The relationship between riboflavin and hypertension with *MTHFR* C677T in older adults in northern China: a case-control study

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Running title: Riboflavin alter hypertension with *MTHFR* C677T

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ABSTRACT

Background and Objectives: The incidence of hypertension is higher in individuals with the methylenetetrahydrofolate reductase (*MTHFR*) 677TT genotype. Riboflavin serves as a coenzyme of MTHFR, but its role in hypertension prevalence is poorly understood. This study aimed to explore the relationship between riboflavin levels and hypertension and the impact of genotype on this relationship. **Methods and Study Design:** The case-control study used data from the Tianjin Elderly Nutrition and Cognition study, with 200 hypertensive patients and 200 matched non-hypertensive controls. It collected questionnaires, clinical data, and measured blood riboflavin levels using the dried blood spot technique. Conditional logistic regression analyzed variables related to hypertension prevalence, with effects assessed by odds ratios (ORs) and 95% confidence intervals (CIs). **Results:** The hypertensive group had significantly lower riboflavin concentration than the non-hypertensive group (p<0.001). The regression analysis indicated that regardless of adjustment for sociodemographic characteristics, riboflavin concentration was negatively associated with the prevalence of hypertension. In the multivariable model, the OR was 0.607 [95%CI, 0.507-0.727], p<0.001. Additionally, we found an interaction between *MTHFR* genotype and riboflavin status. Compared with the combined TT genotype and below-median riboflavin levels as the reference category, the CC genotype and above-median riboflavin levels decreased the prevalence of hypertension (OR, 0.189 [95%CI, 0.071-0.501], p=0.001). **Conclusions:** In conclusion, riboflavin status impacts the development of hypertension. There is also interaction between the *MTHFR* genotype and riboflavin. Specifically, low riboflavin status increases the prevalence of hypertension when combined with the *MTHFR* 677TT genotype.

Key Words: hypertension, riboflavin, *MTHFR* 677TT genotype, older adults, case-control study

INTRODUCTION

It is estimated that approximately 1.28 billion adults aged between 30 and 79 around the world are living with hypertension.[1] Hypertension is considered a significant cause of premature death globally. Moreover, the incidence of hypertension in China is higher than the global average, with rates as high as 44.7% for Chinese adults.[2]

Genome-wide association studies have identified eight genetic locations that affect blood pressure.[3] One of these locations is near the gene for the folate metabolizing enzyme methylenetetrahydrofolate reductase (MTHFR). Several studies have found that the MTHFR

C677TT polymorphism increase the prevalence of developing hypertension, particularly in the Chinese population.[4,5] The carrier of MTHFR C677TT polymorphism cannot effectively synthesize 5-methyl-THF, leading to homocysteine (hcy) accumulation and hyperhomocysteinemia (Hhcy).[6] Hypertension combined with Hhcy has a multiplier effect on stroke.[7]

Riboflavin, as a cofactor of MTHFR, partially compensates for the inactivated enzyme activity.[8] Higher riboflavin concentration can prevent the MTHFR cofactor flavin adenine dinucleotide (FAD) (the main component of riboflavin in the body) from separating from the active site or allow its rapid replacement, thus stabilizing the MTHFR and improving enzyme activity.[9] In recent years, several randomized controlled trial (RCT) trials conducted in the United Kingdom have shown that targeted riboflavin supplementation can effectively reduce systolic blood pressure by 6 to 13 mmHg in people at high prevalence of hypertension.[10,11] However, the relevant reports are limited to Northern Ireland, and the sample size is small. There needs to be more to prove a consistent relationship in the Chinese population. Moreover, the prevalence of Hhcy is higher in the Chinese population due to a higher TT genotype carrying rate. Therefore, it is necessary to explore further the relationship between riboflavin and the TT genotype in hypertensive patients in the Chinese population.

However, the role of riboflavin in the TT genotype hypertension prevalence is unclear, particularly among the Chinese. The case-control study aims to investigate whether the level of riboflavin influences the prevalence of hypertension in individuals with different MTHFR status. The study hypothesizes that the level of riboflavin can modify genetic predisposition, impacting the prevalence of hypertension in individuals.

