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DDAH2 (-449 G/C) G allele is positively associated with leukoaraiosis in northeastern China: a double-blind, intergroup comparison, case-control study

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Graphical Abstract *DDAH2 can contribute to the early detection of leukoaraiosis-susceptible individuals*

The GG genotypes of *DDAH2* (-449 G/C) were more common in the leukoaraiosis (LA) group, but they were the least in the control group. A higher frequency of the G gene and a lower frequency of the C gene had been shown in the LA group. Furthermore, the concentration of asymmetric dimethylarginine (ADMA) in the GG group was statistically higher than that in the CG and CC groups.
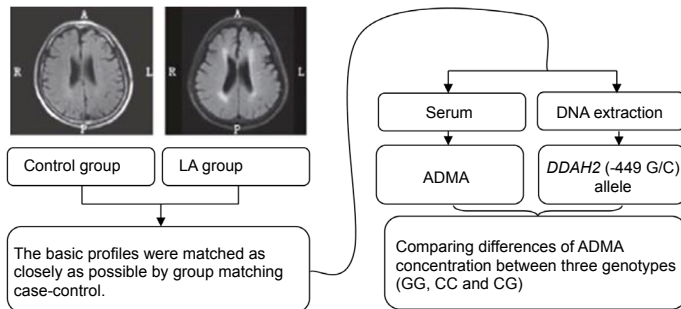
Abstract

Cerebrovascular endothelial dysfunction is involved in the progression of leukoaraiosis. Asymmetric dimethylarginine is a competitive inhibitor of nitric oxide, which is highly expressed in patients with leukoaraiosis. Dimethylarginine dimethylaminohydrolase (DDAH) is a hydrolytic enzyme that is primarily responsible for eliminating asymmetric dimethylarginine, and it plays a role in the pathogenesis of cardiovascular and cerebrovascular diseases. The DDAH2 subtype is expressed in organs rich in induced nitric oxide synthase, including the heart, the placenta, and the cerebral endothelium during cerebral ischemia, in the stress state, or under neurotoxicity. Overexpression of the *DDAH2* gene can inhibit asymmetric dimethylarginine-induced peripheral circulating endothelial cell dysfunction. However, it is unknown whether this polymorphism regulates plasma asymmetric dimethylarginine levels in patients with leukoaraiosis. In this double-blind study, we recruited 46 patients with leukoaraiosis and 46 healthy, matched controls. Plasma asymmetric dimethylarginine levels were determined using enzyme-linked immunoassays. Genomic DNA was isolated from whole blood samples, and polymerase chain reaction, SmaI restriction enzyme digestion, restriction fragment length polymorphisms, and agarose electrophoresis were used to detect *DDAH2* (-449 G/C) gene polymorphisms. The results revealed that 95.65% of leukoaraiosis patients had recessive genetic models (GG and CG), while 89.13% of healthy control subjects had dominant genetic models (CC and CG). There was a significant difference in the genotype composition ratio between leukoaraiosis patients and healthy controls (P = 0.0002). The frequency of G alleles in the leukoaraiosis patients (71.74%) was significantly higher than in healthy controls, whereas the frequency of C alleles was lower (χ^2 = 13.9580, P = 0.0002). Furthermore, asymmetric dimethylarginine concentrations in subjects with the GG genotype were significantly higher than in subjects with the CG and CC genotypes (Kruskal–Wallis H = 24.5955, P < 0.0001). In addition, the GG genotype of *DDAH2* (-449 G/C) was more common in patients with leukoaraiosis. These findings suggest that the G allele of *DDAH2* (-449 G/C) is a risk factor for leukoaraiosis morbidity and is correlated with high levels of asymmetric dimethylarginine. This study was approved by the Institutional Ethics Committee of the 2nd Affiliated Hospital of Harbin Medical University of China (approval No. KY2016-177) on July 28, 2016.

Key Words: leukoaraiosis; dimethylarginine dimethylaminohydrolase 2; gene polymorphism; allele; asymmetric dimethylarginine; nitric oxide; endothelial dysfunction; cerebrovascular diseases; clinical trial

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Introduction

Leukoaraiosis (LA) is a cerebral small vessel disease that can lead to cognitive dysfunction, subcortical dementia syndrome, gait instability, and even symptomatic cerebral hemorrhage (Fierini et al., 2017), thus seriously reducing patients' quality of life. It is generally believed that the main pathogenesis of LA is cerebrovascular endothelial dysfunction (Maccarrone et al., 2017), which results in a series of reactions, such as chronic cerebral ischemia, vascular stenosis, atherosclerosis, and cerebral white matter lesions (Hachinski et al., 1987; Kidwell et al., 2001; Pantoni, 2002; Bian et al., 2019).

