Brief Report

Transcobalamin II G775C Polymorphism and Indices of Vitamin B12 Status in Healthy Older Adults

Running Head: Transcobalamin II Genotype and B12 Status

Joshua W. Miller¹, Marisa I. Ramos², Marjorie G. Garrod², Margaret A. Flynn³, Ralph Green¹

¹Univ. of California, Davis, School of Medicine, Dept. of Medical Pathology
²Univ. of California, Davis, Dept. of Nutrition
³Univ. of Missouri, Columbia, School of Medicine, Dept. of Family and Community Medicine

This study was financially supported by the Wallace Research Fund, Cedar Rapids, IA; the Eshe Fund, New York, NY; and the School of Medicine, Univ. of Missouri, Columbia, MO

Corresponding author and address for reprint requests: Joshua W. Miller, PhD, UC Davis Medical Center, Department of Medical Pathology, Research III – Room 3200A, 4645 Second Ave., Sacramento, CA, 95817.

Phone: 916-734-3532; FAX: 916-734-2698; e-mail: jwmiller@ucdavis.edu

Word Count (abstract): 150

Word Count (text): 1164

Scientific Heading: Clinical Observations

Key Words: Transcobalamin II, vitamin B12, methylmalonic acid, homocysteine
ABSTRACT

A common polymorphism (G775C) in the vitamin B12 transport protein, transcobalamin II (TCII), has been identified in which proline replaces arginine at codon 259. We determined the influence of TCII genotype on indices of B12 status, including total serum B12, the amount of B12 bound to TCII (holoTCII), methylmalonic acid, and homocysteine, in 128 healthy older adults (age 40-88y). Mean total B12 and homocysteine concentrations were not significantly different among the three genotypes. Mean holoTCII concentration was significantly higher in those subjects homozygous for the proline form of TCII (PP) compared with those homozygous for the arginine form (RR) and heterozygotes (PR) ($p \leq 0.006$). In addition, mean methylmalonic acid concentrations were significantly lower in the PP and PR groups compared with the RR group ($p \leq 0.02$). The PP genotype may be more efficient in delivering B12 to tissues resulting in enhanced B12 functional status. TCII genotype may thus influence susceptibility to B12 deficiency.
INTRODUCTION

The serum protein, transcobalamin II (TCII), transports vitamin B12 (B12) from the ileum to the tissues. The B12-TCII complex (holoTCII) is then taken up into cells by receptor-mediated endocytosis. In the 1970’s and 80’s, two research groups independently identified distinct isopeptide forms of TCII by polyacrylamide gel electrophoresis\textsuperscript{1, 2} and isoelectric focusing\textsuperscript{3}. More recently, DNA sequencing has revealed that the isopeptide forms of TCII are the result of single nucleotide polymorphisms\textsuperscript{4-6}. The most common polymorphism in Caucasian populations is a G to C substitution at base position 775 (G775C) which results in the replacement of proline with arginine at codon 259. Recently, the potential influence of the G775C polymorphism on indices of vitamin B12 status has been investigated. Individuals homozygous for the proline form of the protein (PP) tend to have higher holoTCII, but similar total serum B12 concentrations compared with those homozygous for the arginine form (RR)\textsuperscript{7-11}. One group has found that homocysteine, a functional indicator of B12 status, is higher in heterozygous individuals (PR) compared with PP and RR individuals\textsuperscript{8, 11}, but this finding was not confirmed\textsuperscript{9, 10}. Notably, the relationship between TCII genotype and methylmalonic acid, potentially a more specific indicator of B12 status than homocysteine, has not been reported. Therefore, we assessed the relationship between G775C TCII genotype and methylmalonic acid, as well as total B12, holoTCII, and homocysteine, in a cohort of healthy older adults.
MATERIALS AND METHODS

Subjects

The study sample consisted of 108 men and 20 women (mean age: 67y; range: 40-88y).

Subjects are current participants in the Longitudinal Aging Study, initiated in 1969 and continuing through the present at the University of Missouri-Columbia. The study population consists primarily of University of Missouri faculty and staff who are physically active and in apparent good health, with no evidence of decreased intrinsic factor secretion or gastrointestinal B12 malabsorption. The project is approved by the University of Missouri-Columbia Institutional Review Board and all participants have provided their informed consent.