MATERIALS AND METHODS

Participants

This study was conducted using data collected from an ongoing population-based prospective study focusing on the nutritional and cognitive health status of older adults in rural areas of northern China. The study is the Tianjin Elderly Nutrition and Cognition (TENC) Cohort Study and has been registered under the identifier ChiCTR2000034348. Detailed information about the sampling procedures and participant eligibility for this cohort has been published elsewhere.[12] All patients provided signed informed consent, and the Ethical Review Committee of Tianjin Medical University (Ethics No: TMUhMEC2018013) and the Ethics Committee of Baodi Clinical College of Tianjin Medical University (Ethics No: BDYYLL202404) approved all studies.

The TENC cohort study involved 7304 older adults living in rural northern China, all 60 years or older. In 2021, whole blood samples were collected and preserved from 3647 individuals. Out of these, we excluded 2773 individuals from the analysis for the following reasons: missing questionnaire data and additional vitamin supplementation (n=1545), significant diseases (such as Parkinson's disease, Alzheimer's disease, cerebrovascular diseases, cancer, mental illness, or mild cognitive impairment) and those outside the age range of <60, >80 years (n=1228), leaving 874 participants. According to the hypertension diagnostic criteria, 565 individuals were diagnosed with hypertension. From this group, 200 hypertensive patients were randomly selected, and we matched the control group in a 1:1 ratio based on age (±2 years) and sex (Figure 1).

According to the sample size calculation formula for 1:1 matched case-control studies, and in accordance with the relevant parameters obtained from previous literature (OR = 2.10, p_0 = 0.2, $\alpha = 0.05$, $\beta = 0.1$),[13,14] the minimum required sample size was 191 persons per group, and the present study included 200 persons per group, which fulfilled the sample size requirement.

Diagnostic standards for hypertension

The clinician's diagnosis of hypertension, according to the Chinese Guidelines for the Management of Hypertension, is as follows: having a systolic blood pressure (SBP) of ≥140 mm Hg, or diastolic blood pressure (DBP) of ≥90 mm Hg, 15 or SBP <140 and DBP <90 and antihypertensive medication within the past two weeks. The diagnosis of hypertension is registered in the system, and we judge whether it is hypertension based on the system records.

Data collection and blood sample collection

Trained professionals administered questionnaires to collect general demographic information from study participants through face-to-face interactions. The health status data we collected included whether the individuals had been diagnosed with hypertension and diabetes mellitus.

We also obtained information on their smoking and drinking habits through interviews. Both smokers and drinkers were categorized as “never” or “yes”. Dietary intake data were collected using the Food Frequency Questionnaire (FFQ), and then the reported frequency and portion sizes of foods were converted to grams and calculated using information from the Chinese Food Composition Table.[16,17]

Fasting blood samples were collected from the participants and stored in a -80°C refrigerator for testing of blood indices and the genotype.

Whole blood riboflavin detection

An automated high-throughput dried blood spot (DBS)-LC-MS/MS method is used to quantify riboflavin in whole blood. The process involves several steps: whole blood is transferred to the center of the DBS card circle, dried, and stored at -80°C in a sealed foil bag. Thaw in a gradient of -20° and 4° in the refrigerator prior to testing. The cards are then transferred to the internal standard (IS) spray module for uniform spraying, dried, and extracted with the extraction solution. The extracted sample is then injected into LC-MS/MS for analysis and washed several times with extraction buffer and rinse solution. The specific detailed steps have been described in this article.[18]

Serum folate and homocysteine levels detection

Serum folate concentration was determined by a SIEMENS Inmmultie-2000XPi automated chemiluminescence immunoassay analyses and its accompanying kit SIEMENS, USA). The enzyme conversion method was used to determine serum Hcy by DIRUI CS-T300 automatic biochemistry analyzer (China). The kits were provided by Ningbo Medical Systems Biotechnology Incorporated (China).

Genotype identification

Genomic DNA was first extracted from the blood samples of the participants in the study. The *MTHFR* C677T genotype was determined using the TaqMan assay by commissioning Shanghai OE Biotech Co, Ltd.

