AIMS Bioengineering, 8(2): 130–139.
DOI: 10.3934/bioeng.2021012
Received: 07 January 2021
Accepted: 19 February 2021
Published: 03 March 2021

http://www.aimspress.com/journal/Bioengineering

Research article

The association between nicotinamide N-methyltransferase gene polymorphisms and primary hypertension in Chinese Han Population

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Abstract: Although no direct evidence shows that Nicotinamide N-methyltransferase (NNMT) is involved in hypertension to date, many reports have shown that NNMT plays important roles in pathogenesis of metabolic syndrome and in various cardiovascular diseases. Here we explored the genetic association between *NNMT* polymorphisms and primary hypertension. 308 primary hypertension cases (aged < 60 years, blood pressure $\geqslant$ 140/90 mmHg or taking antihypertensive therapies) and 315 controls (aged $\geqslant$ 60 years and without any diagnosed diseases) were recruited from unrelated Chinese Han ethnicity volunteers. Then a case-control study was carried out to explore the genetic association between *NNMT* polymorphism and primary hypertension. A significantly associated SNP (rs1941404) was found in *NNMT* gene ($P < 0.0125$). At this locus, the risks for minor homozygote CC carriers being of hypertension were highly significantly higher than those for the TT + CT carriers (OR = 2.120, $OR_{adjusted}$ = 2.573, P = 0.000, $P_{adjusted}$ = 0.001 and statistical power = 0.997), but the differences between the genotypes CT + CC and TT (dominant genetic model) were not statistically significant (P = 0.321, $P_{adjusted}$ = 0.230 and statistical power = 0.200). In conclusion, rs1941404 in *NNMT* gene sequence is significantly associated with primary hypertension under a recessive inheritance mode. At this locus, the minor homozygote CC carriers are susceptible population and the TT and CT carriers are non-susceptible population.

Keywords: nicotinamide N-methyltransferase (NNMT); cardiovascular diseases; obesity; metabolic syndrome; diabetes; hyperhomocysteinemia

1. Introduction

As one of transmethylation enzymes, NNMT catalyzes nicotinamide methylation using S-adenosylmethionine (SAM) as a methyl donor while producing methylnicotinamide (MNA) and S-adenosyl homocysteine (SAH) [1,2]. As the primary precursor of NAD^+, nicotinamide plays important roles in energy metabolism, and is a promising novel agent in the development of therapies against metabolic disorders [3]. Meanwhile, in the methylation of nicotinamide, SAH is produced, and subsequently broken down to form adenosine and homocysteine (Hcy) [4], which is an independent risk factor for various cardiovascular diseases [5]. Therefore, the activities of NNMT are highly related with metabolic disorders and many cardiovascular diseases, such as coronary heart disease, atherosclerosis and diabetes [3,6]. Although no direct evidence shows that the activities of NNMT are involved in hypertension to date, Giuliante et al. [7] found that the levels of NNMT mRNA, protein and activity were significantly elevated in adipose tissue of the rats with metabolic syndrome, and proposed that NNMT plays important roles in the pathogenesis of metabolic syndrome. As a complication of metabolic syndrome, hypertension is highly associated with obesity, diabetes, and hyperlipidemia. On account of the roles NNMT plays in energy metabolism and cardiovascular diseases, it is reasonable to speculate that the activities of NNMT are involved in hypertension.

