsCRP ($p < 0.001$) (Table 1).

A subgroup analysis assessing for the differences in the two ethnic groups, Indians and Chinese, separately showed that while the Hp 2-2 genotype was associated with higher

hsCRP ($p < 0.05$) and lower Hp levels ($p < 0.05$), association with CIMT did not reach statistical significance ($p > 0.05$) in both ethnic groups (data not shown).

A subgroup analysis of patients with T2DM showed association of the Hp 2-2 genotype with higher hsCRP ($p = 0.001$), lower Hp levels ($p < 0.001$) while association with CIMT did not reach statistical significance ($p = 0.10$) (data not shown). Multivariate analysis in the healthy volunteers was limited by small sample size.

Discussion

In our study, we saw similar frequencies of Hp genes in the two ethnic groups as seen previously in a local study done in 1984. In this study of 870 participants, the frequencies of Hp1, Hp2 and Hp0 were 0.330, 0.670 and 0.029 in

Chinese; 0.298, 0.702 and 0.004 in Malays; and 0.167, 0.833 and 0.009 in Indians, respectively. The distribution of all the Hp genotypes was in HWE in all the population groups studied.[12] In our study, the distribution was not in HWE in the T2DM group, although it was in equilibrium in the healthy individuals. The Hp 2-2 genotype has also been associated with an increased risk of T2DM in northern Chinese patients.[13] It could be due to higher prevalence of T2DM in the Hp 2-2 genotype. Other reviews of the genetic distribution has shown that the highest frequency of the Hp1 allele is found in the African and South American populations, and the lowest frequency is seen among the Southeast Asian populations particularly in India.[14]

We found that the Hp 2-2 genotype was associated with higher hsCRP in the univariate and multivariate analysis. In Hp 2-2 patients, impairment in anti-inflammatory macrophage signalling through a down-regulation of CD163/pAkt/interleukin (IL)-10 signalling axis in T2DM has been seen.[3]

We found a significant correlation with CIMT after adjusting for metabolic and demographic variables. Previous studies reported an association of the Hp 2-2 genotype with carotid atherosclerosis in diabetes mellitus (DM) patients.[5,8] In the Diabetes Heart study, Hp 2-2 genotypes were strongly correlated to CIMT ($p=0.001$).[8] The Strong Heart Study reported odds ratio of CVD two to five times greater in Hp 2-2 genotype than Hp 2-1 DM patients ($p=0.002$).[7]

We also found that the Hp 2-2 genotype was associated with lower Hp concentrations. Hp phenotype dependency has been observed in turbidimetric assays, and the Hp 2-2 genotype is known to be associated with lower plasma Hp concentrations compared to Hp 1-1 genotype.[14]

Our study is limited by the small sample size, its cross-sectional design and the use of only the CIMT and hsCRP as surrogate markers of CVD risk. Despite the small sample size, we found significant differences between the Hp 2-2 genotypes and non-Hp 2-2 genotypes.

Hence, there is a need to conduct large-scale longitudinal studies to confirm this association in multi-ethnic groups, vessels other than the carotid artery and randomised controlled intervention trials to elucidate whether antioxidants or anti-inflammatory agents may specifically ameliorate CVD risk among those with the Hp 2-2 genotype.

Declaration of conflicting interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship and/or publication of this article.

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