Covariates

In our study, we included the following variables as covariates: age (in years), sex (male or female), educational level (categorized as a primary school or below, middle school, or high school and above), smoking status (non-smoker or smoker), drinking status (non-drinker or drinker), self-reported diabetes (yes or no), MTHFR genotype (with categories CC, CT, TT), energy, dietary sodium intake, dietary iodine intake, dietary saturated fatty acid (SFA) intake, degree of saltiness of dietary taste (with categories salty, normal, mild), and riboflavin concentration, which were classified as high or low based on whether they were above or below the median level in all case and control group (the median riboflavin concentration for all participants in the study was 1.566 ng/mL). Additionally, we considered folate and Hcy concentrations in our analysis.

Statistical analysis

The statistical analysis was conducted using SPSS software (version 26) and R (version 4.3.2). The Shapiro-Wilks test was applied to verify the normal distribution of data. Normalized data were expressed using means and standard deviations (SD), and group differences were analyzed using the Student's t-test or two-way analysis of variance. For non-normally distributed data, the Mann–Whitney U test was employed for two-group comparisons. Categorical variables were expressed as frequency (%), and the chi-square test assessed group differences. A *p*-value of less than 0.05 was considered statistically significant. Conditional logistic regression was used to explore variables related to hypertension prevalence, with the significance of effects determined by odds ratios (ORs) and 95% confidence intervals (CIs). The interaction between *MTHFR* genotype (CC/CT/TT) and riboflavin levels (above or below median) was analyzed by dividing into six categories, with the first group (TT genotype + below-median riboflavin level) serving as the reference. The significance of the interaction between genotype and riboflavin level was evaluated using the *p*-value of the product term, adjusting for smoking status, drinking status, education level, folate, Hcy, energy, dietary sodium intake, dietary iodine intake, dietary saturated fatty acid (SFA) intake, degree of saltiness of dietary taste, and self-reported diabetes.

RESULTS

Characteristics of participants

The demographic information and biochemical characteristics of the participants in our case-control study are shown in Table 1. We enrolled 400 participants, 200 in the hypertension group and 200 participants in the non-hypertension group, matched for age and sex. There were no significant differences between the hypertensive and control groups in terms of education level, folate and Hcy concentration, smoking, alcohol, energy, dietary sodium intake, dietary iodine intake, dietary riboflavin intake, dietary thiamine intake, dietary niacin intake, dietary folate intake, dietary SFA intake, degree of saltiness of dietary taste and diabetes status. The *MTHFR* 677C→T polymorphism (TT genotype) was found in 16.5% of the non-hypertensive individuals compared with 34.5% in those with hypertensive. Significantly lower levels of riboflavin ($p<0.001$) were observed in patients with hypertension compared to the non-hypertensive individuals. The comparison of 200 hypertensive patients in this article with the 565 hypertensive patients screened (out of 874) found no significant

differences between the two groups in terms of age, gender, smoking, and alcohol consumption (see Supplementary 1).

The whole blood riboflavin levels were negatively associated with the prevalence of hypertension

In individuals with the same genetic makeup, the analysis indicated that those with hypertension had lower levels of riboflavin compared to those without hypertension. Specifically, hypertensive patients with the *MTHFR* 677TT genotype had significantly lower riboflavin levels compared to non-hypertensive individuals with the same genotype (1.56±1.39 ng/mL vs 3.15±2.98 ng/mL). Moreover, older adults with the *MTHFR* 677TT genotype in the hypertension group had lower riboflavin levels than those with the CC or CT genotype, but there was none significant. Among the six groups studied, hypertensive patients with the *MTHFR* 677TT genotype had the lowest riboflavin concentration (Figure 2).

In Figure 3, it was observed that higher concentration of whole-blood riboflavin were linked to a decreased prevalence of developing hypertension. This negative association persisted even after adjusting for sociodemographic characteristics. Specifically, the univariable model showed an OR, 0.645 (95%CI, 0.550-0.758, p<0.001), and the multivariable model showed an OR, 0.607 (95%CI, 0.507-0.727, p<0.001).

Riboflavin modified the genetic predisposition to hypertension

In comparison to those with TT genotypes and below-median riboflavin levels, Individuals with all other combinations of genotype and riboflavin levels had a lower prevalence of developing hypertension, including CC genotypes and above-median riboflavin levels OR, 0.189 (95%CI, 0.071-0.501, p=0.001); CC genotypes and below-median riboflavin levels OR, 0.327 (95%CI, 0.124-0.859, p=0.023); CT genotypes and above-median riboflavin levels OR, 0.114 (95%CI, 0.050-0.260, p<0.001); CT genotypes and below-median riboflavin levels OR, 0.536 (95%CI, 0.238-1.206, p=0.132); TT genotypes and above-median riboflavin levels OR, 0.303 (95%CI, 0.110-0.836, p=0.021) (Figure 4).

