Impact of FPGS and GGH SNPs on Plasma Folate and Homocysteine Levels in the Singapore Chinese Health Study

A THESIS
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

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June 2010

Acknowledgements

I would like to thank my advisor, Kim Robien and my committee members Jian-Min Yuan and Julie Ross. I would also like to thank other members of my research group, Maki Inoue-Choi and Lori Strayer.

Abstract

Background: Folate has widespread importance to health due to its role in one-carbon metabolism. Folate status and genetic variants in the folate uptake and metabolism pathways have been assessed in relation to numerous disease processes, especially colon cancer and cardiovascular disease. The enzymes folylpolyglutamate synthase (FPGS) and gamma-glutamyl hydrolase (GGH) are essential for maintaining intracellular folate homeostasis. FPGS adds glutamyl groups to the folate molecule, which is essential for cellular retention of folate. The polyglutamted form of folate metabolites is the preferred substrate for many one-carbon metabolism enzymes. GGH removes glutamyl groups, which allows folate to leave the cell. **Objective:** The purpose of this study was to evaluate whether single nucleotide polymorphisms (SNPs) in the FPGS and GGH genes influence plasma folate and homocysteine levels. **Methods:** Study participants were a sub-cohort (n = 484) from the Singapore Chinese Health Study. SNPs were selected using a literature review, HapMap, and SNPPer. Multiple linear regression was used to evaluate the association between the SNPs and plasma folate and homocysteine levels. **Results:** Two FPGS (rs10106, rs1098774) and 9 GGH (rs1031552, rs11545076, rs1800909, rs3758149, rs3780126, rs3824333, rs4617146, rs11545078, rs719235) SNPs were included in the final analysis. None of the FPGS or GGH SNPs were associated with plasma folate levels. Similarly, none of the FPGS SNPs were associated with plasma homocysteine, but three GGH SNPs were associated with plasma homocysteine levels: rs11545076 ($p = 0.02$), rs1800909 ($p = 0.03$), and rs3758149 ($p = 0.04$). In all three, the homozygous variant alleles were associated with lower plasma homocysteine values. **Conclusions:** It appears that genetic variation in GGH may be influential in determining circulating homocysteine levels, and thus, may influence intracellular folate homeostasis rather than circulating folate levels. Since this is one of the first studies to assess these genetic variants in relation to plasma folate and homocysteine, further research is needed to explore these variants and to better characterize the role of genetic variation in intracellular folate homeostasis.

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Introduction

Folates are important for one-carbon metabolism, where they act as one-carbon donors and acceptors. The widespread importance of one-carbon metabolism has made folate a vitamin of interest in the development of many chronic diseases. The relationship between folate and homocysteine is a primary research interest given that homocysteine is a risk determinant of cardiovascular disease. The role of folate in DNA synthesis and repair has also made it a nutrient of interest in cancer research. The level of folate available to tissues influences how well the body can synthesize and repair DNA as folate is required for the production of methionine, purine and thymidine. Most folate research has focused on polymorphisms in other genes in the one-carbon metabolism pathway, such as 5, 10-methlylene-tetrahydrofolate reductase (MTHFR), dihydrofolate reductase (DHFR), and thymidylate synthase (TS). Very few studies have been conducted on the effect of other genes that influence the availability of folate in the cell.

While many of the enzymes in the folate metabolism pathway have relevance for disease risk, it is also important to examine the enzymes that determine the availability of the substrates for the enzymes in these key pathways. This study focuses on the impact of polymorphisms in two key enzymes for intracellular folate homeostasis, folylpolyglutamate synthase (FPGS) and gamma glutamyl hydrolase (GGH). Intracellular folate homeostasis is regulated by the addition or removal of glutamate residues to the γ-carboxylate group of folate (or folate derivatives) (1, 2). Many folate enzymes in the one-carbon metabolism pathway have a higher affinity for folate polyglutamates than for monoglutamtes, thus the conversion between polyglutamate and monoglutamate forms can dictate the availability of substrates for key one-carbon

metabolism enzymes (1, 2). Polymorphisms in these genes, thus, have the potential to influence the availability of folate to act as substrates for these key pathways.

To briefly overview the folate pathway, folate is obtained from the diet (folate and folic acid) and supplements (folic acid). Folic acid, the form used in supplementation and fortification, is readily absorbed, but food folates are generally reduced and polyglutamated (1, 2). In order to be absorbed, these must be cleaved to the monoglutamated forms of folate by folate hydrolase (FOLH1) in the jejunal brush border (1, 2). The reduced folate monoglutamate is taken into the intestinal mucosa via the proton coupled folate transporter (PCFT), where it is converted to the main circulating form, 5-methyl tetrahydrofolate (5-CH_3H_4PteGlu) (2, 3). The 5-methyltetrahydrofolate form is taken into cells either by the reduced folate carrier (RFC1) or the folate receptor (FOLR1) depending on the tissue. Once in the cell, the methylated, fully reduced monoglutamate form must be demethylated by methionine synthase and polyglutamted by FPGS (1, 2). The conversion to the polyglutamated form by FPGS is essential for folate to be retained in the cell. Polyglutamated folates are the preferred substrate for entrance into the methionine, purine and thymidine cycles (1, 2).

