Chapter 4

Nutritional Guidance in Sakado Folate Project

Notification of the C677T Genotype of Methylenetetrahydrofolate Reductase Increased both Serum Folate and the Intake of Green Vegetables

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Abstract

Background: Serum folate levels are lower in TT homozygotes of the single-nucleotide polymorphism (rs1801133) of methylenetetrahydrofolate reductase (MTHFR) than in CC homozygotes and CT heterozygotes.

Objective: To improve folate status, the genotype was notified to each subject to motivate them to eat more green-yellow vegetables.

Design: Genotype, dietary folate intake, and blood biochemistry were determined and statistically analyzed for 404 subjects (109 males, mean 58.9 years; 295 females, mean 61.8 years). Their serum folate and total homocysteine (tHcy) concentrations were measured before and after receiving nutritional guidance and genotype notification.

Results: The frequencies of the CC, CT, and TT MTHFR genotypes were 35.4, 49.7, and 14.8%, respectively. TT homozygote participants significantly increased their intake of green-yellow vegetables ($p < 0.01$) and of food-derived folate ($p < 0.05$) following nutritional guidance. The increase in serum folate ($p < 0.001$) and the decrease in tHcy ($p < 0.001$) in TT homozygotes following nutritional guidance were more than twice that of the CC homozygote and CT heterozygote participants. An increase in broccoli, spinach and Komatsuna intake was observed following nutritional guidance, irrespective of the season.

Conclusion: Genotype notification was effective in increasing the intake of green-yellow vegetables and in improving folate status in TT homozygote participants.

Keywords: folate, homocysteine, genome notification, polymorphism, vegetable

1. Introduction

Widespread commercial genotyping direct to consumers (DTC) is anticipated to be applied to personalized dietary recommendations, but it remains unclear if providing individuals with their personal genetic information changes dietary behavior. The authors of this current chapter have tried for 11 years to increase the intake of green-yellow vegetables by the general Japanese population by providing personalized nutritional guidance with genotype notification to improve folate intake. The international standard or recommended dietary allowance (RDA) of folic acid is 400 μg/day to prevent diseases such as spina bifida, stroke, and dementia. The single-nucleotide polymorphism C677T (rs1801133) of methylenetetrahydrofolate reductase (MTHFR) has the effect that TT homozygotes require a higher folate intake than do CT heterozygotes and CC homozygotes. For this reason, Sakado City and Kagawa Nutrition University, Department of Medical Chemistry, cooperated to implement the "Sakado Folate Project" by genotyping the MTHFR polymorphism of subjects and notified participants of their genotype and urged participants to increase their green-yellow vegetable intake to prevent stroke, dementia, and to reduce medical costs [1]. Here we show that genotype notification of the risky TT homozygote is effective.

1.1. RDA of folate in Japan and by the WHO

The RDA of folic acid for adults is only 240 μg/day in Japan [2], but the World Health Organization (WHO) and the United Nations Food and Agriculture Organization (FAO) recommend a folic acid intake of 400 μg/day worldwide [3, 4]. Folic acid is a common name for the compound pteroyl monoglutamate. On the other hand, "folate" is an umbrella term that refers to many compounds derived from pteroyl monoglutamate that are not chemically well-characterized, including other derivatives of pteroylglutamates. Folates are differentiated by the reduced state of the pteridine ring, one carbon substitution at the N5 and/or N10 positions (formyl, methyl, methylene, and methenyl), and the length of the γ-polyglutamyl residues [3, 4]. Polyglutamyl 5-methyltetrahydrofolate species are the most abundant naturally occurring folates in vegetables [3, 4]. The bioavailability of synthetic folic acid (monoglutamyl pteridine) is approximately 70% better than that of dietary sources of folate (mainly polyglutamyl pteridine), and therefore, dietary folate equivalent (DFE) is generally used [3, 4]. Thus, the nutritional value of any chemical form of "folate" is expressed as folic acid in the Japanese RDA [2]. Dietary reference intake (DRI) for the Japanese population is based on the estimated average requirement (EAR), defined as satisfying the required amount of a nutrient for 50% of the population [2]. Based on the distribution of the required amount measured in a target group, RDA is defined as the amount satisfying the dietary requirements of most people (97–98%) in a specific population. Thus, RDA is calculated using EAR (EAR +2 × standard deviations of EAR) [2]. Most folate in food is present as pteroyl polyglutamate substituted with a one carbon unit, and it is tightly bound to MTHFR as coenzyme. The Japanese RDA of folate is insufficient for persons with polymorphisms in genes involved in folate metabolism and in elderly persons with decreased folate bioavailability [1].