DISCUSSION

The study found that the group with high blood pressure had significantly lower riboflavin concentration than those without high blood pressure. The analysis showed that regardless of adjustments for sociodemographic characteristics, lower riboflavin concentration was linked to a higher prevalence of high blood pressure. When comparing the combined TT genotype

and below-median riboflavin levels to the reference category, having the CC genotype and above-median riboflavin levels was associated with a decreased prevalence of high blood pressure (OR, 0.189 [95% CI, 0.071-0.501]). Low riboflavin levels combined with the *MTHFR* 677TT genotype increase the prevalence of high blood pressure.

Supplementation of riboflavin can impact the activities of *MTHFR* in individuals with the TT genotype. As a coenzyme of *MTHFR*, riboflavin improves enzyme activity and promotes homocysteine metabolism, particularly in individuals with the TT genotype.[19] Previous randomized controlled trials have indicated that riboflavin supplementation effectively manages blood pressure. Among participants with the TT genotype, 16 weeks of riboflavin supplementation reduced daytime systolic blood pressure by 3.8 mmHg.[20] Studies have shown that riboflavin protects against hypertension, and supplements may help lower blood pressure. However, these studies measured riboflavin concentration in the body using methods such as three consecutive 24-hour dietary reviews,[21] which may be affected by recall bias. In our present study, we utilized the DBS-LC-MS/MS method to measure riboflavin levels in whole blood, enabling accurate evaluation of riboflavin nutritional status in the human body. The DBS-LC-MS/MS method for automated extraction and quantifying riboflavin levels in blood provides much more convenience and robustness.[18] Moreover, our study focused on an elderly population in Asia, which differs from earlier research. Furthermore, based on genetic stratification, we explored the link between riboflavin and the prevalence of developing hypertension, which provides a more comprehensive approach than previous studies.

Studies have shown that riboflavin supplementation in patients with hypertension who had the *MTHFR* 677TT genotype was independent of the antihypertensive drug effectiveness. This is in line with our findings that riboflavin levels affect the prevalence of hypertension in individuals with the TT genotype.[22] Because riboflavin acts specifically on genes, additional riboflavin supplementation could be used to treat those with the TT genotype who do not respond well on conventional blood pressure medications. This suggests that personalized non-pharmacological treatment may become available in the future for hypertension.

The cofactor FAD of *MTHFR* seems to protect thermostable enzymes in human from heat destabilization.[23] High levels of riboflavin can prevent the segregation of FAD from *MTHFR*. Additionally, research has found that the blood pressure-lowering effects of riboflavin might be associated with one-carbon metabolic processes and DNA methylation of hypertension-related genes.[24,25] 5-Methyltetrahydrofolate, a product of one-carbon metabolism, modulates nitric oxide (NO), which is a potent vasodilator production, and enhances vascular endothelial

function.[26] Riboflavin supplementation could affect one-carbon metabolic processes, improve concentrations of 5-methyltetrahydrofolate in those with the TT genotype,[27] increase NO bioavailability, and regulate blood pressure levels. However, the exact mechanism has yet to be demonstrated, and further research will be required to explore it.

Limitations

It is important to note that our research has several limitations. Firstly, as it was a case-control study, it could not determine the causal relationship between the factors studied and the outcome. Secondly, it's important to note that our results may lack generalizability since our population consisted of older individuals aged 60 years and above in northern China. Thirdly, we should interpret the results of our study with caution because the sample sizes of each group in our study were relatively small, which could lead to some uncertainty in the results. Lastly, whole blood riboflavin concentration was measured using the dry spot method rather than the gold standard method, the erythrocyte glutathione reductase activation coefficient.

Conclusion

The study concluded that the prevalence of developing hypertension was affected by the interaction between genotype and riboflavin status. Those with low riboflavin concentration were at higher prevalence of developing hypertension, particularly those with the *MTHFR* 677TT genotype. This new gene-nutrient interaction provides a non-pharmacological approach to treatment. In the future, riboflavin could be used as a personalized management technique for hypertensive individuals with this genetic prevalence factor due to its gene-specific effects.

