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ARTICLE

Association of a 31 bp VNTR in the CBS gene with postload homocysteine concentrations in the Framingham Offspring Study

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Elevated total plasma homocysteine concentrations (tHcy), both fasting and post-methionine load, have been established as risk factors for vascular disease. Recently, we described the association of a 31 bp variable number of tandem repeats (VNTR) in the cystathionine β-synthase (CBS) gene with both CBS enzyme activity and tHcy concentrations. In the present study, we determined the 31 bp VNTR genotypes in 2598 individuals of the Framingham Offspring Study and studied the association between this genotype and fasting, 2-h post-methionine load and delta (ie increase upon methionine loading) tHcy concentrations in 1416 subjects. We observed a positive association between the number of repeat units of the CBS 31 bp VNTR and both postload and delta tHcy concentrations. Adjustment for possible effect modifying factors like age, sex and vitamin (B_6, B_{12} and folate) status did not change this observation. We hereby confirm the results of our earlier study, in which we found that this 31 bp VNTR is a genetic determinant of post-methionine load tHcy concentrations. Since also post-methionine load tHcy concentrations are found to be associated with an increased risk for cardiovascular disease (CVD), this 31 bp VNTR may be considered a risk factor for CVD.
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Introduction

In addition to the well-known risk factors for cardiovascular disease (CVD) like an unfavorable lipid profile (ie high LDL, low HDL cholesterol), hypertension, diabetes mellitus and smoking, an elevated total plasma homocysteine concentration (tHcy) has been identified as a risk factor for vascular disease in a number of studies.[1,2] Besides fasting tHcy concentrations, also post-methionine load tHcy concentrations have been reported as a risk factor for CVD.[3–5]

Homocysteine is formed by demethylation of methionine via *S*-adenosylmethionine and *S*-adenosylhomocysteine. Homocysteine can be further metabolized in two directions: it can be remethylated to methionine by methionine synthase (MS or BHMT), or catabolized by the transsulfuration pathway in which cystathionine β-synthase (CBS) irreversibly condensates homocysteine into cystathionine. tHcy levels are influenced by dietary and lifestyle factors, and also by genetic factors can interfere with transsulfuration and remethylation.

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Although CBS deficiency is the most common cause of severe hyperhomocysteinemia,[6] no genetic variants in the CBS gene have been described so far that are associated with hyperhomocysteinemia in vascular disease. However, very recently, we and others described the association of a 31 bp variable number of tandem repeats (VNTR) in the CBS gene with tHcy concentrations.[7,8] In our previous study, we assessed the relationship between the CBS 31 bp VNTR genotype and both fasting and post-methionine load tHcy concentrations in 190 CVD patients and 381 population-based controls, and found a positive association between postload tHcy concentration and increasing number of repeat units of the 31 bp VNTR.[7]

In the present study, we determined the CBS 31 bp VNTR genotype in more than 2500 samples of the Framingham Offspring Study and studied the association between this genotype and fasting, post-methionine load and delta (ie increase upon methionine loading) tHcy concentrations in 1416 subjects. Since vitamin B_6 concentrations in blood were available of this population, we were also able to study possible effect modification by vitamin B_6.

Materials and methods

Subjects were participants in the Framingham Offspring Study; details of the design and methods have been described elsewhere.[9] Starting in 1948, 5209 subjects between the ages of 28 and 62 were enrolled in the cohort study, and beginning of 1971, a total of 5124 of their children and the children's spouses were enrolled.[10] Between 1987 and 1991, blood samples for DNA were collected and DNA was available of 4144 subjects. In the Framingham Offspring Study, fasting and 2-h post-methionine load tHcy concentrations were available of a part of the study group.

