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RESEARCH ARTICLE

Polymorphisms in *transcobalamin II* gene is associated with coronary artery disease in Indian population

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Abstract

Transcobalamin (TCII) is a key enzyme involved in intracellular transport of vitamin B12. We had earlier shown that vitamin B12 levels are associated with Coronary Artery Disease (CAD). Herein, we evaluated the association of four nonsynonymous single nucleotide polymorphisms (SNPs) of *TCII* gene with CAD in 1398 individuals (589 CAD cases and 809 controls). Using logistic regression, we found that three SNPs (G1196A, C776G and C1043T) were significantly associated with CAD and one (G1196A) with vitamin B12 levels even after controlling for confounding factors. Thus, polymorphisms in *TCII* gene may play an important role in the etiology of CAD.

Keywords: Coronary artery disease, vitamin B_{12}, transcobalamin, single nucleotide polymorphism

Introduction

Cardiovascular diseases (CVD) are one of the leading causes of mortality and morbidity worldwide. An estimated 17.1 million people died from CVD in 2004 accounting for 30% of the total global deaths (WHO, 2011). Of these, 7.2 million were caused by coronary artery disease (CAD; Bedi, 2006). Further, it has been reported that low- and middle-income countries are disproportionately affected with almost 82% of deaths caused by CVD (WHO, 2011). CAD is a multifactorial disease that can be influenced by many different environmental factors including dietary habits and genetic risk factors. In terms of diet, it has been reported that the risk of fatalities caused by heart diseases are reduced up to 24% by switching to a vegetarian diet (Key, 1999). This is probably because a vegetarian diet might contain lower levels of some of the classical risk factors for CAD than nonvegetarian diet. If this was universally true, then in a country like India, where a significant proportion of the population adhere to a vegetarian diet due to family conventions or religious doctrines, it would have been expected that the incidence of heart diseases will be low. However, India has one of the highest incidences of CAD and it has been predicted that deaths caused by CAD are likely to be more than any other disease in India (Balarajan, 1991). Further, in Indians, CAD tends to occur much earlier than any other ethnic groups (Key, 1999). A strict vegetarian diet lacks vitamin B_{12}, an important micronutrient that is sourced only from animal products. Deficiency of Vitamin B_{12} leads to elevated levels of homocysteine and cysteine, the two thiol amino acids that have been reported to be associated with CAD (Kumar, 2009). In fact, we have earlier shown that vitamin B_{12} deficiency is associated with CAD in Indian population (Kumar, 2009).

Vitamin B_{12} (Cobalamin, cbl) is only synthesized by microorganisms and mammals have evolved ways for intestinal absorption, transport, and cellular uptake of vitamin B_{12}. Three different proteins—Intrinsic factor (IF), Hepatocorrin (HC) and Transcobalamin II (TC II) are required for the absorption and assimilation of vitamin B_{12} in humans. Initially, Vitamin B_{12} binds to HC in the stomach and after proteolysis in the duodenum

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binds to IF which is then taken up by mucosal cells in the ileum (Quadros, 1991). This complex is then degraded in the enterocyte and vitamin B_{12} is transferred to TCII (Quadros, 1991; Seetharam, 1999). Only about one third of Vitamin B_{12} is bound to TCII (referred to as the biologically active fraction) and this fraction of vitamin B_{12} is delivered to the cells via receptor-mediated endocytosis (Quadros, 1991; Seetharam, 1999). Although about 90% of vitamin B_{12} present in plasma binds to hepatocorrin, it does not enter the cells and can be taken up only in the hepatocytes. Thus TCII is primarily responsible for the concentration of intracellular vitamin B_{12}.

A low level of intracellular Vitamin B_{12} is associated with several disease conditions like megaloblastic anemia, gastrointestinal and neurological disorders, impaired immune defense and as mentioned above, with coronary artery disease (Cooper, 1987; Tuchman, 1998; Kumar, 1999). Low vitamin B_{12} concentrations may be either due to low intake or due to defects in absorption, transport or cellular uptake of the micronutrient or both. For instance, genetic variations in *TCII* could affect the binding of vitamin B_{12} to TCII or intracellular uptake of TC-vitamin B_{12} by the receptor, resulting in lower concentration of intracellular Vitamin B_{12}.