At present, over 200 SNPs have been reported in the DNA sequence of *NNMT* [8], and 4 of them (rs10891644, rs694539, rs194140 and rs22562924) were reported to be associated with many noninfectious chronic diseases (NCDs) or energy metabolism. For example, rs694539 has been shown significantly associated with abdominal aortic diseases [9], migraine [10], congenital heart diseases [11], bipolar disorder [12], nonalcoholic steatohepatitis [13], epilepsy [14], schizophrenia [15] and hyperhomocysteinemia [5]; rs10891644 associated with obesity [16]; rs1941404 associated with type II diabetes [8], hyperlipidemia [17] and schizophrenia [15]. Besides these 3 SNPs, in our previous works, we found a SNP in *NNMT* DNA sequence (rs2256292) significantly associated with sport performance and maximal oxygen uptake, which indicates that *NNMT* gene polymorphism is involved in the regulation of energy metabolism [18]. However, the associations between *NNMT* gene polymorphisms and primary hypertension have not been reported so far. We checked all the existing candidate genetic association studies (CGASs) related to *NNMT* gene and all the existing genome-wide association studies (GWASs) on primary hypertension. The result shows that no CGAS has been performed to explore the genetic associations between *NNMT* gene polymorphisms and primary hypertension to date. GWASs have identified more than three hundred SNPs associated with primary hypertension over the past years, but none of them are located in *NNMT* gene sequence [19]. Nevertheless, the vast majority of these GWASs were performed predominantly in Caucasian populations, which suggests the possible existence of many more undiscovered SNPs or at the very least SNPs unique to other populations which are not from Caucasian ancestry [19]. At the same time, GWASs apply the strictest genome-wide statistical significance criterion to avoid the type I error (false positive, the error of rejecting a null hypothesis when it is actually true), which usually leads to false negative discoveries in statistical analyses. Therefore, to test the association for *NNMT* polymorphisms and primary hypertension, a CGAS is still necessary. In this investigation, the primary hypertension cases and controls were all recruited from unrelated Chinese Han population, and then a case-control study was performed to check the associations between primary hypertension and the 4 known SNPs, rs10891644, rs694539,

rs1941404 and rs2256292, which have been shown significantly associated with metabolic disorders, cardiovascular diseases, energy metabolism, or other NCDs. The positions of these 4 SNPs in *NNMT* gene sequence are shown in Figure 1.

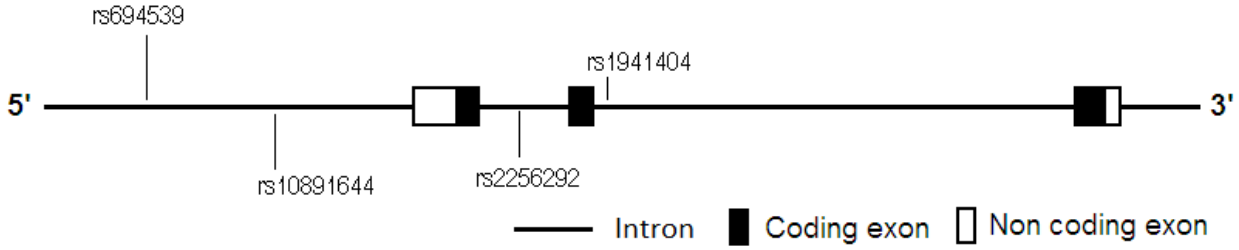

Figure 1. Positions of the 4 SNPs in *NNMT* gene sequence.

2. Subjects and methods

2.1. Subjects

308 primary hypertension cases (aged < 60 years, blood pressure ≥ 140/90 mmHg or taking antihypertensive therapies) and 315 controls (aged ≥ 60 years and without any diagnosed diseases) were recruited from unrelated Chinese Han ethnicity volunteers. Usually, the risk to be of primary hypertension increases with age. Young people without hypertension do not really mean that they are non-susceptible population, but the younger primary hypertension cases mean they are more susceptible to primary hypertension. Thus the healthy controls were selected from volunteers aged ≥ 60 years to make sure that those subjects are real non-susceptible population to primary hypertension, and the primary hypertension cases were selected from the volunteers aged < 60 years to make sure that those subjects are the real susceptible population to primary hypertension. The demographic and clinical characteristics of the 308 patients and the 315 controls are reported in Table 1. The local ethics committee of Jiangxi Normal University approved this investigation, and this investigation conforms to the latest revision of the Declaration of Helsinki.

Table 1. Demographic and clinical characteristics of the hypertension patients and controls.

	Controls (n = 315)	Cases (n = 308)	Significance
Age (years)	71.44 ± 7.91	51.21 ± 8.57	P < 0.001
Gender (male/female)	174/141	162/146	P = 0.52
BMI (kg/m^2)	21.92 ± 4.32	24.55 ± 3.80	P < 0.001
Diabetes, n (%)	0 (0%)	151 (49%)	P < 0.001
hyperlipidemia, n (%)	0 (0%)	85 (28%)	P < 0.001

*Note: BMI, body mass index; Diabetes, fasting plasma glucose ≥ 7.0 mmol/l, and/or plasma glucose ≥ 11.1 mmol/L in 2 h oral glucose tolerance test, or taking anti-diabetic therapies; hyperlipidemia, serum total cholesterol level >220 mg/dL, and/or serum triglyceride level >150 mg/dL.