Most studies on FPGS are centered on methotrexate treatment effectiveness. To date there is only one study that has examined SNPs in FPGS in relation to the potential impact on circulating folate and homocysteine levels. DeVos *et al* examined the association between the FPGS polymorphism rs10106 in relation to plasma folate, homocysteine and DNA uracil concentrations, but did not find any significant associations between the SNP and folate or homocysteine levels (4). An *in vitro* study

by Leil *et al* identified several SNPs and found several that SNPS that led to changes in the half maximal effective dose (EC_{50}) for folate; however, most of the SNPs in the study had little variation or no variation in certain populations (5).

A second important enzyme that determines intracellular folate levels is gamma-glutamyl hydrolase. Folate is converted back to the monoglutamate from by GGH and is then able to leave the cell. Like FPGS, many studies that examine GGH are focused on anti-folate drug treatments. DeVos et al found that GGH -124T>G (rs11545076) homozygous variant allele (GG) was associated with increased DNA uracil content, but was not associated with plasma folate or homocysteine levels. They found no significant differences for the other GGH SNP examined (rs3758149) (4). An *in vitro* study by Chave *et al* found that rs11545076 and rs3758149 increases promoter activity (6). Kawakami *et al* found that low expression of GGH was associated with CpG island methylator phenotype (CIMP+) colorectal cancer, which suggests that a polymorphism in GGH which affects the activity of the enzymes could have important implications (7).

The purpose of this study was to characterize genetic variation in FPGS and GGH in the Singapore Chinese, and to determine if SNPs in either enzyme have any association with variations in plasma folate or homocysteine. A study examining molecular influences on folate levels after leucovorin treatment for colorectal cancer, found that high FPGS activity and low GGH activity were indicators of folate levels after treatment (8). This suggests that genetic variations in these genes may potentially influence the levels of intracellular folate. The core hypotheses for this study were that variant alleles that are thought to cause higher FPGS activity would lead to lower

circulating folate and decreased homocysteine levels. Conversely, variant alleles that result in decreased FPGS expression would result in increased plasma folate and homocysteine. The inverse action of GGH led to the hypothesis that variant alleles that increase GGH activity would lead to higher plasma folate and homocysteine levels while those that decrease GGH activity will result in lower circulating folate and reduced homocysteine production.

Methods

Study Population

This study was conducted among participants in the Singapore Chinese Health Study, a population-based prospective cohort focused on investigating diet and cancer. The complete cohort contains 63,257 Chinese men and women who were 45-74yrs at enrollment and were living in government housing estates. The Singapore Chinese Health Study was approved by the Institutional Review Boards of the University of Southern California, the National University of Singapore and the University of Minnesota.

Data Collection

At the time of enrollment (April 1993 – December 1998), a face-to-face interview was conducted in the participants' homes. In this interview, demographic, lifestyle and dietary data were collected using a structured questionnaire. For the dietary data, participants were asked about usual intake and portion sizes for 165 items in the previous year using a food frequency questionnaire (FFQ) developed for this population (9). In

April 1994, blood and single-void urine specimens were collected from a random 3% sample of study participants (n=509). A 20-mL non-fasting blood sample was collected from each of the 509 participants (221 men, 288 women). One 10-mL plain tube was used for the preparation of serum and one tube with heparin was used for plasma. The tubes were stored on ice until processing and all specimens were held at room temperature for 2 hours prior to separation into plasma, serum, red blood cells and buffy coat. All components were then store at -80°C until analyzed.

Plasma homocysteine was measured using HPLC with electrochemical detection in the laboratory of the Department of Community, Occupational, and Family Medicine at the National University of Singapore. For details of the sample preparation procedure see the report by Hughes and Ong. Plasma folate concentrations were analyzed with the Quantaphase II folate radioimmunoassay kit (Bio-Rad Laboratories Inc, Hercules, CA).

Participants who did not have a plasma folate value (n=15) or had a plasma folate value of >80nmol/L (n=2), participants missing a plasma homocysteine value (n = 6) and participants with energy intakes >5000kcal (n = 1) and <500kcal (n = 1) were excluded. A total of 484 participants were included in the final analysis (215 men, 269 women).