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CONFLICT OF INTEREST AND FUNDING DISCLOSURE

The authors declare no conflict of interest.

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Table 1. The demographic characteristics of hypertensive individuals and controls. (n=200/group)

Characteristics	Controls	Hypertension	p value
Age (mean (SD))	68.55 (2.84)	68.64 (2.60)	
Male	76 (38.0)	76 (38.0)	
Education level (%)			0.834
Primary school and below	129 (64.5)	133 (66.5)	
Middle school	47 (23.5)	42 (21.0)	
High school or above	24 (12.0)	25 (12.5)	
Smoker (%)	43 (21.5)	58 (29.0)	0.107
Drinker (%)	35 (17.5)	38 (19.0)	0.796
Diabetes (%)	44 (22.0)	52 (26.0)	0.412
MTHFR genotype (%)			<0.001
CC	47 (23.5)	41 (20.5)	
CT	120 (60.0)	90 (45.0)	
TT	33 (16.5)	69 (34.5)	
Riboflavin (ng/mL)†	3.12 (0.19)	1.70 (0.10)	<0.001
Folate (mean (ng/mL) †	6.92 (0.28)	6.72 (0.24)	0.238
Hcy (mean (μmol/L) †	13.49 (0.43)	14.80 (0.51)	0.070
Energy (kcal) †	2090.20 (520.82)	2084.87 (559.11)	0.998
Dietary sodium intake (mg) †	682.26(331.68)	683.02 (335.55)	0.827
Dietary potassium intake (mg) †	2155.46 (800.72)	2128.92 (870.25)	0.575
Dietary riboflavin intake (mg) †	0.96 (0.50)	0.95 (0.58)	0.406
Dietary folic acid intake (μg) †	336.43 (123.98)	334.05 (143.91)	0.658
Dietary thiamin intake (mg) †	0.99 (0.32)	0.98 (0.36)	0.790
Dietary niacin intake (mg) †	12.02 (5.38)	12.03 (5.52)	0.947
SFA (g) †	15.38 (5.83)	15.66 (6.79)	0.977
Degree of saltiness of dietary taste (%)			0.082
Salty	63 (31.5)	46 (23.0)	
Normal	77 (38.5)	76 (38.0)	
Mild	60 (30.0)	78 (39.0)	

mean (SD), mean (standard deviation); IQR, Interquartile range; SFA, Saturated fatty acid; Hcy, Homocysteine.
†The non-normal data for riboflavin, folate, energy, dietary sodium intake, dietary potassium intake, SFA and Hcy is expressed as mean ± standard deviation for better readability