Detection of the CBS 31 bp VNTR

Genomic DNA was isolated from peripheral blood leukocytes by standard methods.[11]

CBS 31 bp VNTR genotype analyses were performed by PCR amplification in standard PCR buffer, containing 100 ng forward primer (5′-TGCAGCCGTCAGA-CCAAG-3′) and 100 ng reverse primer (5′-TTAAGTCCCCAAAACACGG-3′), 0.2 mmol/l dNTPs, 1.5 mM $MgCl_2$ and using an annealing temperature of 60°C in the first 10 cycles, followed by an annealing temperature of 56°C in the next 30 cycles. The DNA fragments with sizes ranging from 784 to 908 bp were analyzed on a 2% agarose gel.

We were able to assess the 31 bp VNTR genotype of 2598 individuals and a complete set of genotype data and 2-h post-methionine load tHcy concentrations was available of 1416 individuals.

Statistics

tHcy, folate, vitamin B_6, vitamin B_{12} and creatinine concentrations were logarithmically transformed prior to all statistical analyses. One-way analysis of variance (ANOVA) was used to assess the differences of continuous variables between different genotypes, followed by Bonferroni-corrected *t*-tests. Adjustment for confounding variables (ie age, sex, creatinine and vitamin status) was performed by general linear model analysis. Statistical significance refers to a two-tailed analysis, where $P<0.05$.

Results

The main characteristics of the study population are depicted in Table 1. A complete set of genotype data and post-methionine load tHcy concentrations was available of 1416 individuals. There was an equal amount of men and women in the study population and the mean age was 59 years. Besides fasting and post-methionine load tHcy concentrations, also data about vitamin B_6, B_{12}, folate and creatinine were available. The number of individuals in each genotype group are shown in Table 1. We detected six different alleles ranging from 15 to 21 repeat units. The allele frequencies of alleles 17, 18, 19 and 21 were 10.1, 78.2, 10.5 and 1.2%, respectively, while the frequencies of the alleles 15 and 16 were <0.1%. The genotype distribution of the 31 bp VNTR was in Hardy–Weinberg equilibrium.

Table 1 Characteristics of study population

	n = 2598
Age (years)	59.0 ± 9.8
Gender (% male)	49.4
Fasting tHcy (μmol/l)	9.4 (9.3–9.6)
Post-load tHcy (μmol/l)	23.5 (23.2–23.9)
Vitamin B_6 (pmol/ml)	62.6 (60.5–64.7)
Vitamin B_{12} (pg/ml)	382.8 (375.3–390.5)
Folate (ng/ml)	8.2 (7.9–8.5)
Creatinine (mg/dl)	1.14 (1.13–1.15)
VNTR genotype [*n* (%)]	
15–18	4 (0.2)
16–18	1 (0.04)
16–21	1 (0.04)
17–17	90 (3.5)
17–18	280 (10.8)
17–19	58 (2.2)
17–21	5 (0.2)
18–18	1730 (66.6)
18–19	270 (10.4)
18–21	47 (1.8)
19–19	106 (4.1)
19–21	3 (0.1)
21–21	3 (0.1)

Age is expressed as mean ± SD; homocysteine (tHcy), vitamin B_6, B_{12}, folate and creatinine concentrations are expressed as geometric means (95% CI).

Table 2 Association between CBS 31 bp VNTR genotypes and tHcy, expressed as geometric means (95% CI)