TCII Genotyping

PCR product was amplified using an Eppendorf Mastercycler (Brinkmann, Westbury, NY) in a total reaction volume of 25.0 µl containing 7.0 µl genomic DNA, 1.5 mmol/L MgCl, 0.2 mmol/L dNTP mix, 2.0 µmol/L forward primer (5'-GTC AGG-TGC TGG-AAC-ACC-TAG-3'), 2.0 µmol/L reverse primer (5'-CCA-GCT-GCT GCC-CGT TCT-GAA-3'), 2.5 µl 10x PCR buffer, and 1U Taq polymerase (Gibco BRL/Life Technologies, Rockville, MD). The amplification consisted of: 1) initial denaturation (94 °C, 2 min); 2) 35 cycles consisting of denaturation (94 °C, 1 min), annealing (64 °C, 1 min), and extension (72°C, 2 min); and 3) final extension (72°C, 7 min). PCR product was digested at 37 °C overnight in a total reaction volume of 20.0 µl containing 15.0 µl PCR product, 2.0 µl 10x digestion buffer, and 20U of MVA I restriction enzyme (Roche Molecular Biochemicals, Indianapolis, IN). Digested samples were electrophoresed on 1% agarose, 2% NuSieve GTG (FMC Bioproducts, Rockland, ME) gels and visualized (Figure 1).
Metabolite Assays

Fasting serum B12 and RBC folate were determined by automated chemiluminescence assay (ACS180, Bayer Diagnostics, Tarrytown, NY); methylmalonic acid by gas chromatography/mass spectrometry; homocysteine by HPLC with fluorescence detection; creatinine by standard spectrophotometric assay; and holoTCII by indirect assay utilizing anti-TCII antibodies as follows: Activated Sepharose beads were coupled with polyclonal antibody raised in goats inoculated with rabbit TCII purified by photodissociative affinity chromatography. The antibody shows immunological cross-reactivity with human holoTCII. In the assay, goat anti-rabbit TCII antibody-coated Sepharose beads were washed and resuspended as a 5% mixture in phosphate-buffered saline. One ml aliquots of the washed beads were transferred into microfuge tubes and centrifuged at 3,000xg (5 min). After aspiration of the supernatant, serum samples (500 µl) were added to the bead pellet, mixed for 2h at room temperature, and then centrifuged at 3,000xg (5 min). B12 concentrations were determined in the supernatants by radioassay (Simultrac Radioassay, ICN Pharmaceuticals, Orangeburg, NY). The difference in B12 concentration between untreated serum and bead-treated serum represented the holoTCII level. In this assay, antibody-coated beads consistently remove >98% of $^{57}$Co-cyanocobalamin-labelled holoTCII (data not shown). Mean (±SD) for holoTCII in 22 non-deficient, normal volunteers was 104.1 (±66.7) pg/ml (range: 38-305 pg/ml). Within and between assay CV’s in non-deficient samples were 15% and 17%, respectively.

Statistical Analyses

Means (±SD) for each metabolite were compared by ANOVA, controlling for age, gender, and other covariates as indicated, followed by Scheffe’s F-test.
RESULTS AND DISCUSSION

Characteristics of the study sample divided by TCII genotype are presented in Table 1. The distribution of genotypes among the subjects was 30% PP, 50% PR, and 20% RR, similar to previous reports. No differences among the genotypes were observed for hematocrit and MCV, and no subjects had evidence of macrocytic anemia. The mean holoTCII concentration was significantly higher in the PP subjects compared with the PR and RR subjects, while no differences in mean total B12 were observed. These results are consistent with previous reports. The mean methylmalonic acid concentration was significantly higher in the RR subjects compared with the PP and PR subjects. Taken together, these findings suggest that TCII genotype influences the cellular delivery of B12 and directly impacts one of the two biochemical reactions in which B12 participates as a cofactor, i.e. the conversion of methylmalonyl CoA to succinyl CoA catalyzed by the enzyme methylmalonyl CoA mutase. PP genotype was also associated with a higher percentage of total B12 bound to TC compared with the other genotypes. This suggests that the PP genotype has higher affinity for B12 compared with the RR genotype. This remains to be definitively determined, however.

No differences in mean homocysteine concentrations were observed among the genotypes. This is in contrast to a previous finding that the PR genotype is associated with higher homocysteine than either homozygous genotype, a finding unconfirmed in two other reports. It has been suggested that the discrepancy between the studies with respect to homocysteine levels is related to differences in the age of the study subjects, with younger subjects exhibiting the homocysteine difference and older subjects not. A more likely explanation is that the higher homocysteine observed in PR individuals in the one study was related to some other uncontrolled determinant of homocysteine, such as gender, B vitamin...
levels (folate, B12, B6), kidney function, thyroid function, and other genetic factors. In this regard, methylmalonic acid may be better than homocysteine as an indicator of the effect of TCII genotype on functional B12 status because methylmalonic acid is influenced by fewer confounding factors. Notably, in the present study, significant differences in methylmalonic acid among the genotypes were observed after controlling for age, gender, creatinine, and total B12.