The average folate intake in Japan in 2016 was 277 μg/day, which is higher than the Japanese RDA of 240 μg/day for adults. However, the folate intake of women aged 20–29 years was only 236 μg/day in 2016 [5], which is much less than the RDA of 480 μg/day for pregnant women [2]. One reason for this folate deficiency is the low intake of green-yellow vegetables. The daily Japanese average intake of vegetables and green-yellow vegetables was 265.9 and 84.5 g [5], considerably less than the 350 g (76%) and 120 g (70%), respectively, recommended in the report entitled "Ministry of Health, Labor and Welfare Healthy People 21 Japan" [6]. Folate intake higher than these recommended levels by Japanese was quite effective in preventing cardiovascular diseases, as established by the Japan Collaborative Cohort Study on a total of 23,119 men and 35,611 women [7]. Increased dietary folate intake, from <272 to >536 μg/day, resulted in a 51% reduction in mortality for men from heart failure ($P < 0.01$) [7]. Therefore, a folate intake of 243–253 μg/day by the general Japanese population is insufficient to prevent cardiovascular diseases, and thus higher folate intake will help reduce medical costs [1].

1.2. Folate metabolism and homocysteine

In the United States, folate in food is expressed as pteroyl monoglutamate, which is 1.7 fold more effective than pteroyl polyglutamate in food [4] because most coenzyme-type folate (polyglutamyl tetrahydrofolate and its C-1 derivatives) is liberated from MTHFR during the cooking and processing of foods and by protein digestion in the stomach. The liberated pteroyl polyglutamate is digested into pteroyl monoglutamate by the intestinal microvillous enzyme conjugase [8] and is absorbed from the epithelial cells of the upper part of the small intestine by reduced folate transporter [9]. Therefore, the absorption rate of pteroyl monoglutamate is estimated to be about 50% [2, 4]. On the other hand, synthetic folate contained in supplements is a monoglutamyl folate, and 90% is estimated to be absorbed [4]. These relative bioavailabilities of folate were confirmed using deuterium-labeled monoglutamyl tetrahydrofolates and folate in human subjects [10]. The most accurate dietary folate metabolism technique is the dual isotope method using [^{13}C5] folate and [$^{2}H_2$] folate [11]. The metabolism of [$^{13}C_5$] folic acid in fortified white and whole-wheat bread, rice, pasta, or in solution was evaluated in human subjects injected with [$^{2}H_2$] folate [11]. The results indicated no significant differences in bioavailability among the various fortified foods and the control ($p = 0.607$). However, there are personal differences in folate bioavailability partly caused by polymorphism of MTHFR that can lead to cardiovascular diseases [12].

Homocysteine (tHcy), a factor that causes vascular endothelial damage common to cardiovascular diseases, is an amino acid produced by the metabolism of the essential amino acid methionine [13]. Homocysteine produces reactive oxygen species that impair many cell components. Folate, vitamin B_{12} (VB_{12}) and vitamin B_6 (VB_6) are involved in the metabolism of tHcy and decrease the serum tHcy concentration [13]. Homocysteine metabolism occurs mainly by two pathways. One is a remethylation pathway that converts tHcy into methionine. Vitamin B_{12} acts as a coenzyme in methionine synthase (MS), which catalyzes the methylation of tHcy using 5-methyltetrahydrofolate as a methyl group donor. MTHFR is responsible for the production of 5-methyltetrahydrofolate from methylenetetrahydrofolate [13]. The other pathway for metabolizing tHcy is a sulfur transfer pathway that converts tHcy to cysteine via cystathionine by cystathionine β-synthase, which uses vitamin B_6 as a coenzyme. Therefore, a deficiency in folate, vitamin B_{12}, or vitamin B_6 increases tHcy in the blood [13].

1.3. Genetic polymorphisms related to folate metabolism

The C677T [Ala222Val] mutation (rs1801133) of MTHFR is the single-nucleotide polymorphism of a gene involved in folate metabolism that has the greatest effect on cardiovascular diseases [12, 14] and fetal development [15]. MTHFR (E.C.1.1.1.68) is the enzyme that reduces 5,10-methylenetetrahydrofolate to 5-methyltetrahydrofolate [4]. MTHFR TT homozygote individuals have high serum tHcy [16] and low serum folate levels [16] and have increased risk for cardiovascular diseases [14, 17]. MTHFR encoded by the TT genotype is a homozygote of a point mutation of C677T. This mutant protein is heat-sensitive because one alanine residue is mutated to valine. The enzyme activity of the CT type (heterozygote) is 65% that of the CC genotype (wild-type), and the activity of the TT type (homozygote) is 30% that of the wild type [18]. The mean residual enzyme activities after heat treatment (46°C, 5 min) were 37.0% (34.1–42.6%) in the controls and 15.2 and 15.1% in the two TT homozygotes [18]. The low activity and thermolability of the MTHFR TT homozygote results in decreased production of 5-methyltetrahydrofolate: the pathway from homocysteine to methionine is inhibited, and the tHcy level in the blood rises [18]. A healthy subject has tHcy levels of between 3 and 15 μmol/L, and a tHcy level of 15 μmol/L or higher is referred to as hyperhomocysteinemia [4]. Hyperhomocysteinemia promotes arteriosclerosis via vascular endothelial cell disorder and promotes blood coagulation and smooth muscle cell proliferation [17].