It has been shown earlier that a nonsynonymous single nucleotide polymorphism (nsSNP) in *TCII* gene (C776G) interferes with the availability of cobalamin in the cells (Castro, 2010) Earlier, Afman et al. studied the relationship of five nsSNPs (I23V, G94S, P259R, S348F and R399Q) in the coding region of *TCII* with total TC concentration and found that two SNPs (I23V and P259R) seems to affect the binding of vitamin B_{12} (Afman, 2002). Recently, using simulated molecular dynamics, we have shown that I23V variant might facilitate the binding of vitamin B_{12} to TCII (Silla, 2011).

We hypothesized that nsSNPs in *TCII* gene might result in low intracellular vitamin B_{12} levels and hence may be associated with CAD. Thus in the present study, we genotyped four nsSNPs in *TCII* gene (rs4820889, rs1801198, rs35838082, rs9621049) in 1398 individuals (589 CAD cases and 809 controls) to assess if any of these polymorphisms are associated with vitamin B_{12} levels and consequently with CAD.

Methods

Study participants

A total of 1398 individuals (controls = 809, cases = 589), mainly of Indo-European origin were recruited for this study. All the cases (confirmed after coronary angiography) and 433 controls who tested negative for treadmill test were recruited from the Department of Cardiology, All India Institute of Medical Sciences (AIIMS), New Delhi, India. The negative controls of treadmill test were found with normal blood pressure and heart rate and had normal ST segment deviation during the exercise and recovery. During the exercise, they didn't complain of any chest pain also. The rest of the controls were healthy individuals from various parts of New Delhi (as revealed through questionnaire), of Indo-European origin and did not have a family history of CVD. Individuals below 18 years of age and pregnant women were excluded from the study. All the participants provided their written consent. These individuals were recruited from various parts of the Delhi and the National Capital Region. The study was carried out in accordance with the Principles of the Helsinki Declaration. Study was approved by the ethics committee of both AIIMS and the Institute of Genomics and Integrative Biology. On the day of recruitment, a detailed questionnaire was filled out for each participant. Information regarding participant's dietary preferences, height, weight, and so on was recorded in the questionnaire. Participants who adhere to vegetarian diet and haven't consumed fish or meat during their entire life span were considered as vegetarians.

DNA isolation and PCR

Peripheral blood samples of 10 mL was collected from each participant into plain tubes as well as tubes containing anticoagulant. Serum and plasma was separated within an hour of collection and stored at –80°C until further analysis. Genomic DNA was isolated from blood samples using the modified salting-out method as described earlier (Kumar, 2005) and stored at –20°C until further use. Oligos were designed for PCR and SNaPshot using the primer select module of the software DNASTAR version 5.07 (DNASTAR Inc, Madison, USA) to amplify and score all the four SNPs of *TCII* gene (Supplementary Table 1). PCR was carried out using GeneAmp PCR

Table 1. General/clinical characteristics of the individuals studied.

Parameter	Controls (n = 809)	Patients (n = 589)	Significance
Age (years)	42 (31–52)	54 (46–61)	<0.001
Sex (Females)	235 (29)	71 (12)	<0.001
Diet (Vegetarian)	363 (45)	307 (53)	0.004
DM	85 (11)	114 (20)	<0.001
Hypertension	179 (22)	152 (26)	0.085
BMI (Kg/m^2)	24.5 (21.8–27.3)	24.2 (22–26.8)	0.10
Vitamin B12(pg/mL)	217 (165–280)	198 (150–266)	0.004

Note: Median (Interquartile Range) is shown for continuous variables while number (percentage) is shown for discrete variables. Significance was calculated using Mann-Whitney for continuous and chi square test for discrete variables.
DM, diabetes mellitus; BMI, body mass index.

system 9700 (Applied Biosystems, Foster City, USA), in a total volume of 10 µL. PCR conditions used were 1.5 mM $MgCl_2$, 0.1 mM of each dNTP (Amersham Biosciences, New Jersey, USA), 0.2 picomole/µL of each forward and reverse primer, 0.05 U/µL of Taq DNA polymerase (Merck, India), 1X buffer recommended by the supplier, 2 ng/µL of genomic DNA and 4% of dimethyl sulfoxide. Annealing temperature used for amplification in PCR was 58°C. After PCR, direct precipitation of PCR products using polyethylene glycol 8000 (PEG 8000)-sodium acetate purification protocol was followed for purification of amplicons.