Identification of SNPs

PubMed was used to identify studies in which SNPs in either the FPGS enzyme and/or GGH enzyme that had previously been reported in the literature. Only one article previously assessed this relationship and the SNPs analyzed in the DeVos, et al study were included in this study. These SNPs were included to allow comparisons to previous research findings. Hap Map (http://hapmap.ncbi.nlm.nih.gov/) was used to identify

haplotype tagging SNPs to include in the analysis. (9/13/2009, CHB populations, algorithm-Tagger-PairwiseT, Hap Map Data Rel 27 Phase II + III, Feb09, on NCBI B36 assembly, dbSNP b126). Additionally, dbSNP (entrez SNP: http://www.ncbi.nlm.nih.gov/sites/entrez?db=snp) was used to obtain further information on SNPs and to ascertain if there were other SNPs that may impact enzymes levels due to nonsynonymous changes resulting in potential changes to the protein structure. Finally, SNPPer (http://snpper.chip.org/) was used to identify any additional information or SNPs not detected by the previous two methods, and assess whether other SNPs had relevant minor allele frequencies in the Chinese population. This information was available for a very limited number of variants given the lack of studies on these enzymes. SNPs that did not appear to be present in the Han Chinese population according to population frequency data available on SNPPer and dbSNP were excluded.

A total of 11 FPGS (rs10106, rs41306702, rs10760502, rs11554717, rs17855900, rs10118903, rs34330923, rs2230270, rs35789560, rs34354111, rs10987742) and 21 GGH (rs11545076, rs3758149, rs1800909, rs11545078, rs1031552, rs719235, rs71898601, rs15073, rs11786893, rs11545077, rs3824333, rs12681874, rs13268472, rs13248452, rs3780130, rs13270305, rs12677953, rs10957267, rs16930092, rs3780126, rs4617146) SNPs met the inclusion criteria. Two of the SNPs were not able to be genotyped (rs3780130, rs71898601). For one no adequate primer was found (rs3780130) and the other was a deletion mutation (rs71808601), which was not possible to genotype on the Sequenom platform. DNA was extracted from buffy coats using a Qiage Kit (Qiagen Inc., Valencia, CA). Genotype determinations were performed in multiplex

using the Sequenom MALDI-TOF mass spectrometry system (Sequenom Inc., San Diego, CA). Successful genotype results were obtained for 26 SNPs (11 FPGS, 15 GGH); we were unable to resolve >5% of genotype calls for rs11545077, rs12681874, rs13270305, rs12677953 (all GGH). Quality control repeats for 10% of the genotype determinations showed 100% concordance. Several of the genotyped SNPs had a minor allele frequency (MAF) <0.02 in this population and were excluded from further analysis (FPGS: rs10118903, rs11554717, rs17855900, rs2230270, rs34330923, rs34354111, rs35789560, rs41306702, rs10760502 and GGH: rs11786893, rs15073). Additionally, several of the GGH SNPs were proxies for a tag SNP. If the tag SNP did not properly genotype the closest proxy (r^2) was chosen and all others were excluded from further analysis (GGH: rs10957262, rs13248452, rs13268472, rs16930092). Genotype frequencies for all the SNPs included in this analysis were found to be in Hardy-Weinberg Equilibrium ($p>0.001$) (Table 2). Table 1 outlines the locations and known changes for the SNPs included in this analysis.

Statistical Analysis

All statistical analyses were performed using SAS version 9.2 (SAS Institute Inc, Cary, NC). An initial univariate analysis was performed for all the variables. Based on this analysis, log-transformed plasma folate and homocysteine values and energy-adjusted dietary folate, vitamin B12 and Vitamin B6 values were used in all subsequent analyses. Geometric means and 95% CIs are presented on the tables. The univariate analysis was followed with a single variable comparison analysis to establish any associations between variables. Pearson correlations were used to assess the relationship

between two continuous variables, tabular methods were used for comparing two categorical variables and ANOVA was used for comparisons between a continuous and categorical variable. Multiple linear regression models were used to examine the relationship between genotype (explanatory variable) and plasma folate and homocysteine. Variables were included in the full adjusted models if they were significantly associated with the outcome in the comparison analysis and/or they were known to be potentially relevant, such as age. The final plasma folate multivariate models were included age, gender, smoking status, folate intake (nutrient density-energy adjusted), general supplement use (yes/no), coffee intake, and tea intake by type (<monthly, black, green, or both). Plasma homocysteine models were adjusted for age, gender, smoking status, plasma folate (log-transformed), B_{12} intake (energy-adjusted), B_6 intake (energy-adjusted), and green tea intake.

To assess whether the relationship between genotype and plasma folate or homocysteine differed by level of folate intake, a stratified analysis was performed. Folate intake was categorized into quartiles based on the population percentiles ($<25^{th}$, 25^{th}-50^{th}, 50^{th}-75^{th} and $>75^{th}$). The cut points for these categories (from lowest to highest intake) were (1) ≤78µg/day, (2)>78 to ≤97µg/day, (3)>97 to ≤119µg/day, and (4) >119µg/day.