1.4. MTHFR C677T gene polymorphism and dietary intake of folate

The bioavailability of folate is low in TT homozygotes and CT heterozygotes [16]. Approximately 15% of the Japanese population is C677T MTHFR TT homozygotes [16]. TT homozygotes require 400 μg/day of folic acid to increase the serum folate level to that of CC homozygotes and CT heterozygotes [16]. Folate deficiency among persons with dementia was confirmed by meta-analysis of 31 studies [19] and in particular of studies of Japanese with dementia who are TT homozygotes [20]. The risk of brain infarction is 3.4-fold higher in TT homozygotes compared to that in CC homozygotes [21]. A randomized controlled double blind test was performed to confirm the exact folate requirement of Japanese [22]. There are ethnic differences in the prevalence of MTHFR polymorphism [23].

Although the RDA of folate in Japan is 240 μg/day [2], MTHFR TT homozygotes are folate deficient [16]. It is reported that serum folate levels are significantly lower in the TT type than the CC and CT types, and tHcy is significantly higher even if the intake of folate is 240 μg/day [2] as judged by the tHcy level not exceeding 14 μmol/L [2]. We have conducted intervention studies on folate intake by young Japanese women [1, 16, 22] and shown that an intake of 400 μg/day of folic acid causes differences in serum folate between genotypes of the MTHFR C677T gene polymorphism to disappear, even in the case of TT homozygotes [16].

1.5. The significance of vegetable intake

Compared with the intake of folate alone (e.g., as a supplement), the increase in the intake of vegetables targeted in this study is accompanied by an increase in the intake of various vitamins, minerals, and dietary fiber, thereby greatly improving nutrition overall. Reports from the WHO/FAO [24] and WCRF/AICR [25] summarized the relationship between vegetable intake and disease and showed that increased vegetable intake is effective against obesity, cardiovascular disease, type 2 diabetes, and several types of cancer (mouth, pharynx, larynx, esophagus, stomach). Increased fruit intake has been assessed as "probable decreasing risk" or more, and increased ingestion of vegetables and fruits has been proposed for maintaining and promoting health [24, 25].

1.6. Outline of the Sakado Folate Project

To prevent the above-described diseases caused by folate deficiency, and especially in MTHFR TT homozygotes, Sakado City made an agreement called the "Sakado Folate Project" in 2006 with Kagawa Nutrition University [1]. The project includes lectures, genotyping, blood analysis, nutrition surveys, genotype notification, and guidance on increasing the intake of green-yellow vegetables and folate, based on data from the subjects [1]. The lectures provide the subjects with an overview of available biomarkers (serum folate and homocysteine concentrations) and the interpretation of the significance of these biomarkers across a range of clinical and population-based uses. In the same lecture, we explain the genetic polymorphisms and obtain written informed consent in accordance with the instructions of the Declaration of Helsinki. The study procedures were approved by the Kagawa Nutrition University, Human Subjects and Genome Ethics Committee (approval number; no. 134, 300 G).

One month after taking blood samples from the participants, the genotype was announced by the medical doctor to the subjects who agreed to know their genotype. Furthermore, nutritional and exercise guidance was provided by the registered dietitian. To supply adequate folate easily, we developed "folate-fortified rice" containing (per 100 g rice) folate 26.7 mg, thiamin 187 mg, vitamin B_6 66.7 mg, and vitamin B_{12} 320 μg. This rice was developed in collaboration with House Wellness Foods Corporation Company. Specified amounts of this "folate-fortified rice" were mixed with typical rice and boiled before eating [1]. We also developed a folate-fortified bread called Sakado Folate Bread (folic acid 340 ± 21 μg/100 g; 215 ± 14.7 μg/slice/64.0 g bread) [1].

More important aspects of the project included the health education of 101,513 citizens through volunteers, and wider consumption of folate-fortified food, especially folate-fortified rice. According to the official report issued by Sakado City on the nutritional behavior of the participants, after the start of this project, 80% of the participants were aware of the importance of folic acid, 90% tried to eat more vegetables, and 73% wanted to obtain advice on improving their health. Moreover, both "Folate-fortified rice" and "Sakado Folate Bread" are commercially available, and after participating in this program, citizens can continue to improve their folate status by eating these staples fortified with folate rather than by increasing their intake of green-yellow vegetables [1].

Here, we report the effects of genotype notification on increased intake of green-yellow vegetables and the effect on increased serum folate and decreased serum homocysteine levels.

2. Methods

2.1. Subjects and survey method

The total number of participants was 1008 (mean age 62.82 years): 266 males (mean age 63.56 years) and 742 females (mean age 62.55 years). Of these, 396 subjects (104 males with a mean age of 59.87 and 292 females with a mean age of 61.77 years) who were assessed before and after enrolling in the program were selected by excluding 111 participants who had taken vitamin supplements prior to enrollment. From the 404 subjects, a detailed survey was conducted on 249 subjects (78 males; mean age 58.2 years and 171 females; mean age 60.4 years) regarding their green vegetable intake. The data were collected from the beginning of the project from 2006 to 2012.

