Markers of B-vitamin deficiency and frailty in older women

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Abstract

Objective—This project was designed to follow-up prior evidence that demonstrated a significant association between vitamin B12 transport and metabolism and the frailty syndrome in community-dwelling older women. The cross-sectional relationship between genetic variants within six candidate genes along this pathway with serum methylmalonic acid (MMA) levels and frailty was evaluated in this same population of older women.

Methods—Baseline measures were collected prior to folate fortification from 326 women in the Women's Health and Aging Studies I and II. Odds ratios and statistical tests were estimated for single SNP and haplotype via linear regression models for serum MMA, a marker for available vitamin B12, and in logistic regression models for frailty.

Results—Fifty-six SNPs from CBS, MTHFR, MTR, MTRR, TCN1 and TCN2 genes were genotyped. Several SNPs in MTHFR, MTR and MTRR demonstrated a modest association to elevated MMA, while SNPs in TCN2 showed significant association to the frailty syndrome. TCN2 polymorphisms, particularly one SNP reported to be in perfect LD with functional variant Pro259Arg, were significantly associated with increased odds of frailty, after adjustment for age, presence of cardiovascular disease and elevated MMA (OR = 2.25, p-value = 0.009).

Conclusions—Using MMA as a marker for vitamin B12, these results suggest that TCN2 gene variants may lead to decreased vitamin B12 availability, leading to reduced energy metabolism, ultimately contributing to frailty pathology. Further studies to determine the biological role of functional TCN2 polymorphisms in frailty are needed.

Keywords

transcobalamin-II; vitamin B_{12}; genetic association

INTRODUCTION

The geriatric syndrome of frailty poses a critical public health concern, conferring a significant vulnerability to adverse health outcomes in older persons [1–4]. Age-related declines in energy metabolism, neuroendocrine, musculoskeletal, and immunological systems are likely to contribute to the clinical characteristics of frailty and to the increases in disability, disease and death observed in frail older adults [2,5]. Reduced mitochondrial function and DNA damage have been hypothesized to contribute to these multisystem declines [6]. Similarly, dysregulation of one-carbon transfer triggers activation of inflammatory pathways via

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mitochondrial dysfunction and increased oxidative stress with resulting DNA damage, making these vitamins important targets for studying frailty [7].

One-carbon transfer pathways rely on sufficient stores of vitamin B12 for efficiency of folate-dependent one-carbon transfers. Reduced vitamin availability along with decreased enzyme activity due to genetic variants disrupt these processes, causing increased serum homocysteine (tHcy), DNA, RNA, protein hypomethylation and decreased glutathione production [7]. Genes coding for proteins regulating one-carbon transfer have been associated with reduced methylation, increased DNA damage, and increased risk of cardiovascular disease, osteoporosis and a number of cancers [8–12]. For example, sequence variation in the gene encoding vitamin B12 transport protein has been associated with markers of functional vitamin B12, such as methylmalonic acid (MMA) and may influence vitamin availability to these one-carbon pathways [13,14].

We have previously shown a cross-sectional relationship between increased risk of frailty syndrome and insufficient stores of functional vitamin B12, as measured by elevated MMA levels (OR = 2.33, p-value = 0.02) [15]. Genes that influence one-carbon transfer pathways may help to explain this relationship, possibly by preventing appropriate transport and use of vitamin B12 in one-carbon transfer and propionate metabolism. We examined six genes related to this pathway (MTHFR, MTR, MTRR, CBS, TCN1, and TCN2) for their relationship to serum MMA levels and frailty syndrome in community-dwelling women aged 70–79. We hypothesize that gene variation in vitamin B-dependent one-carbon transfer pathway genes contributes to variation in MMA levels and to risk of the frailty syndrome.