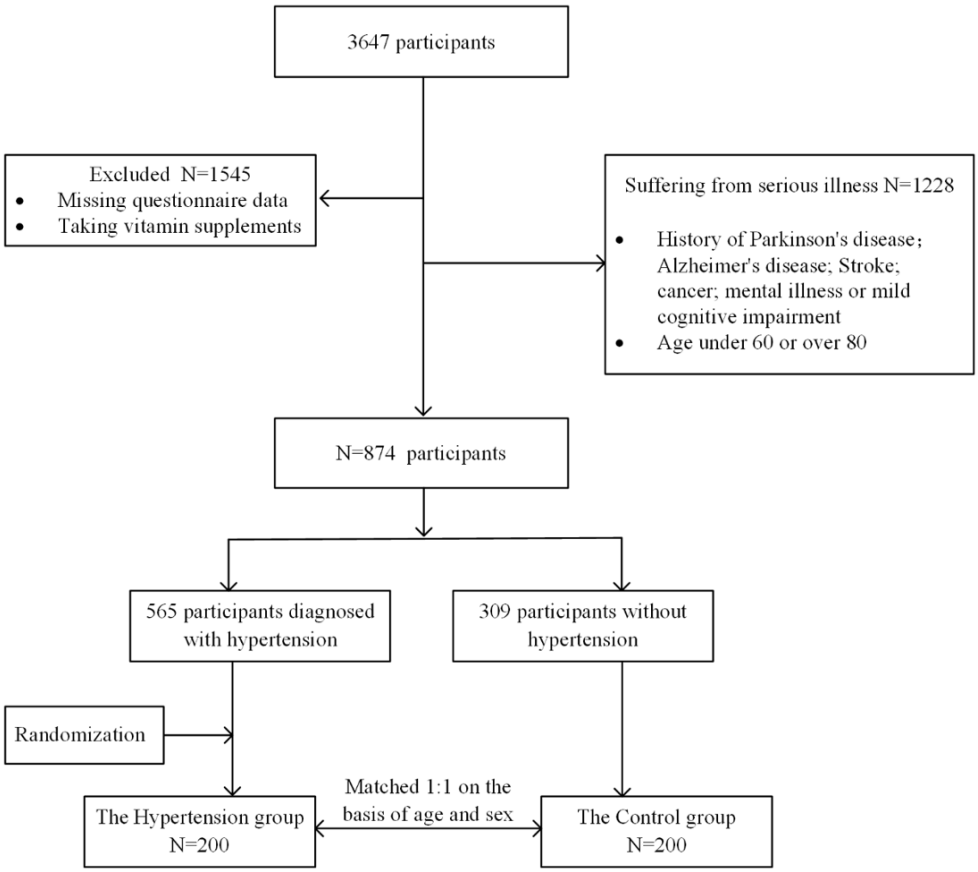

Figure 1. Flowchart of study participants

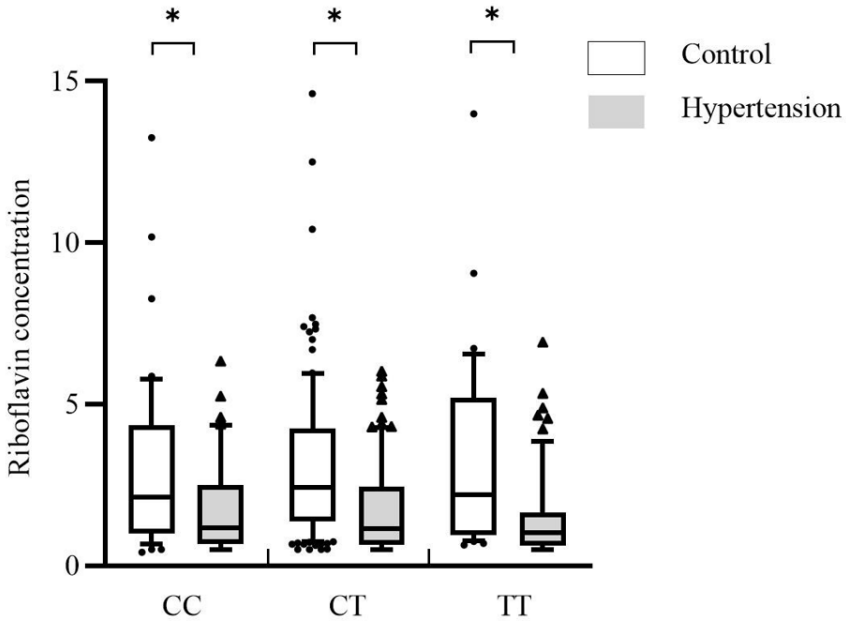

Figure 2. Comparison of riboflavin levels between two groups. * $p<0.05$, indicating a significant difference between the hypertension and non-hypertension groups with the same genotype.