2.2. Detection of NNMT gene polymorphisms

2.2.1. Main instruments and reagents

The main instruments included a Gene Amp PCR system 9600 (Norwalk, CT.06859 USA), an electrophoresis apparatus (Beijing Junyi Electrophoresis Co., Ltd.), an automatic UV-Visible spectrometer, a biological electrophoresis image analysis system (Shanghai Furi Science & Technology Co., Ltd.), and a Sequencer (ABI). The main reagents included Wizard DNA extraction kit (Promega, USA), PCR primers, dNTPs (Shanghai Hanyu Biological Engineering Co., Ltd.), a Taq enzyme system (Shanghai Biowing Applied Biotechnology Co., Ltd.), 1.5-mL eppendorf tubes, pipette tips of all sizes, and 96-well PCR plates (Haimen Yonghui Experimental Equipment Co., Ltd.).

2.2.2. Extraction of the genomic DNA and designation of the primer and probe sequences

Genomic DNA was extracted from ethylenediamine tetra-acetate (EDTA) whole blood using Wizard DNA extraction kit following the manufacturer's instructions, and then stored at a –80 ℃ refrigerator. The information of the 4 selected SNPs and DNA sequence were downloaded from NCBI database. Based on these, the primer sequences and the probe sequences were designed using Primer 3 online (https://bioinfo.ut.ee/primer3-0.4.0/), and shown in Table 2 and Table 3 respectively.

Table 2. Primer sequences.

SNP	Forward	Reverse
rs694539	5'-CAGCCATCTCAAATGGATGC-3'	5'-GTCCTAGAGTCCTAGAATCC-3'
rs10891644	5'-GGAATTGCTTTCCTTTCCAA-3'	5'-AAGAAGCGTGATGGGAGAAA-3'
rs2256292	5'-TAAGGTCTAGGAGAAGGTAA-3'	5' -CCATGTAACAGACTTTCTGG-3'
rs1941404	5'- CCATTACTCTGGTGCACACA-3'	5'-AAGAGAGATGAGATAGGCCC-3'

Table 3. Probe sequences.

SNP	Allele	Sequence
rs694539	A	TTTTTTTTTTTTTGGTTTGGAAAACCCCTCCAACAT
	G	TTTTTTTTTTTTTGGTTTGGAAAACCCCTCCAACAC
rs10891644	G	TTTTTTTTTTTCCGGGTGCAGTGGCTCACGCCTGTC
	T	TTTTTTTTTTTCCGGGTGCAGTGGCTCACGCCTGTA
rs2256292	C	TTTTTTTTTTTTTTTACATCTGGTGTACAGACTGAAG
	G	TTTTTTTTTTTTTTTACATCTGGTGTACAGACTGAAC
rs1941404	C	TTTTTTTTTTTTTTTGAGATAGGCCCATGTGTGTGCG
	T	TTTTTTTTTTTTTTTGAGATAGGCCCATGTGTGTGCA

2.2.3. Genotyping the SNPs

As described previously [8], PCR-LDR (polymerase chain reaction-ligase detection reaction) was used in genotyping the SNPs. The target DNA sequences were amplified by a method of multiplex PCR in the Gene Amp PCR system 9600 firstly. After the amplification, 1 μL of proteinase k (20 mg/ml) was added, then heated for 10 min at 70 ℃, and quenched for 15 min at 94 ℃. LDR was carried out for 2 min at 95 ℃ and followed by 40 cycles (at 94 ℃ for 15 s and at 50 ℃ for 25 s). The LDR fluorescent products were differentiated in ABI sequencer (PRISM 3730). Ten percent of the PCR-LDR reactions in the detection were performed twice and over 99.5% of them had the matching results, which ensures the results of genotyping are reliable.