As outcome measures, we obtained data such as serum folate and homocysteine levels and MTHFR genotype and also analyzed the results of a questionnaire that included food intake, and particularly green vegetable (folate rich vegetables, not green-yellow vegetables) consumption. Following the Sakado Folate Project, we collected the number of green vegetable dishes taken each week in the morning, afternoon, and evening in seven areas of Sakado City using a self-administered questionnaire of monthly intake of green vegetables.

2.2. Blood biochemistry

Venous blood samples were collected in plain and EDTA-containing Venoject tubes from the cubital vein of each participant before breakfast at the beginning and at the end of the Sakado Folate Project [1]. Whole blood was subjected to genomic DNA extraction as described in the next section. The serum was isolated and stored at −80°C until analysis. Serum folate and vitamin B_{12} concentrations were measured at an external laboratory (SRL, Inc., Tokyo, Japan) using a chemiluminescence enzyme immunoassay (Access 2, Beckman Coulter, Inc., CA, USA). Serum total homocysteine concentration was determined by enzyme assay using an Alfressa Auto Hcy kit (Alfressa Pharma, Inc., Osaka, Japan) [26]. In addition to serum folate and vitamin B_{12}, measurements, 28 general biochemistry/hematology parameters were analyzed by SRL Corporation; however, only serum folate and serum homocysteine levels were applicable to this study.

2.3. Genotyping

DNA was extracted from whole blood using a Magtration System (Precision Systems Science Co. Ltd., Chiba, Japan) and magnetic beads [27]. To rapidly and inexpensively genotype a large number of blood samples, we developed an automated genotyping machine using a bead array in a capillary tube [28]. This BIST method specifically genotypes the C677T single-nucleotide polymorphism (rs1801133) in MTHFR using beads in a straw tip [28]. If necessary, DNA was amplified by polymerase chain reaction and analyzed by electrophoresis in a 10% polyacrylamide gel.

2.4. Evaluation of the intake of nutrients/food groups by FFQ, DHQL, and BDHQ questionnaires

Three questionnaires were previously used to collect data regarding meals. These questionnaires were similar and evaluated vegetable and nutrient intake: FFQ (Food Frequency Questionnaire), DHQL (a larger version of a self-administered diet history method questionnaire), and BDHQ (a brief self-administered diet history questionnaire) [29, 30]. We evaluated the data in the three questionnaires in the same manner. The BDHQ and DHQL questionnaires were obtained from EBN Tokyo, Japan, and we requested automatic counting [29, 30]. Use of a calculation program allowed the food intake records of approximately 40 nutrients and 150 food types to be calculated and to output an individualized document for each subject. The BDHQ questionnaire uses a simplified structure, as well as simplified replies and data processing, while maintaining the characteristics of the DHQ questionnaire [29, 30]. The results of a validity study on BDHQ have been reported in a research report. The calculated nutrients were folate, retinol equivalent (vitamin A), vitamin D, vitamin E, vitamin K, vitamin B_1, vitamin B_2, niacin, vitamin B_6, vitamin B_{12}, pantothenic acid, vitamin C, n-3 type fatty acid, and n-6 fatty acid. In addition, the food group could be selected from the following groups: cereals, potatoes, sugar, sweeteners, pulses, green-yellow vegetables, other vegetables, fruits, fish and shellfish, meat, eggs, milk, fats and oils, confections, and seasonings/spices.

2.5. Statistical analysis

Statistical analysis of the current data was conducted using IBM SPSS statistics version 21, and past data were analyzed using programs such as Stat view. The average value and standard deviation were calculated for age and nutrient/food group intake. Multiple regression analysis was used for vegetable intake, continuous variable independent variables (such as folate intake), and continuous variables (such as serum folate). Logistic regression analysis was used as a qualitative dependent variable: for example, in the case of gender, male is 0 and female is 1, and in the case of genetic polymorphism, TT type is 1, CT type is 2, and CC type is 3 as dummy variable calculated.

3. Results

3.1. Genotype distributions of MTHFR polymorphisms

Of the initial 404 subjects, 399 people provided complete data, allowing the frequencies of the CC, CT, and TT MTHFR genotypes to be calculated: 35.4, 49.7, and 14.8%, respectively. Of the 249 people whose vegetable intake was surveyed in detail, the frequencies of the CC, CT, and TT MTHFR genotypes were 36.5, 49.8, and 13.6%, respectively. Prior to obtaining nutritional guidance, the serum folate levels of the subjects increased according to the number of C alleles, and folate utilization was high (correlation coefficient $r = 0.375$, $p < 0.001$), and conversely, homocysteine levels decreased according to the number of C alleles ($r = -0.520$, $p < 0.001$).

Of all the participants (n = 395), only the subjects with TT type polymorphism (n = 57) increased their intake of green-yellow vegetables significantly, from 110.14 ± 4.65 to 139.29 ± 75.74 g after genotype notification and completing the nutritional guidance program (**Figure 1**, $p < 0.01$).