Nitric oxide is mainly produced by nitric oxide synthase (NOS) in endothelial cells, and it acts as an important endogenous vasodilator to regulate cerebrovascular systolic and diastolic function (Gunawardena et al., 2019). Asymmetric dimethylarginine (ADMA) is a metabolic byproduct that was first detected in human plasma in the 1970s (Liu et al., 2014) and is essential for protein methylation. ADMA inhibits the production of nitric oxide by competing with NOS (Dovinová et al., 2018), leading to a series of changes in endothelial function and atherosclerosis in patients with hyperlipidemia. Our previous studies revealed that ADMA levels in patients with LA are significantly higher than those in healthy control subjects (Guan et al., 2017), and that high concentrations of ADMA are associated with cognitive dysfunction in LA (Gao et al., 2015).

Why might patients with LA have higher plasma ADMA levels? Some studies have reported that ADMA is mainly (95%) eliminated through degradation by dimethylarginine dimethylaminohydrolase (DDAH), which is a critical factor in regulating plasma ADMA concentration (Gad et al., 2011; Anavi and Tirosh, 2020). The DDAH2 subtype is expressed in organs rich in induced NOS, including the heart, the placenta, and the cerebral endothelium during cerebral ischemia, in the stress state, or under neurotoxicity (Anavi and Tirosh, 2020). Overexpression of the *DDAH2* gene can inhibit ADMA-induced peripheral circulating endothelial cell dysfunction (Liu et al., 2012), suggesting that *DDAH2* may be involved in the pathogenesis of cardiovascular and cerebrovascular diseases. The core promoter elements of *DDAH2* can influence mRNA transcription and protein translation. The -449 G/C (rs805305) polymorphism is located in the core promoter region of *DDAH2* (**Figure 1**) and affects plasma ADMA levels in patients with heart failure and nephropathy (Rector et al., 1996; Dayal and Lentz, 2005; Stühlinger and Stanger, 2005). However, it is unknown whether this polymorphism regulates plasma ADMA levels in patients with LA. Therefore, this study was conducted to explore differences in the -449 G/C polymorphism of *DDAH2* in patients with LA, and to investigate the relationship between this polymorphism and ADMA levels.
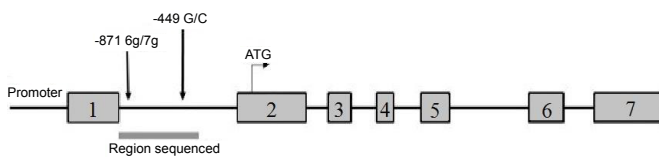
Figure 1 | **The -449 G/C polymorphism is located in the core promoter region of the *DDAH2* gene.**
DDAH2: Dimethylarginine dimethylaminohydrolase 2.

Participants and Methods

Participants
Patients were recruited from the Department of Geriatrics and Neurology, whereas the healthy control participants were recruited from the Physical Examination Center of the 2nd Affiliated Hospital of Harbin Medical University. All participants were recruited from January to July 2016, and came from Heilongjiang Province, Jilin Province, Liaoning Province, or the northeastern Inner Mongolia Autonomous Region. All of these regions are in northeastern China and have long winters that last for approximately 5 months. Participants were eligible for inclusion in the double-blind, case-control study if they met the following criteria: a) at least 40 years of age, without a clear history of heart or brain disease; b) underwent brain magnetic resonance imaging (MRI) examination; and c) were conscious and consented to participate in the study. Participants were excluded from the study if they: a) had acute cerebral or myocardial infarction, hemorrhage, transient ischemic attack, or other neurological disorders (epilepsy, trauma, intracranial or extracranial malignancy, schizophrenia, normal pressure hydrocephalus, depressive and anxiety disorder, Parkinson's disease, or Alzheimer's disease); b) had high signal abnormalities on MRI scans that could be attributed to long-term radiation exposure, carbon monoxide poisoning, or a history of immune system disease (e.g., multiple sclerosis, vasculitis, or leukodystrophy); c) had severe heart, lung, liver, kidney, or thyroid dysfunction; d) had other malignancies or acute inflammatory disease; e) refused to provide the requested information; or f) had blood relationships with each other, were taking hormones, angiotensin-converting enzyme inhibitors, or statins, or lived in provinces other than the aforementioned regions, which might affect ADMA or *DDAH2* levels. All participants signed their informed consent. This study was approved by the Institutional Ethics Committee of the 2nd Affiliated Hospital of Harbin Medical University of China (approval No. KY2016-177) on July 28, 2016 (**Additional file 1**).