SUBJECTS AND METHODS

Study Population

The women included in this study were participants in the Women's Health and Aging Study (WHAS) I and II, companion studies of community-dwelling older women. WHAS I was a prospective cohort of women age 65 years and older designed to sample the 1/3 most disabled women in a 12 zip code area of Baltimore, MD [16]. After screening, 1,002 of 1,409 eligible women consented to participate, 78% of whom consented to baseline blood draw. WHAS II sampled the 2/3 least disabled women, age 70–79, in the WHAS I catchment area using the same sampling frame [17]. There were 436 who consented to participate, 90% of whom consented to baseline anthropometry. Standardized data collected upon interview included demographics, self-report on physical function and prevalent diseases, and performance-based functional measures. Due to the complementary nature of the research designs, the WHAS cohorts can be joined for analysis. Differences in inclusion standards between the cohorts restrict the combined analyses to women between the ages of 70–79 who had a Mini-Mental State Exam (MMSE) above 23 and consented to a blood draw at baseline, resulting in a final sample size of 416 women, 326 Caucasian and 90 African American [18]. All blood was obtained prior to food folate fortification in the United States. Characteristics of these women have been previously presented [15].

Blood Measurements

Non-fasting blood samples were collected, serum prepared and frozen at the Johns Hopkins Core Facility and shipped frozen to the University of Colorado Health Sciences Center for measurement. Serum methylmalonic acid (MMA) was assayed through stable isotope dilution capillary gas chromatography/mass spectrometry with selected ion monitoring [19]. Normal range, previously established using 60 `normal' blood donors age 18–65 with an equal number of males and females, was defined within two standard deviations of the mean after log normalization (73–271 nmol/L) [20]. Serum folate levels were measured at Corning Clinical

Laboratories (Ciba-Corning Diagnostics Corporation, Medfield, MA.). The normal range of folate was reported as 6.8–36.0 nmol/liter. Serum creatinine was also assayed at Corning Clinical Labs by routine methods. The normal range for creatinine was reported as 0.6–1.1 mg/dL for females. Log transformation was carried out to normalize MMA. Biomarkers tested as potential confounders were dichotomized as normal versus abnormal based on reported normal ranges: low serum folate (< 5.7 nmol/L) and elevated creatinine (> 1.2 mg/dL) [19,21].

Phenotype Measurements

Frailty was defined by previously validated five-component criteria that include weight, grip strength, endurance, physical activity and walking speed [2,5]. Women were evaluated at baseline for the following frailty measures: 1) shrinking, defined as either body mass index less than 18.5 kg/m^2 or greater than 10% loss of body weight since age 60; 2) weakness, defined as the lowest quintile of grip strength of the dominant hand assessed with a hand-held JAMAR dynamometer (model BK-7498; Fred Sammons Inc, Burr Ridge, IL); 3) poor endurance and energy, defined as self-report of being either more tired or weaker than usual in the past 30 days; 4) slowness, defined as the lowest quintile in time to complete a 4 m walk; and 5) low activity level, defined by the lowest quintile of self-report of weekly activity determined by a subset of questions from the Minnesota Leisure Questionnaire. Lowest quintile cutoffs were established using the female population of the Cardiovascular Health Study (CHS) cohort [2]. Frailty was defined as the presence of three or more of these five criteria, while women with one or two criteria were considered prefrail, or intermediate, and women with no criteria were labeled not frail, or robust.

Gene Variant Measurement and Statistical Analysis

Six candidate genes related to one-carbon metabolism were included in this study: MTHFR, MTR, MTRR, CBS, TCN1 and TCN2. Single nucleotide polymorphisms (SNPs) within each gene were selected using information from available databases at the time of design (August, 2005) [22,23]. A final list of SNPs was determined based on high probability of being successfully genotyped (> 0.80), heterozygosity (> 0.10), and dense coverage across gene (5 kb density in regions of low linkage disequilibrium (LD) (solid spine of LD D' < 0.80) and 20 kb density in regions of high LD (D' > 0.80)).

DNA samples were extracted from whole blood using Puregene isolation kits (Gentra Systems, Inc.) and stored in a −80°C freezer. Sample plating and genotyping were carried out as part of a larger frailty candidate gene SNP project of the Claude B. Pepper Older Americans Independence Center at Johns Hopkins University using Illumina BeadArray technology [24, 25].