VNTR genotype	*Fasting tHcy (μmol/l)*	*Postload tHcy (μmol/l)*	*tHcy increase (μmol/l)*
15/18	9.7 (5.4–17.4)	16.1 (10.5–24.8)	6.4 (5.3–7.8)
16/18	10.1	—	
16/21	7.0	17.4	10.4
17/17	8.8 (8.2–9.4)	20.7 (19.4–22.2)	11.6 (10.5–12.7)
17/18	9.2 (8.8–9.7)	22.1 (21.1–23.2)	12.7 (11.9–13.5)
17/19	9.0 (7.8–10.3)	19.3 (17.3–21.6)	9.8 (8.6–11.3)
17/21	11.6 (8.0–16.8)	24.5 (23.5–25.6)	12.6 (10.0–15.9)
18/18	9.4 (9.2–9.6)	24.1 (23.6–24.6)	14.1 (13.8–14.5)
18/19	9.4 (9.0–9.9)	22.6 (21.6–23.7)	12.8 (12.0–13.7)
18/21	8.4 (7.4–9.6)	25.8 (23.1–28.8)	17.2 (14.8–19.9)
19/19	9.4 (8.9–10.0)	22.3 (20.9–23.8)	12.3 (11.2–13.6)
19/21	8.3 (3.8–17.8)	18.9 (2.9–120.6)	9.8 (6.7–14.1)
21/21	10.7 (0.4–253)	25.1 (13.9–45.4)	18.0
Total	9.4 (9.3–9.6)	23.5 (23.1–23.9)	13.5 (13.2–13.8)
Total (*n*)	1617	1416	1414
P ANOVA	0.624	<0.001*	<0.001

**P* adjusted for folate, vitamin B_6 and B_{12}, and creatinine <0.001.

Table 2 shows the associations of the 31 bp VNTR genotypes with tHcy concentrations (2-h post-methionine load and increase upon methionine loading). Fasting tHcy concentrations (Table 2) as well as vitamin B_6, folate, vitamin B_{12} and creatinine concentrations (data not shown) did not differ between genotype groups. Post-methionine load tHcy concentrations, however, did show an association with the genotypes of the 31 bp VNTR, as did the increase upon methionine loading (for both associations ANOVA: $P<0.001$; Table 2). Adjustment for effect modifying factors like age, sex and vitamin (B_6, B_{12} and folate) status did not change the association found between the 31 bp VNTR and both postload and increase upon methionine loading tHcy concentrations. The observed association between the genotypes of the 31 bp VNTR and post-methionine load tHcy concentrations was similar in men and women (data not shown). Statistically significant linear trends were found when the homozygous genotypes were considered separately (Figure 1). When the compound heterozygotes, carrying allele 18 in combination with one of all possible alleles, were considered separately, we also detected significant linear trends (Figure 2). These results support our hypothesis that there is an association between the number of repeat elements and elevated postload plasma tHcy levels.

Discussion

In this study, we investigated whether the 31 bp VNTR in the CBS gene is associated with tHcy concentrations in a population of over 1400 subjects of the Framingham Offspring Study. We found a significant positive association between the number of repeat units of this 31 bp VNTR with both 2-h postload and increase upon methionine loading tHcy concentrations, while no association with fasting tHcy concentrations was observed. This significant association with postload as well as increase upon methionine loading tHcy concentrations was not affected by possible effect modifying factors like age, sex and vitamin (B_6, B_{12} and folate) status.

Besides mildly elevated fasting tHcy concentrations, also elevated post-methionine load tHcy concentrations have been shown to be an independent risk factor for CVD.[3,5,12] Although CBS deficiency is the most common cause of severe hyperhomocysteinemia,[6] heterozygosity for CBS deficiency is not a significant determinant of mildly elevated tHcy concentrations in patients with vascular disease.[13,14] However, an involvement of mildly impaired CBS function in hyperhomocysteinemia and vascular disease is not excluded. A 68 bp insertion (844ins68) in the CBS gene was thought to be a possible pathogenic variant, but individuals carrying the insertion showed normal CBS enzyme activity[15] and normal tHcy concentrations,[15,16] although there are also reports on lower post-methionine load increase of tHcy in carriers of the 844ins68 insertion.[17,18] Recently, we described the association of a 31 bp VNTR in the CBS gene with post-methionine load tHcy concentrations.[7] In a Dutch population, of 190 vascular patients and 381 population-based controls, we distinguished five different alleles, that is, consisting of 16, 17, 18, 19 or 21 repeat units of 31 bp and observed higher 6-h post-methionine load tHcy concentrations with increasing number of repeat units. This observation was further substantiated by the observation of decreased CBS activities in fibroblasts of individuals with genotypes that include a high number of repeat elements. We postulated that, by the presence of multiple potential splice donor sites, alternative splicing occurs, which was demonstrated by RT-PCR experiments.