A double-blind method was adopted in this experiment. All participants were unaware of their grouping. The MRI diagnostic physicians were only responsible for analyzing the MRI scans and were unaware of the grouping, and the testers of plasma ADMA and *DDAH2* (-449 G/C) alleles were also unaware of the grouping.

The diagnostic criteria for LA were that the lateral ventricle or centrum semiovale white matter contained diffuse high signals on T2-weighted or fluid-attenuated inversion recovery imaging (T2/FLAIR), observable by MRI (Hachinski et al., 1987). Patients who had normal imaging findings were enrolled as controls. To match the case and control groups, if the numbers of cases were different between the two groups, then cases were excluded using a random number table.

Clinical parameters and biochemical measurements
Our descriptive study collected clinical and demographic data, including age, sex, and history of smoking, hypertension, and diabetes. Blood samples (2 mL) were drawn from all participants' elbow veins after overnight fasting for at least 8 hours. Serum levels of fasting blood glucose, triglycerides, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, uric acid, and creatinine were determined using an automatic biochemistry analyzer (HCP-7600; Hitachi, Tokyo, Japan) in a clinical laboratory.

Plasma ADMA measurements
The blood samples were centrifuged at 1500 × *g* for 20 minutes using a 5810R centrifuge (MLX-206; Crystal Technology & Industries, Inc., Dallas, TX, USA). The serum was stored at −80°C for the enzyme-linked immunoassay. According to the kit instructions, serum concentrations of ADMA were determined using enzyme-linked immunoassays (Human ADMA Enzyme-linked Immunoassay Kit; Sinobest Biotechnology Co., Shanghai, China) (Liu et al., 2012). A monoclonal antibody specific to ADMA was precoated onto a microplate. A competitive inhibition reaction was initiated between biotin-labeled ADMA and unlabeled ADMA (standards or samples) with the pre-coated antibody specific to ADMA. After incubation, the unbound conjugate was washed off. Avidin conjugated to horseradish peroxidase was then carefully added to each microplate and incubated.

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The amount of bound horseradish peroxidase conjugate was inversely proportional to the concentration of ADMA in the sample. After adding the substrate solution, the optical density (OD) value was measured at a single wavelength of 450 nm using an iMark microplate absorbance reader (Bio-Rad, Hercules, CA, USA). The intensity of the resulting color was inversely proportional to the ADMA concentration in the sample.

DNA isolation, genetic variant selection, and genotyping

Genomic DNA was isolated from whole blood samples using the QIAamp DNA Blood Mini Kit (GenStar Biosolutions Co., Ltd., Beijing, China) in accordance with the manufacturer's instructions. DNA was extracted from 200 µL of whole blood using the spin columns provided. The isolated DNA was stored at −20°C and the optical density values at 260 nm ($OD_{260\ nm}$) and 280 nm ($OD_{280\ nm}$) were determined. The concentration and purity of the extracted DNA were calculated according to standard formulas, and the OD260/OD280 was between 1.7 and 1.9, indicating that the DNA purity of the extracted samples was high. The primers for the DDAH2 -449C/G (rs805305) polymorphism were: forward: 5′-CTA CCC ACC TCT CTC CCC TCT CTC-3′ and reverse: 5′-CAC TCT ACC CAG CAC CCT CAG AG-3′. The polymerase chain reaction (PCR) conditions consisted of one cycle of 5 minutes at 94°C; 35 cycles of 30 seconds at 94°C, 30 seconds at 55°C, and 30 seconds at 72°C; followed by 10 minutes at 72°C. A GeneAmp PCR 2720 (Applied Biosystems, Foster City, CA, USA) was used. The amplification was performed using a 20 µL reaction volume containing 5 µL of isolated DNA, 1 µL of each upstream and downstream primer, 10 µL of 2× Taq Mix (GenStar Biosolutions Co., Ltd., Beijing, China), and 3 µL of double-distilled H_2O. The primer concentration was 10 µM. The amplified fragment was the target band (330 bp). The electrophoresis gel consisted of 0.5 g of heated agarose and 40 mL of 0.5× Tris–borate–ethylenediaminetetraacetic acid. After the gel cooled to 50°C, 10 µL of ethidium bromide was added to form an electrophoresis mold. A well comb was inserted into the mold and removed after 30 minutes. Next, 10 µL of the PCR product or DNA marker was added to the gel and the electrophoretic reaction was initiated (100 V, 60 mA, 60–80 minutes). Finally, the PCR product was detected using a gel imaging system. Gene Tools software (Gene Tools, LLC, Philomath, OR, USA) was used to identify single nucleotide polymorphisms (SNPs). The position of the nucleotide sequence was based on the reference sequence obtained from the National Center for Biotechnology Information (NCBI) SNP database (http://www.ncbi.nlm.nih.gov/SNP/).