Since SNP allele frequencies and LD characteristics may differ by self-reported race, all statistical analyses in this report were stratified by race. Allele and genotype frequencies were calculated and tests of Hardy-Weinberg equilibrium were performed for each SNP per race. Pairwise LD values (D' and r^2 statistics) were calculated for each pair of SNPs within a particular gene.

Multivariate linear regression models were used to assess the relationship between individual SNPs and ln(MMA), adjusted for age and low serum folate. Multinomial logistic regression models evaluated the association between SNP and frailty (3 levels: robust, pre-frail, frail), adjusting for age, presence of CVD and elevated MMA. Inheritance model-independent analyses were first tested in which heterozygous genotypes and homozygous rare allele genotypes were separately compared to homozygous common allele genotypes. Statistical significance was assessed via 2-df likelihood ratio tests (LRT) comparing models with both heterozygous and homozygous indicator variables to those where genotype effects were

constrained to zero. Regression estimates from statistically significant tests were used to infer and refit appropriate genetic inheritance models such as dominant, recessive or additive inheritance. Effect modification by relevant vitamin deficiencies per candidate gene was also assessed. Single SNP tests were carried out using STATA version 9.0 [26].

Haplotype block structure for each gene was estimated from the observed data using the Haploview program [27]. Haplotype blocks were defined according to the solid spine of LD model (D' > 0.80), and haplotypes within each block with estimated frequencies < 1% were pooled for analysis. Adjusted haplotype association tests were performed using the Haplo.Stats (haplo.glm) software in the R statistical environment [28,29] which employs generalized linear models to estimate regression coefficients of serum MMA or frailty on haplotype pair status. Global score statistics were calculated using the haplo.score function. Since multinomial logistic models are not accommodated in current haplotype software, frailty was evaluated via two independent binary logistic regression models, the first testing the odds of being prefrail compared to robust and the second testing the odds of being frail compared to robust.

RESULTS

Given the extremely small set of African American participants (n=90), this report focuses on results from 326 Caucasian women. On average the WHAS Caucasian women were 74.1 years of age, were diagnosed with 2 chronic conditions, and had mean serum MMA of 238.7 nmol/ L (SD = 157.1, range of values= 79.0–1,427.0). Elevated MMA (> 270 nmol/L) was observed in 25.2% of women, while low serum folate and elevated creatinine were present in 17.5% and 11.0% of subjects, respectively.

There were 55 SNPs successfully genotyped in the six candidate genes. The average distance between SNPs was 3.75 kb per gene. Two MTR SNPs (rs10925250, rs10925257) were slightly out of Hardy-Weinberg equilibrium (p-values = 0.03), but were included in subsequent analyses. Haplotype block length was highly variable, ranging from 0.2 to 106.6 kb.

Significant associations between ln(MMA) and SNPs in genes MTHFR, MTR and MTRR were observed in our multiple linear regression models (Figure 1), adjusted for age and low serum folate. The common functional variant C677T SNP of MTHFR best fit a recessive model, with the TT genotype significantly associated with 56.9 nmol/L lower MMA compared to CT and CC individuals (p-value = 0.005). Likewise, the G allele of MTRR functional variant A66G carried a 55.7 nmol/L increase in MMA compared to AA individuals under a dominant model (p-value = 0.009). Across the MTR gene, heterozygotes at multiple SNPs high LD with each other had a 38–42 nmol/L decrease in serum MMA, compared to either type of homozygote.

We next examined whether variation in these vitamin B-dependent one-carbon pathways genes contributes to risk for frailty. SNPs in the TCN2 gene showed significant association with frailty syndrome, after adjustment for age, presence of cardiovascular disease and elevated MMA (Figure 1). In particular, in an additive genetic model rs2267163 demonstrated a 2.29 increased odds of being frail compared to being robust (p-value= 0.006) (Figure 2). Other typed SNPs in high LD with rs2267163 were observed to have similar effect sizes (Figure 2). A non-significant decrease in odds of being prefrail compared to robust was also observed for SNP rs22671634 (OR = 0.76, p-value = 0.13). Two SNPs in the MTRR gene also showed 2–4 times greater odds of being frail compared to robust, although these associated SNPs appeared to be unrelated to the MTRR SNP associated with MMA levels. No evidence of effect modification by MMA or low serum folate was observed with TCN2 or MTRR gene SNPs. Results of global and haplotype specific regression models for ln(MMA) and frailty were consistent with single SNP tests (results not shown).