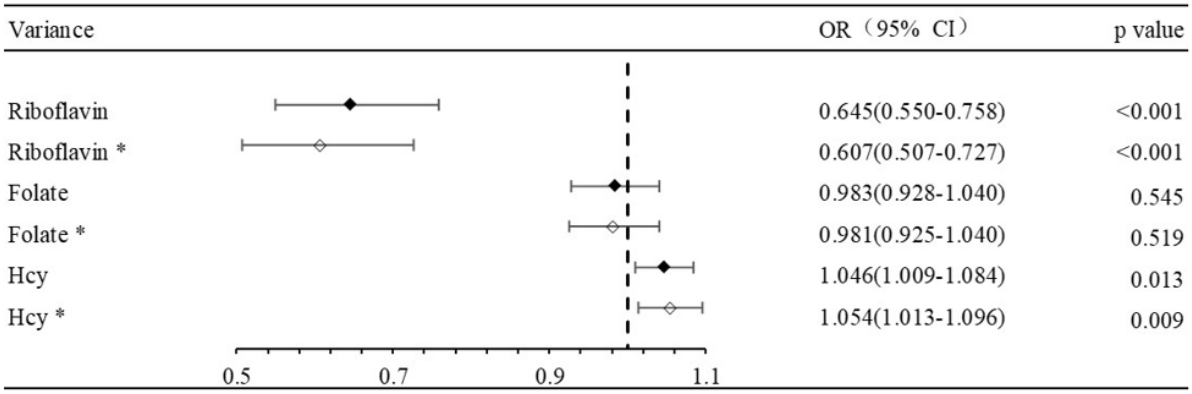

Figure 3. Odds ratios for risk factors associated with hypertension. * Model adjusted for smoking status (yes or no), drinking status (yes or no), education (primary school and above, middle school, high school and above), energy, dietary sodium intake, dietary iodine intake, dietary saturated fatty acid (SFA) intake, degree of saltiness of dietary taste, and self-reported diabetes (yes or no). OR, odds ratios

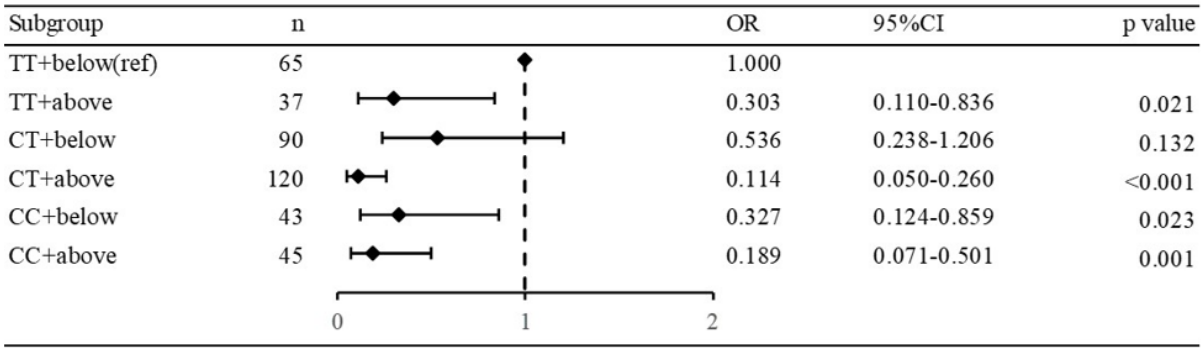

Figure 4. Influence of MTHFR genotype and riboflavin status on the risk of hypertension. Adjusted for smoking status (yes or no), drinking status (yes or no), education (primary school and above, middle school, high school and above), folate, hcy, energy, dietary sodium intake, dietary iodine intake, dietary saturated fatty acid (SFA) intake, degree of saltiness of dietary taste, and self-reported diabetes (yes or no). CI confidence interval, OR odds ratio

Supplementary Table 1. The demographic characteristics of hypertensive patients in the included in the analysis (n=200) and total hypertensive patients (n=565)

Characteristics	Hypertensive patients included in the analysis	Total hypertensive patients	p value
Sample size	200	565	
Age (mean (SD))	68.64 (2.60)	69.07 (3.48)	0.114
Sex (%)			
Male	76 (38.0)	232 (41.1)	0.500
Female	124 (62.0)	333 (58.9)	
Smoke (%)			
None	142 (71.0)	369 (65.3)	0.167
Yes	58 (29.0)	196 (34.7)	
Drink (%)			
None	162 (81.0)	443 (78.4)	0.501
Yes	38 (19.0)	122 (21.6)	
Genotype (%)			
CC	41 (20.5)	118 (20.9)	0.881
CT	90 (45.0)	263 (46.5)	
TT	69 (34.5)	184 (32.6)	
Folate (mean (ng/mL) †	6.72 (3.33)	7.23 (3.79)	0.095
Hcy (mean (μmol/L) †	14.80 (7.15)	14.14 (6.76)	0.245

SD, standard deviation; Hcy, Homocysteine

†The non-normal data for riboflavin, folate, and Hcy is expressed as mean ± standard deviation for better readability.