Results

Singapore does not require folate fortification of grain products in contrast to the US. Thus, their dietary folate consists of that from whole foods and contains little, if any folic acid. Folate intake in this population was very low in comparison the US RDA (400µg) and still low compared to Singapore's recommended dietary allowances

(200µg). The mean intake was 101.96 µg/day with a range from 33.0 – 267.7 µg/day. Tea intake was relatively high with 48.6% of the population consuming some type of tea at least weekly. Unlike many other parts of Asia, this population drank similar amounts of black (weekly: 18.4%) and green tea (weekly: 18.6%). There were low rates of smoking and low alcohol intake and rates differed significantly between men and women. Men were more likely to smoke with 44.2% of men being never smokers compared to 95.5% of women. Alcohol consumption is very low among the women in this cohort (92.7% never consume alcohol) and regular consumption is also uncommon in men (69% never consume alcohol). Further differences between men and women included that men were slightly older and women had a higher folate intake, were less likely to consume tea, and had lower levels of education. Additionally, women had a higher mean plasma folate value and a lower mean homocysteine level (Table 2)

Plasma folate ($r = 0.18$, $p < 0.001$) and homocysteine ($r = -0.17$, $p<0.001$) were significantly correlated with folate intake. Plasma folate and homocysteine were negatively correlated with each other ($r = -0.47$, $p<0.001$), and homocysteine was correlated with age ($r = 0.31$, $p<0.001$). Homocysteine was significantly inversely correlated with B_6 intake ($r = -0.10$, $p = 0.03$), but not with B_{12} intake ($r = -0.06$, $p = 0.20$). Smoking was negatively associated with plasma folate levels ($p<0.001$) and positively associated with homocysteine levels ($p<0.001$). Coffee consumption was associated with plasma folate levels ($p = 0.002$) with those with the highest coffee intake having lowering folate levels, but coffee consumption was not associated with folate intake ($p = 0.47$). Alcohol intake was not associated with plasma folate ($p = 0.47$) or

homocysteine (p =0.78), however, alcohol intake was generally low in this population with only 15 participants consuming alcohol daily and 82% being non-drinkers. While supplement intake was generally low it was associated with plasma folate levels (p = 0.01). Green tea intake was associated with homocysteine levels (p = 0.03), but there was no clear linear pattern with increasing intake.

Table 3 summarizes the findings of the genetic analyses. Two of the GGH SNPs (rs11545078 and rs719235) had small numbers of participants who were homozygous for the variant allele, and thus, results for these SNPs should be interpreted with caution. Neither of the two FPGS SNPs were associated with plasma folate or homocysteine levels (Table 4). Those homozygous for the variant rs10106 allele appear to have slightly higher plasma folate values, but the association was not statistically significant (p =0.16).

None of the SNPs for GGH were significantly associated with plasma folate levels (Table 5); however, several were significantly associated with plasma homocysteine levels. Two SNPs (rs11545076 and rs3758149) were significantly associated with homocysteine levels and one was borderline significantly associated with homocysteine levels (rs1800909). One SNP (rs11545078) was borderline significant, but had low numbers in the homozygous variant group and the heterozygous genotype in this SNP was associated with the lowest homocysteine level while the mean homocysteine levels for the homozygous variant and wild-type alleles were relatively similar (Table 5). For all three SNPs significantly associated with plasma homocysteine, values the homozygous wild-type have the highest values with similar values for heterozygous and homozygous variant.

An additional analysis was performed to assess whether any of the genetic variations had any differential effect with higher verses lower folate intakes. Studies have found that the effects of some mutations in the MTHFR gene differ depending on the level of folate intake, and thus, it may be important to assess this potential interaction (10). However, we did not have sufficient numbers for stratification. When stratified by dietary folate, the sample size became relatively small for most of the SNPs, leading to wider confidence intervals of plasma folate and homocysteine and inconsistent results (data not presented).

Discussion

To our knowledge this is only the second study to assess SNPs in the folate pathway enzymes, FPGS and GGH, in relation to plasma folate and homocysteine levels. In this study the two FPGS SNPs analyzed in this study do not appear to influence plasma folate or homocysteine levels. For rs10106 this is consistent with finding from DeVos *et al*. This study did not find rs10106 to be associated with plasma folate, homocysteine or DNA uracil content (4). These two studies represent different populations with different dietary patterns, so further study is warranted. To our knowledge no other studies have evaluated the effect of rs10987742 on plasma folate or homocysteine levels.

GGH genetic variants were not associated with plasma folate levels in this study; however, three SNPs did appear to alter plasma homocysteine levels (rs11545076, rs1800909, rs3758149). Only rs11545706 has been assessed for potential influence on plasma homocysteine in a previous study. DeVos *et al* did not find rs11545076 to be associated with homocysteine, but they did find it was associated with increased DNA uracil (4). An *in vitro* study found that the variant alleles in rs11545076 and rs3758149

increase promoter activity, suggesting that both have the potential to influence enzyme levels, and ultimately, intracellular folate homeostasis (6). The third SNP, rs1800909, is located in an exon and results in a missense mutation (11). Thus, it seems plausible that individuals homozygous for the variant rs1800909 allele (CC) could have altered GGH expression, however, bioinformatics prediction suggests that this is a benign mutation (PolyPhen: available at: http://genetics.bwh.harvard.edu/pph/data/index.html, accessed 4/19/10). In this study, individuals homozygous for the variant allele actually had lower levels of plasma homocysteine than those homozygous for the wild-type or heterozygous. Based on findings from the cell culture study by Chave *et al*, it appears that the variant alleles for rs11545076 and rs3758149 increase GGH expression, and higher GGH expression may result in lower homocysteine levels (6).