Alternative splicing as a result of the presence of multiple alternate splice sites had been refuted earlier by Yang *et al*,[8]

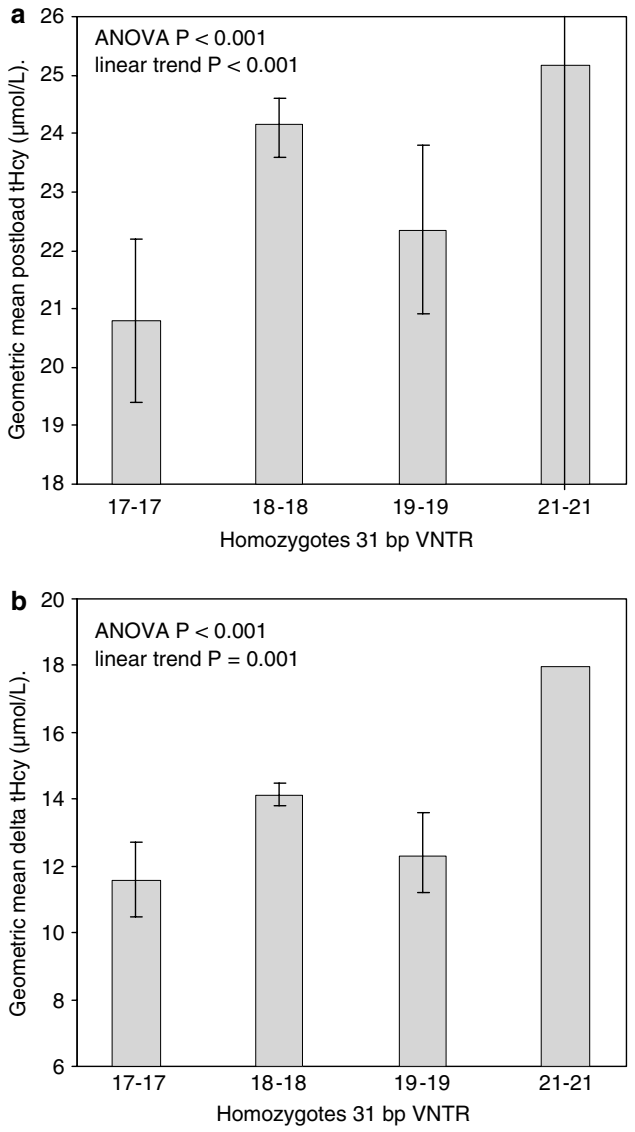

Figure 1 Association between CBS 31 bp VNTR homozygotes and (**a**) 2-h post-methionine load and (**b**) delta (ie increase upon methionine loading) tHcy concentrations (error bars represent 95% CI).

who studied this CBS variant in relation to fasting and post-methionine load tHcy concentrations. They named their alleles according to the number of repeat units observed in each allele, with allele 17 being the most common. However, we observed that this most common allele in fact consist of 18 repeat units, resulting in a different numbering in which our allele 18 is the most common.[7] They reported that the genotypes 17–18 and 18–19 had lower delta (ie increase upon methionine loading) tHcy concentrations that the most common 18–18 genotype, and that this observation is probably caused by linkage disequilibrium with upstream transcriptional regulatory elements. They argued that the occurrence of alternative splicing is prevented by a G to A substitution at the exon–intron border of the second repeat unit. Although this G to A substitution is absent from all subsequent repeat units, they assumed that alternative splicing does not occur at those distal sites due to the lack of exon 13 sequences not contained in the repeat elements but needed for the binding of spliceosomes. Of course, it could be speculated that the alleles of the 31 bp VNTR may be in linkage disequilibrium with other, yet unkown, functional variants, which could explain the observed association with plasma tHcy concentrations. However, we do not consider this option very likely, since is does not explain the linearity of the observed