The PCR products were digested with SmaI restriction enzymes for 10–14 hours in a water bath set to 30°C, and the products were detected using agarose electrophoresis. The length of the DDAH2 rs805305 (-449 G/C) fragment obtained from PCR was 330 bp. There were two products from the Sma I enzyme digestion: two bands at 103 and 228 bp for gene locus C, or one band at 330 bp for gene locus G. Thus, the CC genotype had two bands, at 103 and 228 bp; the GG genotype had only one band, at 330 bp; and the CG genotype had three bands, at 103, 228, and 330 bp (Xuan et al., 2016).

Statistical analysis

According to the formula for calculating the sample size of simple random samples, $N = Z^2 [P(1 − P)] / \delta^2$, with confidence of 95% and error of not more than 4%, the estimated sample size should be 625. Considering losses from follow-up and halfway withdrawal from the study, it is appropriate to increase the sample size by 20%, so the ideal final sample size is approximately 750. Analyses were performed using SPSS software version 19.0 (IBM, Armonk, NY, USA). All data were double checked. Student's t-tests and one-way analysis of variance were performed for values with a normal distribution, which were expressed as the mean ± standard deviation (SD). Values with a non-normal distribution were expressed as the median and quartile spacing (P25, P75) and compared using the rank-sum test. The distributions of the categorical variables were expressed as frequencies and percentages, and comparisons were performed using the least significant difference test with the chi-squared test. The association between ADMA and the risk of LA was estimated by calculating the odds ratio and its 95% confidence interval. Multivariate unconditional logistic regression was used to estimate odds ratios and 95% confidence intervals after adjusting for age; sex; history of hypertension, diabetes, and smoking; fasting blood glucose; total cholesterol; fasting blood glucose; high-density lipoprotein cholesterol; low-density lipoprotein cholesterol; uric acid; and creatinine. The Hardy–Weinberg equilibrium was calculated using a Q test with one degree of freedom (Xuan et al., 2016). We examined the differences in ADMA concentrations between the three genotypes (GG, CC, and CG) using the Kruskal–Wallis H test. If there was statistical significance, the Nemenyi test was used for comparisons between any two genotypes. All reported P values were two-sided, and $P < 0.05$ was considered statistically significant.

Results

Clinical characteristics

The selection and inclusion of patients and controls are shown in **Figure 2**. The characteristics of the 46 LA patients and 46 control participants are summarized in **Table 1**. Although there were no differences in sex between the two groups, the LA patients were older ($P < 0.0001$) and had a higher prevalence of hypertension ($P = 0.0101$) than the controls. There were no differences between the two groups in their smoking or diabetes history ($P > 0.05$). Thus, the basic profiles between the two groups were matched as closely as possible.