Intake was increased by 29.15 g (+26.5%) by the TT group, exceeding the national target of 120 g [6], while the increase was 10.44 g (+7.9%) for the TC group and there was no increase by the CC group. Moreover, the intake of food-derived folate also increased only in subjects with the TT type genotype, from 321.22 ± 101.94 to 348.71 ± 100.23 μg after genotype notification and nutritional guidance (**Figure 2**, $p < 0.05$). The increment in the increase in folate intake by subjects with the TT type genotype was 29.15 μg (+8.6%), exceeding the Japanese RDA of 240 μg [2] but not the WHO RDA of 400 μg [3, 4].

Previous studies [6, 16] reported that subjects with the TT genotype showed lower serum folate levels than subjects with the CT and CC genotypes prior to nutritional guidance (**Figure 3**). However, following genotype notification and nutritional guidance, there was a significant increase in serum folate (**Figure 3**, $p < 0.001$ in the TT, $p < 0.01$ in the TC, and $p < 0.01$ in the CC genotypes) due to increased green vegetable intake by all subjects, regardless of genotype (n = 399).

In contrast to the low serum folate level of subjects with the TT genotype prior to nutritional guidance, subjects with the TT genotype showed the highest serum folate levels, ranging from 6.75 ± 4.40 to 11.80 ± 7.88 ng/ml among three genotypes following nutritional guidance

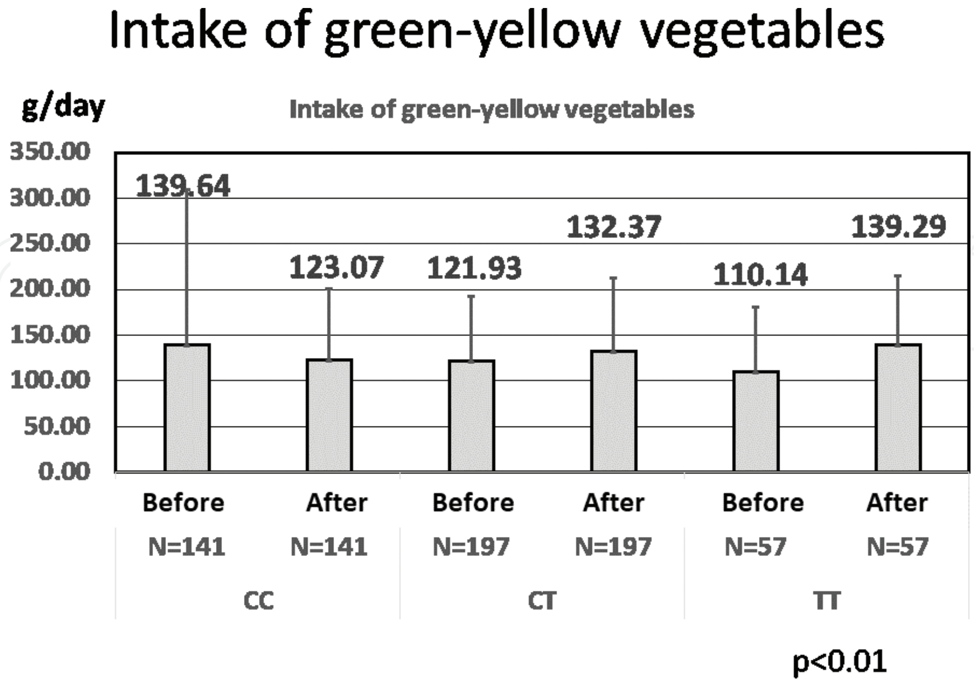

Figure 1. Effect of genotype notification on the intake of green-yellow vegetables by individuals with three MTHFR genotypes: Before: before nutritional guidance. After: after nutritional guidance.