2.3. Statistics

Statistical power computation and sample size estimation were carried out using Power and Sample Size Calculation (PS, Ver. 3.0). The allele and genotype frequency distributions and Hardy-Weinberg equilibrium were analyzed with SHEsis online version [20]. In genotype effect analyses, the recessive genetic model and the dominant genetic model were tested using two classification logistic regressions. Statistical P values (two-sided) less than 0.05 were thought as statistical significance. Bonferroni corrections were used in the analyses of the allele and genotype frequency distributions. The corrected statistical significance level was $P < 0.0125$, which was computed as 0.05/4, for 4 SNPs were studied in this investigation. All subjects of the case group were aged ≥ 60 years and all subjects of the control group aged < 60 years, therefore adjustment for age was inappropriate. The gender ratios of the case group and the control group were almost equal, so adjustment for gender was unnecessary. To exclude the influences from the complications of metabolic syndrome, the adjustments were done for BMI (Body mass index), diabetes and hyperlipidemia in analyses of the genotype effects.

3. Results

3.1. Genotype and allele frequency distributions

The genotype and allele frequency distributions of the 4 SNPs are shown in the Table 4. Three SNPs (rs10891644, rs2256292 and rs1941404) were qualified in Hardy-Weinberg equilibrium tests ($P > 0.05$), but only 1 SNP (rs1941404) showed the significant allele differences ($P < 0.0125$, the corrected significance level) and the significant genotype differences ($P < 0.0125$) between the primary hypertension case group and the control group. The primary hypertension case group exhibited a significantly higher frequency of allele C (the minor allele) and a significantly higher frequency of genotype CC than the control group at this locus (Table 4). At the other 3 loci (rs694539, rs10891644 and rs2256292), the allele and genotype frequency distributions did not show statistically significant differences between the primary hypertension case group and the control group after Bonferroni correction ($P > 0.0125$) (Table 4). Therefore, the subsequent analyses of genotype effects were primarily focused on rs1941404.

3.2. Genotype effects of rs1941404 variation on primary hypertension

As shown in Table 4, the genotype and allele frequency distributions of rs1941404 demonstrated that allele C (the minor allele) and genotype CC had the higher risk to be primary hypertension than allele T and genotypes (TT + CT), respectively, which suggests that the genotype effects of rs1941404 variation might be under a recessive inheritance mode. Then the recessive genetic model (CC vs. TT + CT) and the dominant genetic model (CC + CT vs. TT) were used to analyze the genotype effects of rs1941404 variation on primary hypertension. The results showed that the risks for genotype CC carriers to be primary hypertension were highly significantly higher than those for genotype TT + CT carriers (OR = 2.120, $OR_{adjusted}$ = 2.573, P = 0.000, $P_{adjusted}$ = 0.001 and statistical power = 0.997) (Table 5). However, the differences between genotypes CC + CT and genotype TT (CC + CT vs. TT, the dominant genetic model) were not statistically significant (P = 0.321, $P_{adjusted}$ = 0.230 and statistical power = 0.200) (Table 5). Therefore, the variation at this SNP locus (rs1941404) is significantly associated with primary hypertension under a recessive inheritance mode, and genotype CC (the minor homozygote) carriers are the susceptible population and genotypes CT and TT (CT + TT) carriers are the non-susceptible population.

Table 4. Frequency distributions of the primary hypertension cases and controls.

SNPs	Allele			*P*	Genotype			HWE	*P*
rs694539	Case	A: 210(0.35)	G: 386(0.65)	0.534	AA: 40(0.13)	AG: 130(0.44)	GG:128(0.43)		0.045
	Ctrl	A: 210(0.34)	G: 416(0.67)		AA: 25(0.08)	AG: 160(0.51)	GG:128(0.41)	0.009	
rs10891644	Case	G: 404(0.66)	T: 204(0.34)	0.255	GG:126(0.41)	GT: 152(0.50)	TT: 26(0.09)		0.015
	Ctrl	G:426(0.69)	T: 188(0.31)		GG:154(0.50)	GT: 118(0.38)	TT: 35(0.12)	0.090	
rs2256292	Case	C: 254(0.42)	G: 346(0.58)	0.089	CC: 48(0.16)	CG:158(0.53)	GG:94(0.31)		0.206
	Ctrl	C: 236(0.38)	G: 392(0.62)		CC: 38(0.12)	CG: 160(0.51)	GG:116(0.37)	0.127	
rs1941404	Case	C: 300(0.51)	T: 288(0.49)	0.004*	CC: 84(0.29)	CT: 132(0.45)	TT: 78(0.26)		0.0008*
	Ctrl	C: 270(0.43)	T: 360(0.57)		CC: 50(0.16)	CT: 170(0.54)	TT: 95(0.30)	0.07	