Biochemical parameters

The biochemical parameters for all participants at baseline are summarized in **Table 2**. LA patients had higher ADMA levels than the control subjects ($P < 0.05$). There were no differences between the two groups in fasting blood glucose, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, uric acid, or creatinine.
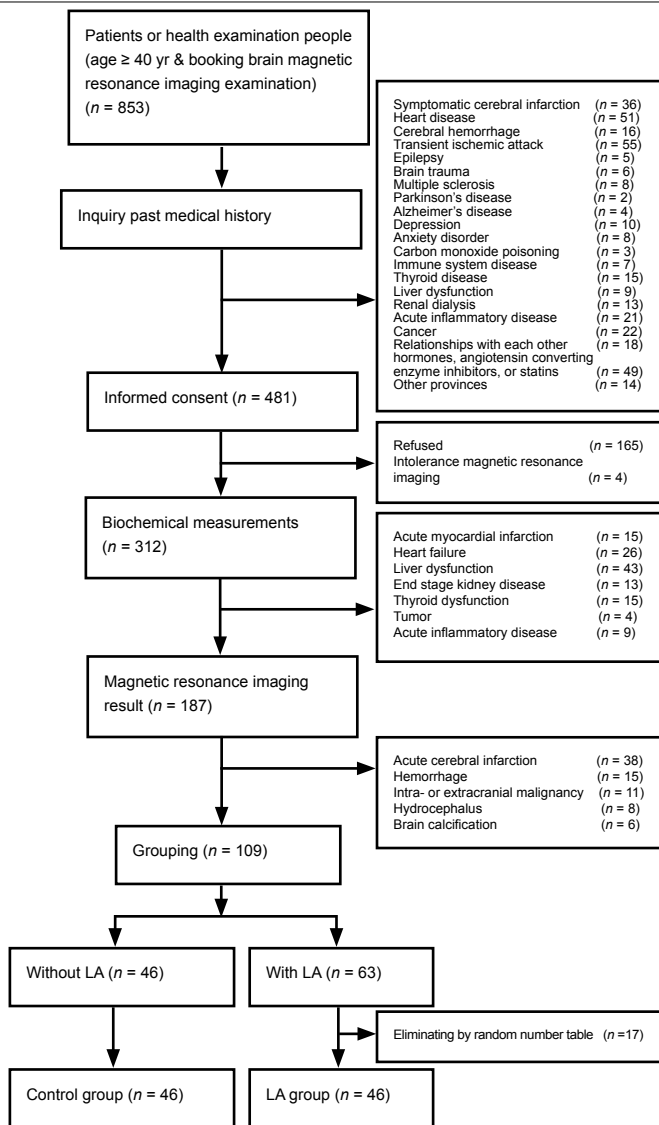
Next, LA morbidity was used as the dependent variable. The most significant parameters and the regression equation were determined using multivariate logistic stepwise regression analysis. Age and ADMA exhibited statistical significance as independent variables (**Table 3**).

Gene polymorphisms of *DDAH2*

The rs805305 (-449 G/C) SNP of *DDAH2* was genotyped in all participants. The genotypes of the LA patients and control subjects are summarized in **Table 4**. In the LA group, the GG and CG genotypes were the most common, each accounting for 48% of all patients. In contrast, the GG genotype was the least common in the control group, accounting for just 11% of the control participants. The difference in genotype composition ratio between the two groups was statistically significant ($P = 0.0002$).

Allele frequency of *DDAH2* (-449 G/C) in LA patients

The frequency distributions of the C and G alleles were 55% and 45%, respectively, which was consistent with the Hardy–Weinberg equilibrium ($P > 0.05$). Compared with the results from the control group, the LA group had a higher frequency of the G allele and a lower frequency of the C allele. The difference in allele frequency between the two groups was statistically significant ($P = 0.0002$; **Table 5**). The sensitivity and specificity of the G allele for LA were 72% and 55%, respectively.

Patients or health examination people (age ≥ 40 yr & booking brain magnetic resonance imaging examination) (*n* = 853)

Symptomatic cerebral infarction (*n* = 36)
Heart disease (*n* = 51)
Cerebral hemorrhage (*n* = 16)
Transient ischemic attack (*n* = 55)
Epilepsy (*n* = 5)
Brain trauma (*n* = 6)
Multiple sclerosis (*n* = 8)
Parkinson's disease (*n* = 2)
Alzheimer's disease (*n* = 4)
Depression (*n* = 10)
Anxiety disorder (*n* = 8)
Carbon monoxide poisoning (*n* = 3)
Immune system disease (*n* = 7)
Thyroid disease (*n* = 15)
Liver dysfunction (*n* = 9)
Renal dialysis (*n* = 13)
Acute inflammatory disease (*n* = 21)
Cancer (*n* = 22)
Relationships with each other (*n* = 18)
hormones, angiotensin converting
enzyme inhibitors, or statins (*n* = 49)
Other provinces (*n* = 14)