SNaPshot

Four nsSNPs were genotyped using single base primer extension reactions (SNaPshot kit™ ddNTP primer extension kit, Applied Biosystems, Foster City, CA) as described earlier (Kumar, 2011).

Vitamin B_{12}

Serum Vitamin B_{12} was measured by electro chemiluminescence immunoassay using elecsys immunoassay analyzer (Roche, USA). The elecsys vitamin B_{12} assay employs a competitive test principle using intrinsic factor specific for vitamin B_{12}. Vitamin B_{12} in the sample competes with the added vitamin B_{12} labeled with biotin for the binding site on the ruthenium-labeled intrinsic factor complex.

Statistical analysis

Genotypic as well allelic frequencies were calculated using the standard procedure. Genotypes were checked for the conformance of Hardy-Weinberg equilibrium using chi square test. SNPs were checked for linkage disequilibrium (LD) using Haploview version 4.2 programme (www.broad.mit.edu/mpg/haploview/). Nonparametric test was performed to evaluate the difference in distribution of vitamin B_{12} in different genotypes among CAD case controls because of skewed distribution of vitamin B_{12} concentrations and median values were reported. Frequency distribution of genotypes between cases and controls was analyzed by using chi-square test. Regression analysis was performed to analyze for dependent variable and adjusting for various confounding factors. All the statistical analysis was performed using Statistical Package for Social Sciences, Windows version 17 (IBM Corporation, USA). Power calculation was done using an online statistical tool (http://www.dssresearch.com/toolkit/spcalc/power_a1.asp)

Results

In the present study, we genotyped four nsSNPs in *TCII* gene to assess if these SNPs are associated with vitamin B_{12} levels and CAD. The general/clinical characteristics of the individuals involved in the study are shown in Table 1.

Genotypic as well as allele frequency of the 4 SNPs are shown in Table 2. The minor allele frequencies (MAF) of three SNPs (G1196A, C776G, C1043T) were found to be comparable with the MAF reported in dbSNP database while the MAF of C643T was lower in this population (0.03) as compared to dbSNP (0.09; Table 2). The genotype distribution conformed to HWE in all the SNPs both in patients and controls ($p > 0.05$). The four SNPs present were also checked for their LD. None of the SNP showed any LD pattern (Supplementary Figure 1). With the available sample size, we have more than 90% power to capture the difference of 0.2 standard deviation vitamin B_{12} levels between different genotype groups.

CAD patients and controls were then analyzed for the four nsSNPs (G1196A, C776G, C643T, C1043T) to evaluate if these SNPs are associated with CAD. In the binary logistic regression taking CAD status as the dependent variable, three SNPs, that is, G1196A (rs4820889), C776G (rs1801198) and C1043T (rs9621049) showed significant association with CAD (p value = 0.001–0.003, Table 3). Even after controlling various confounding factors like age, sex, diet and diabetes, all the three SNPs remained significant (p value = 0.01–0.04, Table 3). Carriers of minor allele were found to be more in patients as compared to the controls. Only one of the SNPs tested, rs35838082, did not show any significant association with CAD.

We have earlier shown that vitamin B_{12} levels are significantly associated with CAD in Indian population (Kumar, 2009) which can be seen from Table 1 also. We therefore evaluated if the SNPs in *TCII* studied are also

Table 2. Genotypic and allele frequency in the studied individuals.