DISCUSSION

We have shown association between genetic variation in genes that influence one-carbon metabolism and vitamin B12 availability, as measured by MMA. Polymorphisms in the MTR gene and the G allele of MTRR's functional A66G variant were associated with higher MMA, representing poorer vitamin B12 status and consistent with previous findings [30,31]. However, the TT genotype of MTHFR's functional C677T polymorphism was associated with decreased MMA, interpreted as better B12 status, a finding contrary to previous findings [8, 10]. None of these polymorphisms appear to be related to frailty, suggesting that the folate-dependent one-carbon metabolism does not play a role in frailty pathogenesis.

MTRR and TCN2 polymorphisms exhibited significant association with odds of frailty, after adjusting for age and presence of CVD. TCN2 SNP genotypes that were associated with increased odds of frailty also demonstrated a 15–22 nmol/L increase in MMA levels (Figure 2), although these associations did not reach statistical significance (p-values = 0.10). However, our study is likely to be underpowered to detect a modest effect difference among genotype groups. The trend in the data may suggest that vitamin B12 transport and absorption mechanisms contribute decreased vitamin B_{12} availability and to increased risk to the frailty syndrome. MTRR polymorphisms did not seem to demonstrate any consistent relationship with MMA levels.

The transcobalamin-II (TC) protein plays a critical role in vitamin B_{12} transport and absorption and TC deficiency has been linked to megaloblastic anemia and neurological disorders [7]. Failure to bind to and utilize available vitamin B_{12} could impair the conversion of methylmalonyl-CoA to succinyl-CoA in propiate metabolism, leading to increased levels of circulating MMA, particularly in skeletal muscle [32]. In a methylmalonyl-CoA mutase knockout mouse model, Chandler et al observed significant increases in MMA over time in methylmalonyl-CoA mutase knock-out mice compared to wild-type mice [32]. This increase of MMA in skeletal muscle could represent a considerable decrease in energy metabolism, ultimately leading to the activation of inflammatory pathways that contribute to frailty syndrome.

Interestingly, previous work has observed an association between a coding SNP of TCN2 (G775C) that substitutes arginine (R) for a proline (P) at codon 259 and lower serum MMA, higher TC and higher tHcy concentrations in older adults [13,33]. In those studies, proline (P) carriers had lower serum MMA and TC and higher tHcy concentrations, suggesting more efficient vitamin B_{12} transport and binding mechanisms versus R allele homozygotes who may be more susceptible to vitamin B_{12} deficiency. This coding SNP is completely correlated ($r^2=1$) with our SNP (rs2267163) most strongly associated with frailty, suggesting this coding SNP may be the functional variant in our study. While the P259R variant is not in a region hypothesized to form the hydrophobic pocket for vitamin B_{12} binding, protein structure algorithms predict that it may affect binding through alteration of the secondary structure of TC [34,35]. Additionally, replacement of neutral amino acid, proline, by positively charged arginine may inhibit TC expression [36]. Further research should focus on genotyping this variant.

The cross-sectional findings of this study were observed in a cohort of community-dwelling Caucasian women between the ages of 70 and 79 and should be carefully interpreted. While serum MMA has been shown to be a good marker of vitamin B_{12} status, we did not have cobalamin-related dietary data or holo-transcobalamin II (holoTC) complex measurements, which could have improved measurement further. Since candidate genes selection was based on a priori scientific evidence regarding one-carbon metabolism and pathophysiology of the frailty syndrome, interpretation of each statistical test must allow for qualitative differences

among tests, based on causal reasoning [37]. Therefore, we did not correct further for multiple testing. Nonetheless, independent replication of the association between TCN2 gene variation and frailty syndrome in another cohort is necessary. Structural equation modeling could also be used to test the relationships among TCN2 gene variation, serum MMA and frailty. Lastly, a larger sample is needed to evaluate these candidate genes in African Americans. Despite these limitations, our results suggest genes of vitamin B_{12} transport may be important in the availability of vitamin B_{12} and can ultimately contribute to the risk of frailty via mechanisms of reduced energy metabolism. Furthermore, the results of this study may help identify valuable targets for pharmacogenetic interventions in frailty.