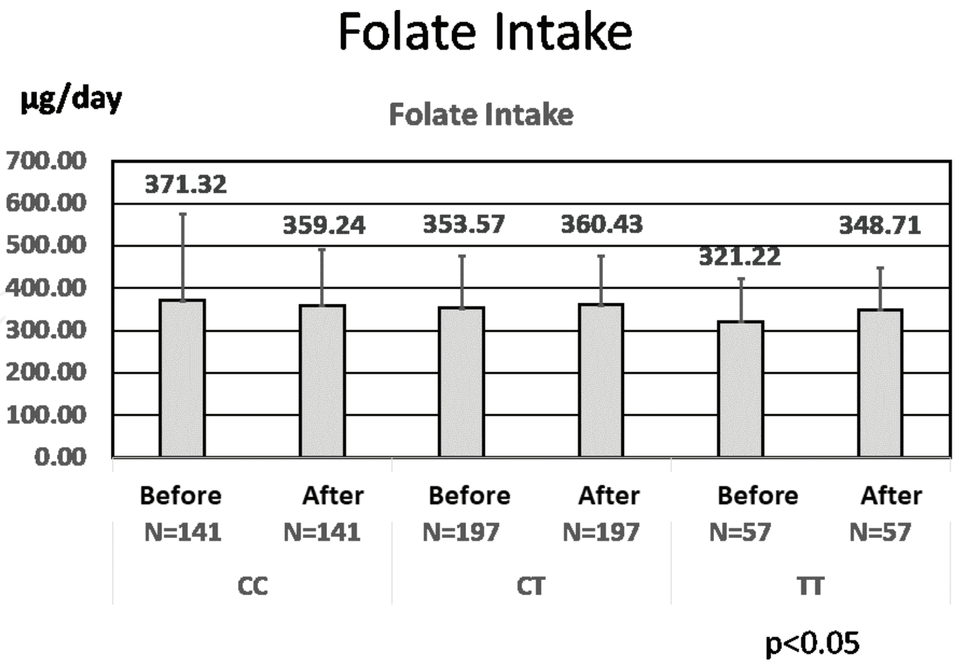

Figure 2. Effect of genotype notification on the intake of dietary folate by individuals with three MTHFR genotypes. Before: before nutritional guidance. After: after nutritional guidance.

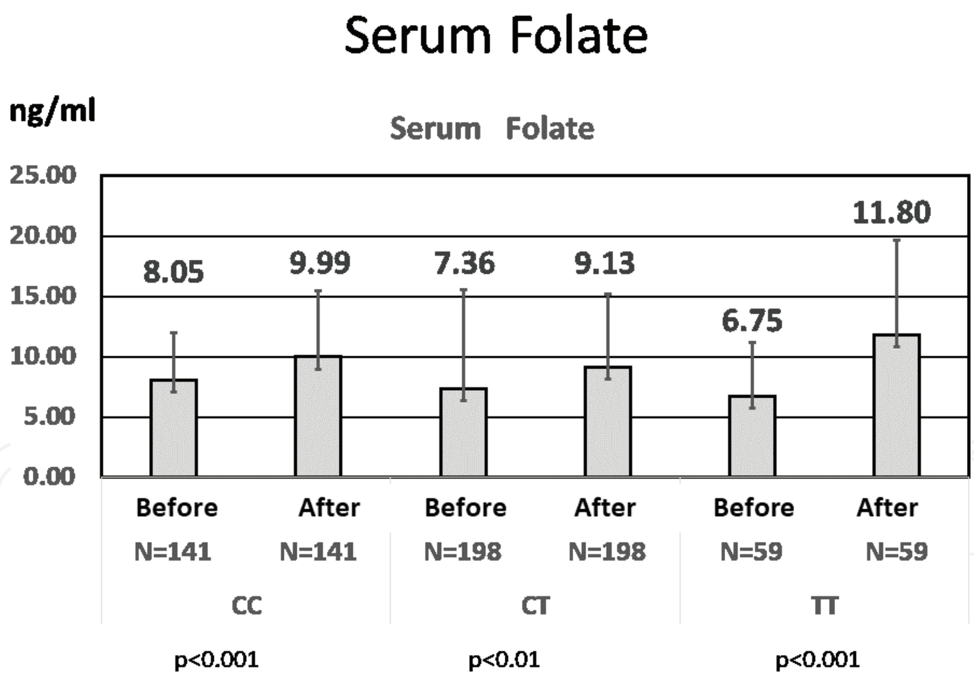

Figure 3. Effect of genotype notification on serum folate levels in individuals with three MTHFR genotypes. Before: before nutritional guidance. After: after nutritional guidance.

(**Figure 3**). Serum folate levels in subjects with the TT genotype showed an increase in serum folate of 5 ng/ml or more in both males and females, more than double that observed in the CC type and CT type groups (**Figure 3**, $p < 0.001$). The decrease in serum homocysteine (**Figure 4**, $p < 0.001$) following genotype notification and nutritional guidance regarding