				Genotypes frequency			Allele frequency		
SNP	Base change	AA change	Status	WW	WM	MM	W	M	Rep MAF
rs4820889	G1196A	R399Q	Control	713 (88.6)	88 (10.9)	04 (0.5)	0.94	0.06	0.04
			Patient	487 (83.1)	91 (15.5)	08 (1.4)	0.91	0.09	
rs1801198	C776G	P259R	Control	324 (40.2)	368 (45.7)	113 (14.0)	0.63	0.37	0.39
			Patient	199 (34.1)	273 (46.8)	111 (19.0)	0.58	0.42	
rs35838082	C643T	R215W	Control	751 (94.8)	40 (5.1)	01 (0.1)	0.97	0.03	0.09
			Patient	524 (94.1)	33 (5.9)	0 (0)	0.97	0.03	
rs9621049	C1043T	S348F	Control	614 (76.5)	179 (22.3)	10 (1.2)	0.88	0.12	0.08
			Patient	403 (69.2)	164 (28.2)	15 (2.6)	0.83	0.17	

Note: Number (%) of genotypes is shown.
AA, amino acid; WW, major homozygous genotype; WM, heterozygous genotype; MM, minor homozygous genotype; W, major allele; M, minor allele; Rep MAF, minor allele frequency reported in dbSNP database.

Table 3. Association of polymorphisms with CAD.

	Unadjusted		Adjusted	
Polymorphism	OR (CI 95%)	*p* value	OR (CI 95%)	*p* value
rs4820889	1.55 (1.17–2.06)	0.002	1.49 (1.09–2.03)	0.01
rs1801198	1.25 (1.08–1.46)	0.003	1.24 (1.05–1.5)	0.01
rs35838082	1.12 (0.70–1.78)	0.62	1.18 (0.7–2.0)	0.54
rs9621049	1.42 (1.14–1.77)	0.001	1.28 (1.0–1.62)	0.04

Note: Unadjusted *p* values calculated using binary logistic regression where single SNP was the predictor. Adjusted *p* values were calculated using binary logistic regression where single SNP was the predictor and adjustment for age, sex, diet and diabetes status was made.
OR, odds ratio; CI, confidence interval.

Table 4. Genotypic association analysis with vitamin B12.

	Genotype frequency				
Polymorphism	Adjusted WW	WM	MM	β value (CI 95%)	*p* value
rs4820889	214 (163–285)	189 (140–234)	170 (113–284)	−29.8 (−47.7 to −11.9)	0.001
rs1801198	211 (163–288)	206 (157–275)	213 (158–259)	−9.7 (−19.3 to −0.04)	0.05
rs35838082	210 (158–280)	204 (166–270)	—	−2.5 (−31.2 to 26.3)	0.87
rs9621049	212 (160–282)	200 (156–269)	178 (133–263)	−12.8 (−26.6 to 1.1)	0.07

Note: Median (Interquartile Range) is reported.
WW, major homozygous genotype; WM, heterozygous genotype; MM, minor homozygous genotype. Beta, unstandardized beta coefficient; CI, confidence interval, *p* value, calculated using linear regression adjusting for age, sex, diet, diabetes, hypertension and CAD status.

associated with vitamin B_{12} levels. Of the SNPs tested, only one G1196A (rs4820889) was found to be significantly associated with the Vitamin B_{12} levels. Individuals with major homozygous genotype were having the highest concentration of vitamin B_{12} followed by individuals with heterozygous genotype and minor homozygous genotypes. Even after correcting for age, sex, diet, diabetes, hypertension and CAD status this SNP remained significant with an adjusted *p* value of 0.001 (Table 4). The SNP at C776G was also marginally significant ($p = 0.05$) after adjustment.

Discussion

The results in this study indicate that SNPs in *TCII* gene are associated with CAD and one of these (G1196A) is also associated with vitamin B_{12} levels. We and others have earlier shown that a significant proportion of Indians have low vitamin B_{12} levels presumably due to consumption of a strict vegetarian diet (Satoskar, 1961; Kumar, 2009). Vitamin B_{12} acts as cofactors for the enzymes methionine synthase (methyl Cbl) and methyl malonyl-CoA mutase (Ado-Cbl). Methionine synthase catalyses the conversion of homocysteine to methionine and hence deficiency of vitamin B_{12} will result in elevated levels of homocysteine, a thiol amino acid, which is considered to be an independent risk factor of CAD. In general, once homocysteine is formed it is either remethylated to methionine by methionine synthase or is converted to cystathionine and then to cysteine via the transsulfuration pathway. Thus, it can be perceived that under the conditions of low vitamin B_{12}, the flux of homocysteine to transsulfuration pathway will increase leading to increased concentration of cysteine, which is also now considered to be a risk factor for CAD (El-Khairy, 2003). Polymorphisms in *TCII* gene may be responsible for CAD due to the fact that such polymorphisms might restrict the internalization of vitamin B_{12} resulting in low intracellular levels of this micronutrient which in turn will result in higher concentrations of homocysteine and cysteine.