Acknowledgments

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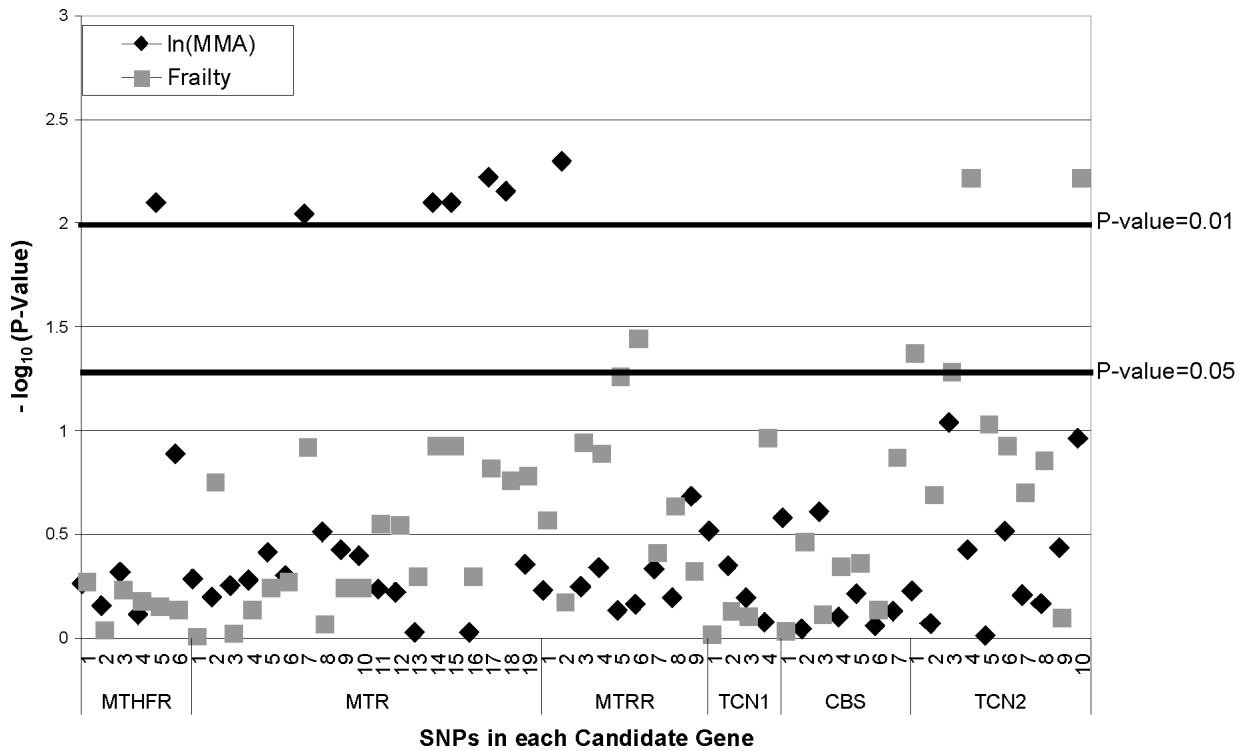37. Goodman SN. Multiple comparisons, explained. Am J Epidemiol 1998;147(9):807–12. [PubMed: 9583709]discussion 815