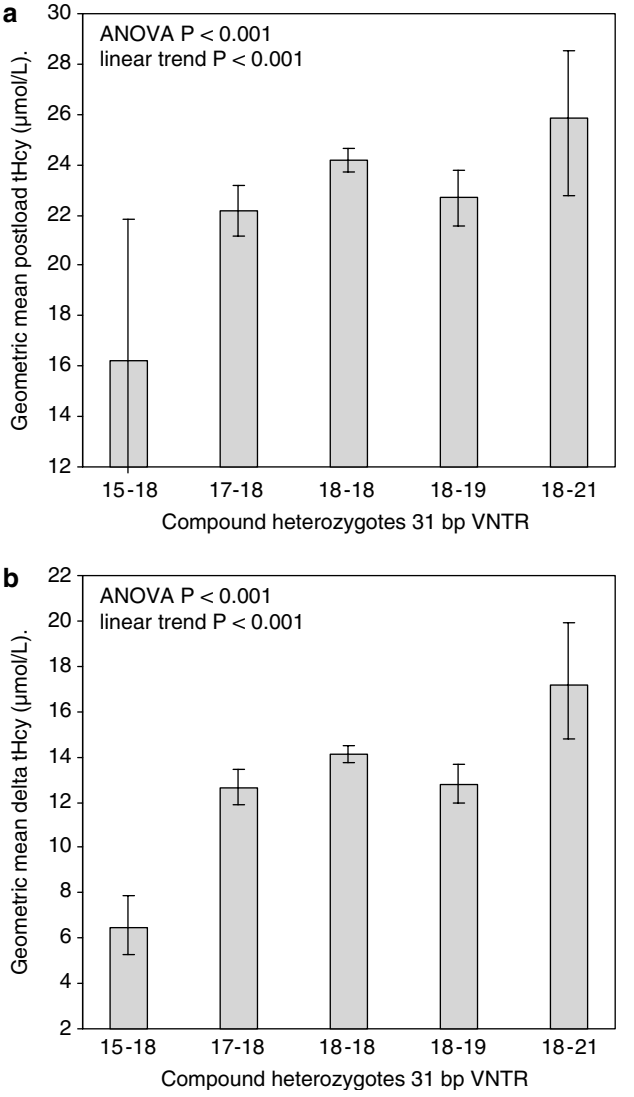

Figure 2 Association between CBS 31 bp VNTR compound heterozygotes and (**a**) 2-h post-methionine load and (**b**) delta tHcy concentrations (error bars represent 95% CI).

observation. Furthermore, we provided evidence that alternative splicing does occur, since the first part of intron 13 is still present in a significant proportion of the CBS mRNA population.[7] Still, we do not know whether the entire intron 13 is retained in some mRNAs, which would lead to a premature stop codon, or that alternative splice donor sites downstream of this repeat are also used, which will cause frame shifts.

In the present study, we observed six different alleles, of which alleles 16 to 21 showed similar allele frequencies compared with those in our previous report of a Dutch population[7] and those of earlier reports.[8,19] Allele 15 was not reported before, probably because its frequency is rather low.

In the present study, 2-h post-methionine load concentrations were available, while in our previous study an association between genotype and 6-h post-methionine load was reported.[7] We were able to confirm the observed positive association between the number of repeat units and 6-h post-methionine load tHcy concentrations in the present study with 2-h post-methionine load concentrations.

In conclusion, the results of this study confirm our earlier results that the 31 bp VNTR is a genetic determinant of post-methionine load tHcy concentrations and show that this association is not influenced by vitamin, in particular B_6, concentrations. Since also post-methionine load tHcy concentrations are found to be associated with an increased risk for CVD,[3,5,12,20] this 31 bp VNTR may be considered a risk factor for CVD. Since it is not known whether intron 13 is partially or completely spliced out further studies are warranted to elucidate the influence of the number of repeat elements on splicing of the CBS mRNA and subsequently CBS enzyme activity and tHcy concentrations.

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