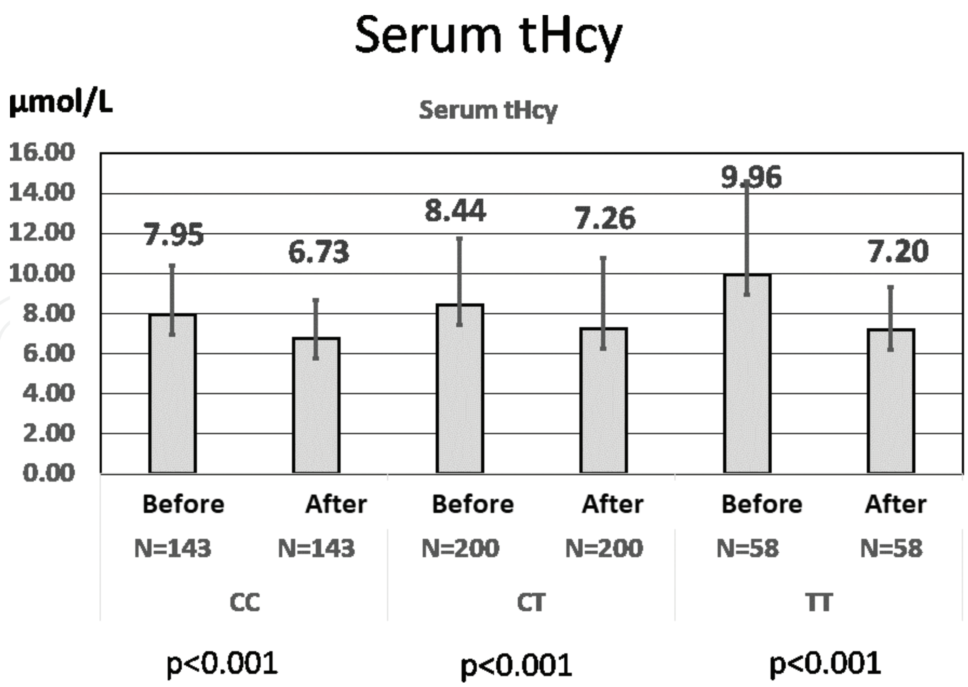

Figure 4. Effect of genotype notification on serum homocysteine levels in individuals with three MTHFR genotypes. Before: before nutritional guidance. After: after nutritional guidance.

green vegetable intake was significant in all three genotype groups. Subjects with the TT type genotype showed the largest reduction in homocysteine levels, from 9.96 ± 4.65 to 7.20 ± 2.11 µmole/L (**Figure 4**, $p < 0.001$). Males showed lower serum folate concentrations and higher serum homocysteine levels than females.

Total vegetable intake was highest in the TT homozygotes, from 244.85 ± 134.00 to 273.21 ± 107.29 g, but the increase did not reach significance levels.

There was a significant inverse correlation between serum folate and homocysteine levels prior to nutritional guidance, as demonstrated by the correlation coefficient ($r = -0.37$), and this correlation has a definite genetic polymorphism influence. However, the number of times green vegetables were taken in the morning, afternoon, and evening did not reach significance levels, and no change was seen in serum folate or serum homocysteine levels.

The frequency of green vegetable intake during the morning, afternoon, and evening showed an increasing trend in all areas of the city following nutritional guidance. The frequency of green vegetable intake was about 7.5–11 times per week for males and females prior to nutritional guidance, corresponding to about 126.4 g green-yellow vegetables per serving. The total average frequency of green vegetable intake in seven city districts by TT homozygotes increased from 9.0 to 12.0 in males ($p < 0.05$) but only from 2.8 to 3.9 in females (not significant). The intake of broccoli, Komatsuna, spinach, and vegetable juice increased the most following nutritional guidance. The type of vegetables consumed differed slightly depending on the season and location (farmlands and cities). We confirmed that the major source of vegetables was supermarkets, regardless of season and location. Since the number of men participating in the study was small, we confirmed the increase in green vegetable intake following nutritional guidance by totaling the vegetable intake in each of the three daily meals (breakfast, lunch, and dinner). In women, a significant increase was observed throughout the day and at dinner, but the TT type group was as small as nine subjects and the genotype-specific increase did not reach significance levels.

4. Discussion

According to the National Health and Nutrition Survey of 2016 [5], the average daily vegetable intake in Japan was 265.9 g (males: 272.3 g, females: 260.4 g). In the present study, the daily vegetable intake prior to nutritional guidance was 282.2 ± 202.0 g, which is similar to the national average [5]. However, the daily intake of green-yellow vegetables in Japan was on average 84.5 g (83.4 g for males, 85.4 g for females) [5] and for the 60–69 year age group, the average intake reported in the National Health and Nutrition Survey of 2016 was 97.5 g (94.7 g for males, 99.9 g for females) [5]. Prior to nutritional guidance, the folate intake was 126.4 g ± 116.2 g by subjects in the entire city of Sakado. We believe that there is a growing interest in vegetable consumption. As mentioned in the previous section, after the start of this project 11 years ago, 80% of Sakado citizens were aware of the importance of folic acid, 90% tried to eat more vegetables, and 73% wanted to obtain advice on improving their health. The average Japanese daily folate intake was 277 μg (283 μg for males, 272 μg for females) and 322 μg (328 μg for males, 317 μg for females) for Japanese in the 60–69 year age group as reported in the National Health and Nutrition Survey of 2016 [5]. The daily intake of green-yellow vegetables by the residents of Sakado City was already 355.4 ± 154.4 g prior to nutritional guidance because Sakado residents had received nutritional guidance for 11 years during the Sakado Folate Project [1]. The frequency of green vegetable intake was about 7.5–11 times per week for males and females prior to participants attending our lectures, corresponding to about 126.4 g of green-yellow vegetables per serving. Since there is no proportional relationship between the number of dishes served at a meal and the amount of vegetables taken, it is difficult to compare accurately, but the number of intake of green vegetables can be judged to be simple and useful as well as this previous study. In addition, the self-descriptive simple survey table for vegetable intake of the kind used in this study is widely used in the United States and is called "a rapid food screener" [31]. This screener is a useful tool for quickly monitoring patients' diets and the health care provider can use it as a prelude to brief counseling or as the first stage of triage.

Unfortunately, Japan's National Health and Nutrition Survey does not quantify serum folate or serum homocysteine, in contrast to surveys in other countries, so it cannot be compared with this study. However, the accurate blood analysis values obtained in this study showed that both serum folate and serum homocysteine levels were significantly improved following nutritional guidance for all genetic polymorphism groups.