Figure 1.
Summary of results of single SNPs for all candidate genes for ln(MMA) and frailty. The -log$_{10}$(p-value) values are based on multivariate Wald statistic tests comparing regression models with confounders and genetic variables to model with confounders. Candidate genes include 5,10-methylenetetrahydrolfolate reductase (MTHFR), methionine synthase (MTR), methionine synthase reductase (MTRR), transcobalamin I (TCN1), cystathionine-beta-synthase (CBS) and transcobalamin II (TCN2).
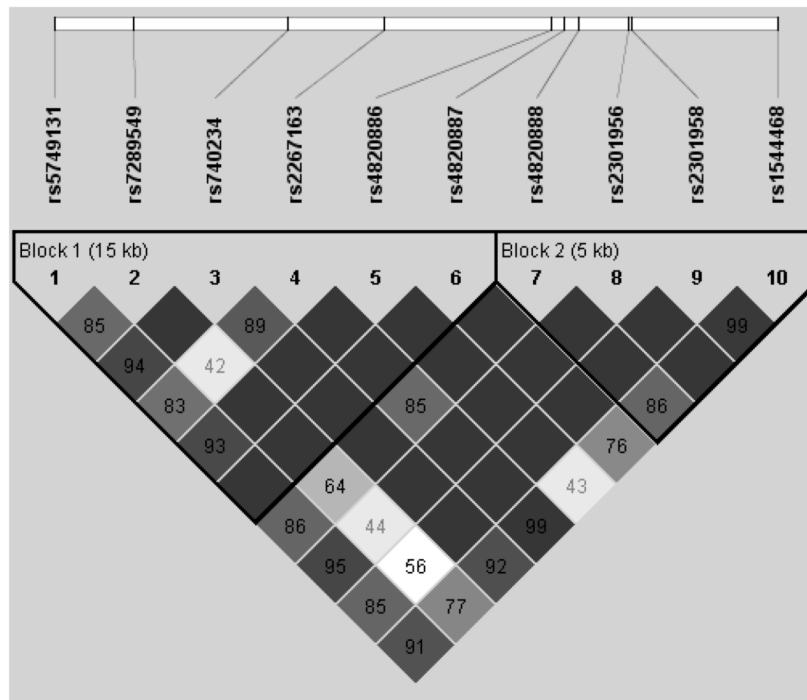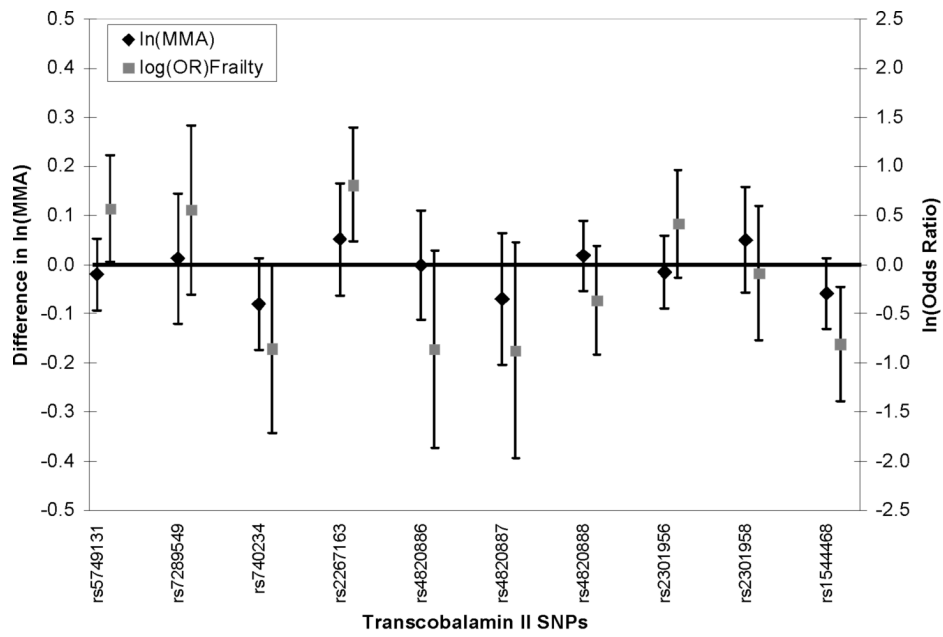
Figure 2.
Beta estimates with 95% confidence intervals for TCN2 for change in ln(MMA) and ln(odds ratio) of frailty with corresponding LD plot.