Inquiry past medical history

Informed consent (*n* = 481)

Refused (*n* = 165)
Intolerance magnetic resonance imaging (*n* = 4)

Biochemical measurements (*n* = 312)

Acute myocardial infarction (*n* = 15)
Heart failure (*n* = 26)
Liver dysfunction (*n* = 43)
End stage kidney disease (*n* = 13)
Thyroid dysfunction (*n* = 15)
Tumor (*n* = 4)
Acute inflammatory disease (*n* = 9)

Magnetic resonance imaging result (*n* = 187)

Acute cerebral infarction (*n* = 38)
Hemorrhage (*n* = 15)
Intra- or extracranial malignancy (*n* = 11)
Hydrocephalus (*n* = 8)
Brain calcification (*n* = 6)

Grouping (*n* = 109)

Without LA (*n* = 46)

With LA (*n* = 63)

Eliminating by random number table (*n* = 17)

Control group (*n* = 46)

LA group (*n* = 46)

Figure 2 | Selection of the LA and control groups.
LA: Leukoaraiosis.

DDAH2 gene polymorphism and ADMA concentration

ADMA concentration levels were highest in GG homozygotes [1.61 (1.18, 1.93) μM] and lowest in CC homozygotes [0.34 (0.27, 0.67) μM]. ADMA was detectable at intermediate levels in heterozygotes [0.47 (0.29, 1.37) μM] (**Figure 3**). The ADMA concentrations in the three genotypes were significantly different according to the Kruskal–Wallis H test (*H* = 24.5955, *P* < 0.0001). Furthermore, the least significant difference test was used to compare ADMA concentrations between the three genotypes. ADMA concentration levels in the GG group were significantly higher than in the CG and CC groups (both *P* < 0.05).

Discussion

With the growing popularity of imaging methods, including computed tomography and MRI, LA diagnoses have increased substantially, especially in the older population. However, specific blood markers for the diagnosis of LA are still lacking. Previous studies have reported that higher concentrations of the endogenous NOS inhibitor, ADMA, are associated with the incidence of LA (Janatuinen et al., 2003; Tsikas et al., 2018).

Numerous studies have reported that lipids such as creatinine, uric acid, and urea nitrogen are associated with ADMA concentrations. ADMA can be excreted in the urine and may

Table 1 | General characteristics of the control and leukoaraiosis groups

Characteristics	Control (*n* = 46)	Leukoaraiosis (*n* = 46)	*P*-value
Gender (male/female)[&]	25/21	23/23	0.6767
Age (yr)[*]	58.50±3.85	69.00±5.46	0.0002
Smoking[#]	10 (22)	14 (30)	0.3440
Hypertension[#]	11 (24)	23 (50)	0.0109
Diabetes mellitus[#]	11 (24)	7 (15)	0.4224

[&]Categorical variables are expressed as the numbers of male and female participants. The *P*-values of the categorical variables were calculated using the chi-squared test. [*]Continuous variables are expressed as the mean ± SD. The *P*-values of the continuous variables were calculated using the unpaired *t*-test. [#]Categorical variables are expressed as the number (percentage). The *P*-values of the categorical variables were calculated using the chi-squared test.

Table 2 | Correlations of biochemical parameters in the control and leukoaraiosis groups

Factor	OR (95% CI)	*P*-value
Fasting blood glucose	0.701 (0.495, 0.992)	0.0449
Total cholesterol	0.991 (0.644, 1.526)	0.9675
High-density lipoprotein cholesterol	0.490 (0.170, 1.409)	0.1857
Low-density lipoprotein cholesterol	0.794 (0.468, 1.348)	0.3935
Uric acid	1.003 (0.998, 1.008)	0.1910
Creatinine	1.020 (0.995, 1.045)	0.1190
Asymmetric dimethylarginine	970.375 (36.538, 25673.1249)	< 0.0001

P-values of the discontinuous variables were calculated using univariate logistic regression analysis. CI: Confidence interval; OR: odds ratio.

Table 3 | Correlation of leukoaraiosis morbidity

Factor	Wald χ^2	OR (95% CI)	*P*-value
Age (yr)	4.9577	1.136 (1.015, 1.271)	0.0260
Asymmetric dimethylarginine (μM)	16.8971	970.375 (36.538, 25673.1249)	< 0.0001

Discontinuous variables were calculated using multivariable logistic regression analysis. CI: Confidence interval; OR: odds ratio.