There is some evidence from drug studies to suggest that alterations in FPGS and GGH function may alter cellular retention of folate. A study by Sadahiro *et al* that suggests variation in FPGS and GGH can influence folate levels after leucovorin treatment for colorectal cancer (8). Additionally, studies examining FPGS and GGH activity in relation to methotrexate (MTX), which is chemically similar to folate and taken into cells by the same mechanism as folate, suggest that decreased FPGS activity and increased GGH activity are associated with MTX resistance by limiting the amount of MTX retained in the cells (12, 13). These studies provide some evidence that SNPs in FPGS and GGH that alter enzyme activity could influence intracellular folate homeostasis.

Several studies with disease outcomes have evaluated SNPs in the FPGS and GGH genes. Two previous studies examined a FPGS (rs10106) SNP in relation to Non-Hodgkin's Lymphoma risk (14, 15). One case-control study by Lim *et al*, using the SEER data, found that those homozygous for the variant allele (CC) may have an increased risk (OR= 1.58, 95% CI = 1.00, 2.49) (14). However, the second study by Lee *et al* in Australia did not find any association (OR = 0.95, 95% CI = 0.69, 1.39) (15). A study by Figueiredo *et al* examined several GGH SNPs that were included in this study (rs11545078, rs3758149, rs3789126, rs4617146) and no statistically significant associations with risk of colorectal cancer or microsatellite instability (16).

Previous studies in the Singapore Chinese Health cohort have examined two of the enzymes in the folate metabolism pathway (17, 18). Saw *et al* examined the association between MTHFR polymorphism C677T and homocysteine concentrations (17). They found that subjects homozygous for the variant allele (TT), had the highest concentrations of homocysteine (30% increase, $p<0.0001$) when folate intake was low, but when folate intake was adequate MTHFR genotype did not appear to influence homocysteine levels. The interaction between MTHFR genotype and plasma folate levels on homocysteine concentration was significant ($p=0.02$). The second study by Trinh *et al* explored a thymidylate synthase (TS) polymorphism as a potential genetic determinant of homocysteine levels (18). The polymorphism was a tandem repeat in the 5' untranslated region where the number of repeats appear to affect TS activity. They found that TS 3/3 (homozygous triple repeat) was associated with a reduction in plasma folate and increased homocysteine levels.

There were some limitations in this study. This study only evaluates genetic variation in FPGS and GGH, but there are many other enzymes and transport proteins that could influence the levels of plasma folate and homocysteine. It is possible that these other variables may be more influential or that additional genetic variants in other pathways interact with SNPs in these enzymes. This study evaluated circulating plasma folate and not intracellular folate concentrations, where FPGS and GGH have greater biological relevance. Erythrocyte folate concentration data was not available, but would likely be more indicative of relative FPGS and GGH activity. It is possible, especially for FPGS, that plasma folate is not the best indicator of enzyme activity. FPGS is a limiting enzyme that is found in relatively small amounts in cells, and requires methionine synthase to remove methyl groups before polyglutamation (2). Thus, it is possible that the rate of this enzyme has minimal effect on plasma folate levels in comparison to other variables such diet and folate absorption. Additionally, erythrocyte folate provides an actual picture of intracellular folate which would be more reflective of how these SNPs influence intracellular levels. This measurement would also allow for a more complete picture of long-term status then a single measurement of plasma folate.

Both dietary intake data and the biospecimen were collected as single, cross-sectional measurements in the Singapore Chinese Health Study. Having only a one-time biochemical measurement does not account for variations in diet and plasma levels over time. FFQs are often criticized for not gathering detailed enough information to accurately analyze specific nutrients (19, 20). In this study, we are assessing the influence of only a few variables in the pathway that contribute to folate markers and homocysteine

levels and it is likely that each variable may only contribute a small change in the overall picture. It is possible that lack of specificity about certain variables could diminish the ability to detect smaller changes (20).

Additionally, this study did not have sufficient numbers of participants to assess whether a certain genotype was more influential under higher or lower folate intake levels. Previous, studies on MTHFR C677T have shown that the variant T-allele is detrimental when folate intake is low, but may be protective against colon cancer with sufficient folate intake (10). Thus, it may be important to take into consideration how genotype might interact with of a person's dietary patterns. Another limitation is that this study involves multiple comparisons so it is possible that significant results occurred by chance. Finally, very few studies have explored how the FPGS and GGH genetic variants influence enzymatic transcription and activity, and only one other study has assessed the potential impact of FPGS and GGH SNPs on plasma folate and homocysteine. This limits the conclusions that can be drawn from the results of this study and illuminates some areas for future research.