We conclude that TCII G775C genotype significantly influences tissue B12 delivery and functional B12 status. Because none of the subjects in the study sample exhibited evidence of hematological abnormalities, the differences among the genotypes in methylmalonic acid and holoTCII may represent pre-clinical alterations in B12 status and function. It remains to be determined if TCII genotype ultimately influences the susceptibility of individuals to develop the overt clinical manifestations of B12 deficiency, including hematological and neurological sequelae.
ACKNOWLEDGEMENTS

We thank at the University of Missouri, Columbia: Genevieve Hill for coordinating subject participation and Wally Thomas for supervision of phlebotomy and blood processing. We thank at the University of California, Davis: Lisa M. Rogers, PhD, Rebecca F. Cotterman, and Autumn Nguyen for conducting methylmalonic acid and holoTCII assays; and Angela Devlin, PhD, for her assistance in developing the TCII genotyping assay.
REFERENCES


Figure 1. Representative gel illustrating PCR and MVA I digestion products indicative of the three G775C TCII genotypes. The size of the PCR product is 218 bp. Complete digestion of the PCR product with MVA I leads to two fragments with sizes of 128 and 90 bp. Complete digestion of the PCR product (2 bands) is indicative of the PP genotype (sample #1), partial digestion (3 bands) is indicative of the PR genotype (sample #2), and no digestion (1 band) is indicative of the RR genotype (sample #3).
Table 1: Characteristics of Study Sample by Transcobalamin II Genotype*

<table>
<thead>
<tr>
<th>Transcobalamin II Genotype</th>
<th>PP</th>
<th>PR</th>
<th>RR</th>
</tr>
</thead>
<tbody>
<tr>
<td>N</td>
<td>39</td>
<td>63</td>
<td>26</td>
</tr>
<tr>
<td>Gender (men/women)</td>
<td>33/6</td>
<td>54/9</td>
<td>21/5</td>
</tr>
<tr>
<td>Age (y)</td>
<td>66 (±11)</td>
<td>67 (±11)</td>
<td>67 (±11)</td>
</tr>
<tr>
<td>B12 (pg/ml)</td>
<td>433 (±177)</td>
<td>415 (±168)</td>
<td>383 (±191)</td>
</tr>
<tr>
<td>HoloTCII (pg/ml)</td>
<td>150 (±81)</td>
<td>113 (±56)</td>
<td>104 (±83)</td>
</tr>
<tr>
<td>% of Total B12 on TCII¶</td>
<td>34.3 (±9.5)</td>
<td>27.8 (±9.9)</td>
<td>24.6 (±10.1)</td>
</tr>
<tr>
<td>Methylmalonic Acid (nmol/L)</td>
<td>208 (±96)</td>
<td>206 (±80)</td>
<td>264 (±138)</td>
</tr>
<tr>
<td>Homocysteine (µmol/L)</td>
<td>10.3 (±2.6)</td>
<td>10.7 (±2.4)</td>
<td>11.2 (±2.8)</td>
</tr>
<tr>
<td>RBC Folate (ng/ml)</td>
<td>363 (±97)</td>
<td>378 (±106)</td>
<td>398 (±106)</td>
</tr>
<tr>
<td>Creatinine (mg/dL)</td>
<td>1.1 (±0.9)</td>
<td>1.1 (±1.0)</td>
<td>1.1 (±0.6)</td>
</tr>
<tr>
<td>Hematocrit (%)</td>
<td>43.4 (±3.2)</td>
<td>43.8 (±3.5)</td>
<td>43.6 (±3.3)</td>
</tr>
<tr>
<td>MCV (µ³)</td>
<td>93.9 (±3.2)</td>
<td>92.9 (±3.2)</td>
<td>92.2 (±5.1)</td>
</tr>
</tbody>
</table>

*Values represent means (±SD).

¶“% of Total B12 on TCII” was calculated using the equation: 100 x (holoTCII/total B12).

1Significantly less than PP genotype after controlling for potential confounding by age, gender, and total B12 (p ≤ 0.006).

2Significantly greater than PP and PR genotypes after controlling for potential confounding by age, gender, total B12, and creatinine (p ≤ 0.02).
Transcobalamin II G775C Polymorphism and Indices of Vitamin B12 Status in Healthy Older Adults

Joshua W Miller, Marisa I Ramos, Marjorie G Garrod, Margaret A Flynn and Ralph Green