Table 4 | Genotype frequencies [*n* (%)] for the rs805305 (-449 G/C) polymorphism of *DDAH2* in control and leukoaraiosis subjects

Group	CC	CG	GG	χ^2	*P*-value
Control (*n* = 46)	10 (22)	31 (68)	5 (11)	17.5653	0.0002
Leukoaraiosis (*n* = 46)	2 (4)	22 (48)	22 (48)		

Discontinuous variables are expressed as the number (percentage). The *P*-values of the categorical variables were calculated using the chi-squared test. DDAH: Dimethylarginine dimethylaminohydrolase.

Table 5 | Allele frequencies [*n* (%)] for the rs805305 (-449 G/C) polymorphism of *DDAH2* in control and leukoaraiosis subjects

Group	C	G	χ^2	*P*-value
Control (*n* = 46)	51 (55)	41 (45)	13.9580	0.0002
Leukoaraiosis (*n* = 46)	26 (28)	66 (72)		

Discontinuous variables are expressed as the number (percentage). The *P*-values of the categorical variables were calculated using the chi-squared test. DDAH: Dimethylarginine dimethylaminohydrolase.

accumulate in the bodies of patients with renal insufficiency. Similarly, plasma creatinine, uric acid, and urea nitrogen also accumulate in the body. In addition, hyperlipidemia is considered to be a risk factor for atherosclerosis (Tarnoki et al., 2012). Several studies have confirmed that ADMA concentrations are correlated with insulin resistance in diabetes and diabetic complications, especially vascular complications (Namiranian et al., 2005; Marra et al., 2013). In the present study, the blood glucose levels in the LA group

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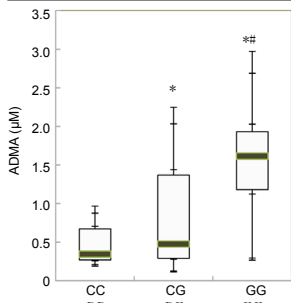
Figure 3 | Comparison of ADMA concentrations in different *DDAH2* (-449 G/C) genotypes.
P-values of the discontinuous variables were calculated using the Nemenyi test. *P < 0.05, *vs.* CC; #P < 0.05, *vs.* CG. ADMA: Asymmetric dimethylarginine; DDAH2: dimethylarginine dimethylaminohydrolase 2.

matched those in the control group. In addition, individuals with severe diabetic complications were excluded from both groups to avoid interference from diabetes-induced serious vascular lesions.

The removal of ADMA mainly occurs through degradation by DDAH, and it is then eliminated via glomerular filtration. There are two known isoforms of DDAH, which have similar molecular structures but different substrate-binding domains. The *DDAH2* gene, located on chromosome 6p21.3, is expressed in induced NOS-rich organs, including the heart, placenta, and immune system, and is a key determinant of plasma ADMA concentrations (Yokoro et al., 2017). There are individual differences in DDAH2 expression, which may be related to gene polymorphisms. A *DDAH2* gene mutation (6G/7G) in the core promoter element -871 during coding transcription can affect subsequent mRNA transcription and protein translation, thus inhibiting protein expression and function. DDAH2 activity is higher at sites that express 7G than at sites that express 6G, and its activity has a strong effect on ADMA metabolism (Yokoro et al., 2017). In the present study, the highest ADMA concentrations occurred with the GG genotype of *DDAH2* (-449 G/C), followed by CG and CC, indicating that polymorphisms in *DDAH2* (-449 G/C) affect plasma ADMA concentrations. This is consistent with previous results regarding ADMA levels and *DDAH2* (-449 G/C) polymorphisms in patients with heart or kidney failure (Zoungas et al., 2006; Marra et al., 2013).

The G allele affects *DDAH2* mRNA transcription and protein translation, reducing the hydrolysis of ADMA and increasing plasma ADMA concentrations. *DDAH2* (-449 G/C) gene polymorphisms are risk factors for multiple diseases, including chronic kidney disease, hypertension, and coronary heart disease (Bode-Böger et al., 2003; Zoungas et al., 2006; Xu et al., 2012; Marra et al., 2013; Hallmark et al., 2019). To the best of our knowledge, there are no previous reports of LA and DDAH2 gene polymorphisms. In the current study, there was a significant difference in *DDAH2* (-449 G/C) genotype between the control and LA groups. Genotypes in the control group were mostly CG and CC (67.39% and 21.74%, respectively), while GG was the least common. The prevalence of LA was associated with *DDAH2* (-449 G/C) gene polymorphisms; the G allele may be a susceptibility factor for LA.