This study also had several strengths. This was only the second study to assess the potential impact of SNPs in FPGS and GGH on plasma folate and homocysteine. The role these two enzymes have in intracellular folate homeostasis make them important for the availability of substrates for one-carbon metabolism pathways, thus contributing to our understanding of genetic differences in these genes is valuable. This study was conducted in a different population than the DeVos *et al* study and there is limited genetic information on the Singapore Chinese, so this study contributes to our knowledge

of genotypes in the folate pathway in this population. Unlike DeVos *et al*, instead of looking at many genes, we assessed multiple SNPs within only two genes, which allowed us to assess the potential influence multiple SNPs for each gene.

More research is needed to explore how these SNPs change the gene expression and enzyme activity. This will also help clarify which variants should be explored for potential impact on health outcomes. Future studies should aim to explore the impact of these genetic variants on long term status by using erythrocyte folate or multiple folate measurements, since it is likely long term status that influences disease risk. It has been reported that expression of GGH is tissue specific (21), so when examining GGH and FPGS polymorphisms it may be important to take this into consideration. Additionally, further research should include haplotype analysis to explore whether there are certain genotype patterns that are more influential compared to a single mutation.

Conclusions

It appears that GGH expression levels or activity may be influenced by several SNPs (rs11545076, rs1800909, rs3758149) and that this altered expression may affect impact on plasma homocysteine levels. Further studies are needed to explore these SNPs to better characterize these enzymes, their potential to influence intracellular folate homeostasis, and ultimately, their potential influence on disease risk.

Tables and Figures

Table 1-FPGS and GGH SNP Potential Functional Relevance

Ref SNP #	Location	Change	Findings
Folylpolyglutamate Synthase (9q.34.1)			
rs10106	3'UTR	---	-No effect on uracil content (3) -No effect on plasma folate or homocysteine (3)
rs10987742	Intron 1	---	-No Studies
Gamma Glutamyl Hydrolase (8q.12.23)			
rs1031552	Intron	---	-No studies
rs11545076	5'UTR	---	-Increased promoter activity (6) -Increased DNA uracil content (3) -No effect on plasma folate and homocysteine(3)
rs1800909	Exon 1	Nonsynonymous, Missense->Arg6Cys	-Only assessed in drug studies -Predicted to be a benign change (PolyPhen)
rs3758149	5'-Near Gene	---	-Increased promoter activity (6) -No effect on DNA uracil content (3) -No effect plasma folate and homocysteine (3)
rs3780126	Intron 1	---	-No studies
rs3824333	Intron 2	---	-No studies
rs4617146	Intron 2	---	-No studies
rs11545078	Exon 3	Nonsynonymous, Missense->Ile151Thr	-Only assessed in drug studies -Predicted to be possibly damaging change (PolyPhen)
rs719235	5'UTR	---	-No studies

Table 2-Cohort Demographics			
	MEN (N = 215)	**WOMEN (N = 269)**	**P -value**
Age (yrs)	56.4 ± 7.6	54.9 ± 7.9	**0.03**
BMI (kg/m^2)	22.8 ± 3.0	23.0 ± 3.1	0.41
Caloric Intake (kcal/day)	1699.0 ± 488.6	1401.5 ± 465.9	**<0.01**
Supplement Use (%)	5.6	7.4	0.41
Smoker (%)	---	---	**<0.01**
Never	44.2	95.5	---
Past	25.1	0.8	---
Current	30.7	3.7	---
Alcohol Intake (%)	---	---	**<0.01**
Never	69.0	92.7	---
Monthly	12.0	3.7	---
Weekly	14.0	2.6	---
Daily	5.0	1.0	---
Folate Intake (µg/day)[1]	97.8 ± 27.9	105.3 ± 34.1	**<0.01**
Vitamin B_{12} Intake (µg/day)[1]	1.5 ± 0.5	1.5 ± 0.56	0.89
Vitamin B_6 Intake (mg/day)[1]	0.7 ± 0.2	0.7 ± 0.2	0.68
Coffee Intake (%)	---	---	**0.02**
Never	16.3	17.5	---
Monthly/Weekly	15.8	11.9	---
1 cup/day	27.9	42.4	---
2-3 cups/day	36.3	26.0	---
4+ cups/day	3.7	2.2	---
Tea Intake (%)	---	---	**<0.01**
None/Monthly	31.6	45.7	---
Black Tea	10.2	13.4	---
Green Tea	28.0	23.8	---
Both	30.2	17.1	---
Education (%)	---	----	**<0.01**
No Formal Education	12.6	37.5	---
Primary School	47.4	36.8	---
Secondary School	27.4	21.2	---
A Level/Vocational	8.4	4.5	---
University	4.2	0	---
Plasma Folate (nmol/L)[2]	12.1	15.1	**<0.01**
Plasma Homocysteine (nmol/L)[2]	11.3	9.0	**<0.01**

[1]Energy adjusted intake.
[2] Geometric Mean.