This conclusion is supported by the finding that folate rice was taken more frequently in the previous study compared with other folate sources, including vegetables. In addition, the intake of green-yellow vegetables increased significantly in both male and female TT homozygote subjects following nutritional guidance (**Figure 1**). The correlation coefficient between each indicator is high ($r = 0.358$) according to the number of C alleles with high folate-utilizing ability, as evidenced by the previous study by our laboratory [16], and serum folate increases and homocysteine decreases according to the number of C alleles ($r = -0.52$). In addition, serum folate concentration and serum homocysteine inversely correlate ($r = -0.37$) since folate deficiency accompanies an increase in homocysteine. However, there was no significant correlation with green vegetable intake in the morning, afternoon, and evening ($r = -0.04$). Rice folate, green tea, laver, and other foods may be additional sources of folate.

Although genotype notification in the Sakado Folate Project was effective in motivating folate intake, especially by those with the TT genotype, the increase in serum folate (from 17.4 to 22.5 nmol/L, 129%, averaged data from 2006 to 2012) was less than that observed following compulsory folate fortification in the United States (from 12.1 to 30.2 nmol/L, 149.6%) [1].

In general, even following nutritional guidance, it is difficult to change behavior such as increasing the number of green vegetable dishes, but among all subjects, both males and females with the TT polymorphism most significantly improved their green-yellow vegetable intake following gene notification (**Figures 1** and **2**). Although the total folate intake exceeded 400 μg, it is necessary to supplement various vitamins, minerals, dietary fiber, and antioxidant compounds found in green vegetables. It is therefore desirable to improve nutritional guidance to further increase green-yellow vegetable intake.

4.1. Efficacy of genotype notification for promoting healthy habits

The widespread commercial application of genotype notification, called direct to consumer (DTC), may be effective for encouraging healthy lifestyles. A meta-analysis of eight papers from seven different studies revealed a significant impact of genetic notification on smoking cessation in comparison to controls (clinical risk notification or no intervention) in short-term follow-ups of less than 6 months (RR = 1.55, 95% CI 1.09–2.21) [32]. In addition, genotype notification was associated with short-term increased depression and anxiety [32]. However, genotype notification is not always effective [33]. The effects of genotype notification of an oncogene (L-myc) genotype to smokers on their ability to quit smoking were tested [33]. Some smokers were allocated to the genotype notification group (intervention group) and the rest served as controls. Twenty-two of the 276 smokers in the control group stated that they quit smoking (8.0%) and 15 (5.8%) in the 257 genotype-notified group quit, providing an odds ratio (OR) of cessation for the intervention of 0.64 (95% confidence interval, 0.32–1.28). It was concluded that more smokers might quit if better methods explaining the need to quit and for notifying participants of their genotypes were employed [33].

Genotype notification of the risky mutant homozygote of the fatty acid Δ5 desaturase 1 (FADS1) gene resulted in increased intake of eicosapentaenoic acid (EPA) ($p = 1.0 \times 10^{-4}$) [34]. Red blood cell content of EPA also increased. The notified group showed increased awareness of EPA by the end of the study, but during the 12-week genotype notification period notification did not appear to influence intake [34].

The prevention of Alzheimer's disease and cardiovascular diseases might be influenced by genotype notification. According to the report of Hietaranta-Luoma et al. [35], subjects notified of the ApoE Ɛ4+ genotype and of the Ɛ4- genotype were compared with a control group with regard to their changes in diet (e.g., fat quality, vegetables), alcohol consumption, and exercise. Dietary fat quality improved more in the Ɛ4+ group than in the Ɛ4- and control groups after obtaining genotype-based health advice, but only for a short time [35].

These unsuccessful examples of nutritional guidance with genotype notification highlight five reasons for the success of the Sakado Folate Project [1].

1. Effective organization of volunteers commended by the government for their effectiveness in promoting health.
2. Invention of a rapid and inexpensive genetic polymorphism analyzer.
3. Involvement of a well-trained registered dietician for overseeing the nutritional survey, providing advice on promoting folate intake, and for addressing anxiety.
4. Cooperation of the local government and the mayor of Sakado City.
5. Development of two folate-fortified foods: Sakado Folate Bread and Folate Rice.

To date, in addition to the successful increase in folate status, there have been no reports of depression or anxiety by the participants because of the well-trained staff providing *nutrigenomic* guidance.

5. Conclusion

The effectiveness of genotype notification was demonstrated in the case of MTHFR polymorphism. Of all the participants following nutritional guidance, only subjects with the TT genotype significantly increased their intake of both green-yellow vegetables (**Figure 1**, $p < 0.01$) and food-derived folate (**Figure 2**, $p < 0.05$). The increase in serum folate (**Figure 3**) and decrease in homocysteine (**Figure 4**) levels were greatest in subjects with the TT genotype, and these changes were confirmed in subjects with the other genotypes.

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Disclosures

The authors report no conflicts of interest with respect to this study.

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