The mutation frequency of the G locus in *DDAH2* (-449 G/C) in the LA group was 72%, which is 5–10% higher than that reported in the general population in Hunan and Guangdong provinces, as well as in other regions in southern China (He, 2009; Xu et al., 2012).

There are several potential explanations for this finding. First, the random selection of LA subjects in the present study was limited by our strict standards, and there may therefore be sampling bias. Second, the subjects selected in our study mainly lived in Heilongjiang, Jilin, Liaoning, and northeastern Inner Mongolia. These four regions are in northeastern China, where winters last a long time (approximately 5 months) and the climate is cold and dry. Studies have shown that cold environments may cause gene mutations as a result of adaptations to the environment (Kim et al., 2015;

Łaczmański et al., 2015; Leipold et al., 2015; Tao et al., 2018). We therefore speculate that environmental factors may influence gene mutations at the G locus and increase mutation rates. Cold environmental factors may also affect the mRNA involved in protein translation after transcription (Kim et al., 2015; Leipold et al., 2015), resulting in changes in protein function and increased ADMA concentrations (Zoungas et al., 2006). Some studies have also suggested that dietary factors are associated with genetic mutations (Tanner et al., 2012; Hallmark et al., 2019). A high-salt diet is prevalent in northeastern China, and preserved foods are eaten more frequently than in southern and southeastern China. A high frequency of G allele mutations as a result of diet and other factors may influence ADMA concentrations during protein translation. However, based on the results of this study, there is no clear experimental evidence for a relationship between mutation rates at the G locus and environmental factors. Further experimental studies are therefore needed to compare population genotypes in northern and southern China.

Nucleosomes, the basic unit of chromatin composed of core DNA, account for 75% of the eukaryotic genome and control protein-binding sites in gene-coding transcription. The presence or absence of nucleosomes near functional gene sites, or the different positions of nucleosomes, can affect gene expression regulation for a variety of reasons (Segal et al., 2006). In a large number of consecutive repeated sequences, nucleosomes are located in preferential sequence regions, which may depend on the A + T and G + C contents. A CpG island, which is a DNA region that has high GC content, has high transcriptional activity (Jones and Takai, 2001). In mammals, CpG islands are often associated with gene promoters (Metushi et al., 2016) that have multiple transcription start sites without common core promoter elements, such as the TATA box. Acetylation and methylation often occur on gene promoters with active transcription (Corsini and Bortolini, 2013; Adalsteinsson and Ferguson-Smith, 2014). In the present study, the ADMA hydrolase *DDAH2* rs805305 (-449 G/C) allele was located in the promoter CG sequence, which may be the active transcription start site. After gene mutation occurs, the frequency of G alleles increases, causing an increase in the transcription of the resting gene promoter containing the A/T gene locus. This results in an increase in the expression of DDAH2 proteins; however, the expressed proteins are less functional, leading to higher blood ADMA in LA patients.

In our study, the exclusion criteria were relatively strict. The subjects had no relationship to one another, and the control group was selected so that the history of the patients matched as closely as possible between the two groups. The sample size was therefore relatively small, and results from a larger study are required to confirm these preliminary findings.

In summary, we demonstrated that the GG genotype of *DDAH2* (-449 G/C) is more common in LA patients than in healthy controls. We also revealed that the G allele is a risk allele that is positively associated with LA in northeastern China, and may increase the levels of ADMA in LA patients. Mutations from the wild-type C to the G allele may alter the function of DDAH2 and reduce the hydrolysis of ADMA, thereby competitively reducing nitric oxide, leading to endothelial vasodilatory dysfunction and, indirectly, to the formation of cerebrovascular lesions. This may result in reduced white matter perfusion and facilitate the development of LA. Therefore, the detection of gene polymorphisms in *DDAH2* may be beneficial for the early detection of LA-susceptible individuals, as well as for early interventions to delay the development of LA.

Additional files:
Additional file 1: Hospital ethics approval (Chinese).

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