Table 3-Genotype and Allele Distributions

SNP	Alleles	Genotype Frequencies[1] Wild Type	Heterozygous	Variant	MAF	HWE[2]	Selection Reason
Folylpolyglutamate Synthase (FPGS)							
rs10106	G/A	229	195	40	0.30	Yes	Tag SNP[3]
rs10987742	C/T	257	186	36	0.27	Yes	Tag SNP[3]
Gamma Glutamyl Hydrolase (GGH)							
rs1031552	C/T	202	221	54	0.34	Yes	Tag SNP[4]
rs11545076	T/G	303	149	21	0.20	Yes	Literature
rs1800909	T/C	306	142	21	0.20	Yes	Literature
rs3758149	C/T	299	152	23	0.21	Yes	Literature
rs3780126	C/T	190	230	57	0.36	Yes	Tag SNP[3]
rs3824333	A/G	204	219	54	0.34	Yes	SNPPer[5]
rs4617146	C/T	172	225	81	0.40	Yes	Tag SNP[3]
rs11545078	C/T	381	88	7	0.11	Yes	Literature
rs719235	G/T	387	85	7	0.10	Yes	SNPPer[5]

[1]The most common allele was designated as wild-type.
[2]HWE was defined as P>0.001
[3]HapMap Data Phase III/Rel#2, FEb09, on NCBI B36 assembly, dpSNP b126, 2/3/10, CHB
[4]Tag SNP did not genotype, Proxy to Tag SNP, $r^2 = 1.0$.
[5]SNPs ascertained from SNPPer 9/11/09.
[6]Tag SNP did not genotype, Proxy to Tag SNP, $r^2 = 0.975$.

Table 4-FPGS: Mean Plasma Folate and Homocysteine by Genotype

	Plasma Folate (nmol/L)[1]		Plasma Homocysteine (nmol/L)[2]	
SNP	Mean (95% CI)[3]	p-value	Mean (95% CI)[3]	p-value
rs10106		0.16		0.41
GG	13.7 (12.6, 14.9)		10.2 (9.3, 11.1)	
AG	13.4 (12.2, 14.7)		10.0 (9.6, 10.5)	
AA	15.4 (13.3, 17.9)		9.7 (9.3, 10.2)	
rs10987742		0.50		0.32
CC	13.5 (12.5, 14.7)		9.7 (9.3, 10.2)	
CT	13.4 (12.3, 14.7)		10.1 (9.6, 10.6)	
TT	14.7 (12.6, 17.1)		10.0 (9.1, 10.9)	

[1]Linear Regression-Adjusted for Age, Sex, Folate Intake (energy-adjusted), Smoking, Tea Intake, Coffee Intake, Supplements.
[2]Linear Regression-Adjusted for Age, Sex, Plasma Folate (log-transformed), B12 Intake (energy-adjusted), B6 Intake (energy-adjusted), Smoking, Tea Intake.
[3]Geometric Mean.

Table 5-GGH: Mean Plasma Folate and Homocysteine by Genotype				
	Plasma Folate (nmol/L)[1]		Plasma Homocysteine (nmol/L)[2]	
SNP	Mean (95% CI)[3]	p-value	Mean (95% CI)[3]	p-value
rs1031552		0.93		0.15
CC	13.9 (12.7, 15.1)		9.6 (9.2, 10.1)	
CT	13.7 (12.6, 15.0)		10.0 (9.6, 10.5)	
TT	13.5 (11.8, 15.5)		10.2 (9.4, 10.5)	
rs11545076		1.00		**0.01**
TT	13.7 (12.6, 14.8)		10.2 (9.7, 10.6)	
GT	13.6 (12.4, 15.0)		9.4 (9.0, 9.9)	
GG	13.6 (11.3, 16.4)		9.3 (8.3, 10.4)	
rs1800909		0.58		**0.05**
TT	13.5 (12.4, 14.7)		10.1 (9.7, 10.6)	
CT	14.1 (12.7, 15.6)		9.5 (9.0, 10.0)	
CC	13.9 (11.5, 16.8)		9.6 (8.5, 10.7)	
rs3758149		0.99		**0.02**
CC	13.6 (12.4, 14.7)		10.1 (9.7, 10.5)	
CT	13.4 (12.2, 14.8)		9.5 (9.0, 10.0)	
TT	13.6 (11.3, 16.3)		9.3 (8.4, 10.4)	
rs3780126		0.40		0.62
CC	13.2 (12.1, 14.4)		9.9 (9.4, 10.4)	
CT	13.7 (12.6, 15.0)		9.8 (9.4, 10.2)	
TT	12.8 (11.3, 14.5)		10.2 (9.4, 10.9)	
rs3824333		0.81		0.18
AA	13.8 (12.6, 15.0)		9.6 (9.2, 10.1)	
AG	13.5 (12.4, 14,7)		10.1 (9.6, 10.5)	
GG	13.2 (11.6, 15.1)		10.1 (9.3, 10.9)	
rs4617146		0.47		0.08
CC	13.3 (12.1, 14.6)		9.9 (9.4, 10.4)	
CT	13.9 (12.8, 15.2)		10.1 (9.7, 10.6)	
TT	13.2 (11.8, 14.7)		9.4 (8.8, 10.0)	
rs11545078		0.77		0.08
CC	13.6 (12.6, 14.7)		10.0 (9.6, 10.4)	
CT	13.8 (12.3, 15.4)		9.4 (8.8, 10.0)	
TT	12.3 (9.0, 16.8)		10.3 (8.5, 12.5)	
rs719235		0.11		0.42
GG	13.5 (12.5, 14.6)		9.8 (9.5, 10.2)	
GT	14.7 (13.1, 16.4)		10.0 (9.4, 10.7)	
TT	11.1 (8.1, 15.3)		8.8 (7.2, 10.7)	

[1]Linear Regression-Adjusted for Age, Sex, Folate Intake (energy-adjusted), Smoking, Tea Intake, Coffee Intake, Supplements.