However, in general, we did not find striking association of *TCII* gene polymorphisms with vitamin B_{12} levels with only one of the SNPs showing association. This could be because, of the total circulating vitamin B_{12}, only a small proportion (about 30%) is bound to TCII and is internalized (Silla, 2011). This fraction is also known as the biologically active fraction. Thus, it has generally believed that total plasma vitamin B_{12} level may not a true reflection of cobalamin deficiency. It has been recently shown that serum holoTC was a better predictor of cobalamin deficiency than serum cobalamin itself (Valente, 2011). Likewise, there are other reports suggesting that holoTc may be a better marker for vitamin B_{12} deficiency. Thus, better measures of circulating vitamin B12 like holo TC may improve the association with *TCII* polymorphisms.

Among the SNPs in *TCII* gene, the SNP C776G is the most widely studied and has been reported to be associated with several diseases. In a recent study, Godbole et Al. reported that maternal *TCII* C776G polymorphism was strongly predictive of neural tube defect in the offspring (Godbole, 2011). The G allele of this polymorphism has also been reported to be associated with unfavorable lipoprotein profile (Semmler, 2010). Mothers and children with homozygous minor genotype (GG) have been reported to contribute to the risk of congenital heart diseases particularly when the levels of maternal vitamin B_{12} are low (Verkleij-Hagoort, 2008). In contrast, a significant over transmission of the C allele was observed at the polymorphism C776G to the offspring affected with cleft palate (Martenelli, 2006). This polymorphism has also

been shown to be a risk factor for Alzheimer's disease (McCaddon, 2004). In this study, we for the first time show that not only C776G but also two other SNPs in this gene G1196A and C1043T are associated with CAD. To the best of our knowledge this is the first report showing that these SNPs are associated with CAD.

There are conflicting reports on the association of C776G with vitamin B_{12} levels. Although some studies have reported significant association with Vitamin B_{12} (Gale, 2006; Garrod, 2010) others did not find any such association of SNP with this micronutrient (Aléssio, 2007; Castro, 2010). Our results also indicate that the SNP C776G is not associated with vitamin B_{12} levels and among the SNPs tested; only G1196A is significantly associated.

We have earlier shown using molecular simulation studies that C776G may not affect the binding of vitamin B12 to TCII (Silla, 2011). In that study we showed that the amino acid change due to this SNP bears marginal deviation of His-173, when compared to the wild-type structure. His-173 directly coordinates with the cobalt ion of cobalamin (vitamin B_{12}) and is thus important for the binding of vitamin B_{12}. Further, from dynamic cross-correlation map anlaysis, we had found that the correlated motion due to this variation was similar to the wild type and principle component analysis indicated that no substantial motional changes were induced between the two domains due to this variation. This was in agreement with the findings of Wuerges et al. (Wuerges, 2007) who clearly showed that the variation C776G is in the flexible loop between helices and hence is not likely to influence the binding of vitamin B_{12} to TCII (Wuerges, 2007). Thus, from the above, it could be perceived that the polymorphism at C776G is unlikely to influence the binding of vitamin B_{12} to transcobalamin. However, this polymorphism is believed to exert significant influence on vitamin B_{12} cellular delivery (presumably having an effect on the receptor mediated endocytosis of TC-vitamin B_{12} complex) although it may not have effect on plasma vitamin B_{12} levels (Quadros, 1991; Seetharam, 1999).

Conclusion

Thus, in the current study, we have shown that at least three SNPs in *TCII* gene (G1196A, C776G and C1043T) are associated with CAD in Indian population and one of these SNPs G1196A is also associated with vitamin B_{12} levels. In a country like India where a significant proportion of the population adhere to a vegetarian diet, it may thus be pertinent to include vitamin B_{12} levels for routine test along with lipid profile and so on to assess the risk of a person towards developing CAD. However, larger cross-sectional studies are necessary before concrete steps can be taken in this direction.

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Declaration of interest

The authors report no conflict of interest.

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