[2]Linear Regression-Adjusted for Age, Sex, Plasma Folate (log-transformed), B12 Intake (energy-adjusted), B6 Intake (energy adjusted), Smoking, Tea Intake.

[3]Geometric Mean.

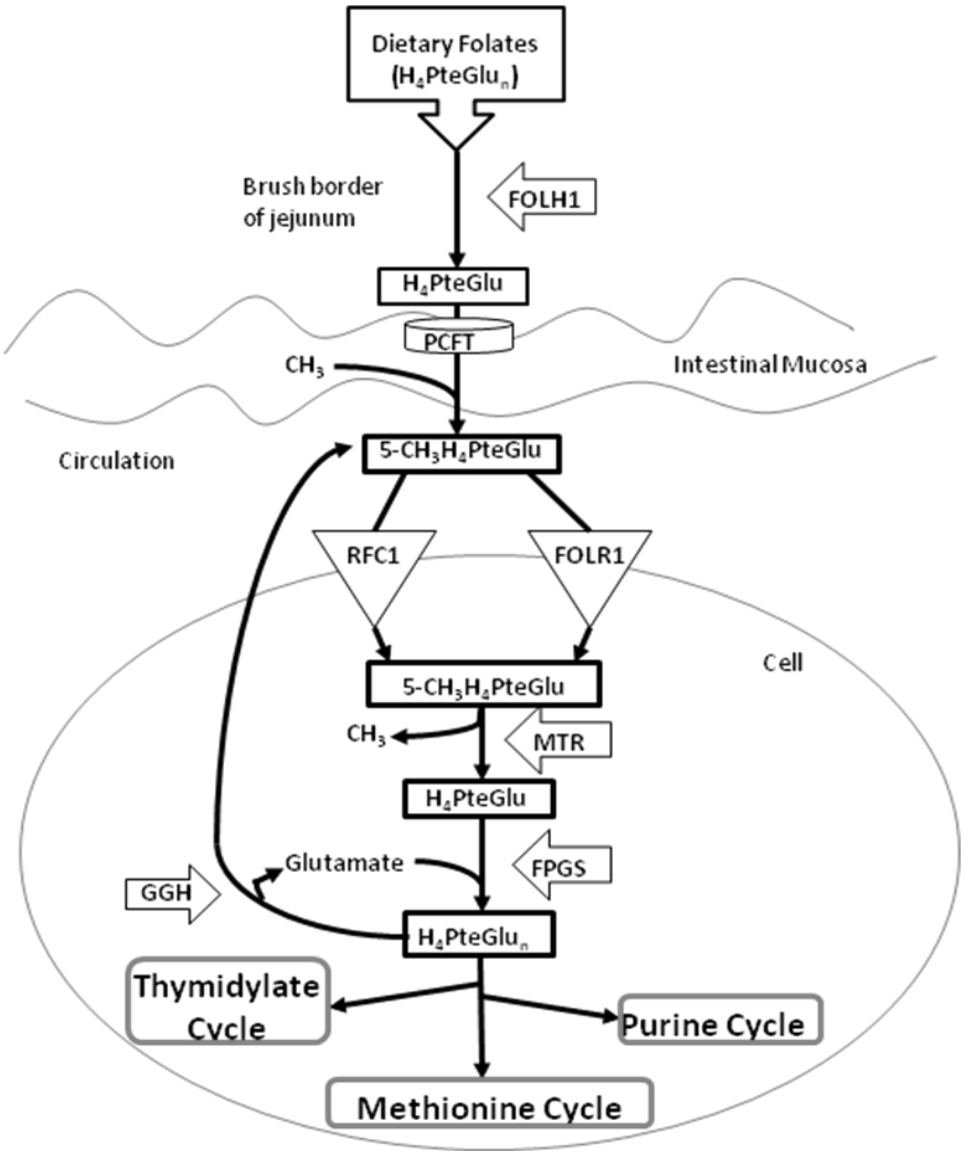

Figure 1- Folate Absorption, Cellar Uptake and Retention- Folate hydrolase 1 (FOLH1), proton-coupled folate transporter (PFCT), folate receptor 1 (FOLR1), reduced folate carrier 1 (RFC1), methionine synthase (MTR), Folylpolyglutamate synthase (FPGS), gamma glutamyl hydrolase (GGH).

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