*Note: Case, the hypertension group; Ctrl, the control group; HWE, P value of Hardy-Weinberg equilibrium test on the control group; the values of allele and genotype are the number of individuals (frequency); *, P < 0.0125.

Table 5. Genotype effects of rs1941404 variation.

Model	Genotype	Case	Control	OR (95%CI)	Adjusted OR (95%CI)	P($P_{adjusted}$)	Statistical power
Recessive	CC	84(0.62)	50(0.38)	2.120	2.573	0.000**	0.997
	TT + CT	210(0.44)	265 (0.56)	(1.429, 3.144)	(1.511, 4.384)	(0.001)**	
Dominant	CC + CT	216(0.50)	220(0.50)	1.196	1.393	0.321	0.200
	TT	78(0.45)	95(0.55)	(0.840, 1.703)	(0.810, 2.393)	(0.230)	

*Note: Case, the hypertension group; Control, the control group; the values of the case and control are the number of individuals (frequency); OR, odds ratio; CI, confidence interval; Adjusted, adjustment for BMI, diabetes and hyperlipidemia; **, P < 0.01.

4. Discussion

Primary hypertension (also called essential hypertension or idiopathic hypertension), which is a form of hypertension with no identifiable causes, is the most common type of hypertension, accounting for 95% of the hypertensive patients [21]. By definition, primary hypertension has no identifiable causes, but it tends to be familial and age-related, and frequently coexists with obesity, diabetes and hyperlipidemia. To exclude the influences from obesity, diabetes and hyperlipidemia, we did adjustment in the genotype effect analyses of rs1941404, and the adjusted statistical results still indicated that rs1941404 variation significantly increases the risk of hypertension under a recessive inheritance mode.

As mentioned above, although no direct evidence shows that the activities of NNMT are involved in hypertension to date, the existing reports have shown that NNMT's activity is highly related with metabolic disorders and many cardiovascular diseases, such as diabetes, atherosclerosis and coronary heart disease [3,6]. It is reasonable to speculate that the activities of NNMT are involved in hypertension. Genetic association studies have shown that 4 SNPs, rs694539, rs10891644, rs2256292 and rs1941404, in DNA sequence of *NNMT* gene are significantly associated with various NCDs. However, in this investigation, only rs1941404 showed the significant associations between the hypertension case group and the control group after Bonferroni corrections. In previous studies, rs1941404 variation has shown significant associations with schizophrenia [15], hyperlipidemia [17] and T2D [8]. Primary hypertension frequently coexists with T2D and/or hyperlipidemia, especially T2D, which seems to share common pathophysiological pathways with hypertension [22]. Evidences show that hypertension is present in more than 50% of people with diabetes [23] and approximately 50% of people with hypertension show insulin resistance [24]. Hypertension also can be caused by insulin resistance. Bonora et al. [25] reported that insulin resistance is independently associated with blood pressure. Therefore, it is not surprising that rs1941404 variation increases the risk of hypertension under the recessive inheritance mode, because Li et al. have reported that the variation at this locus also significantly increases the risks of T2D [8] and hyperlipidemia [17] under the recessive inheritance mode.

Additonally, Hcy is a risk factor for hypertension, although the exact mechanism is poorly understood [26]. The role of NNMT in Hcy synthesis partly explains the association between *NNMT* gene polymorphisms and hypertension. As mentioned above, in the methylation of nicotinamide, which is catalyzed by NNMT, SAH is produced, and subsequently broken down to form Hcy. Hidru et al. [27] found that elevated Hcy confers a corresponding increase in diastolic blood pressure (DBP), systolic blood pressure (SBP), and prevalence of hypertension in the middle-aged and elderly Chinese population. Shi et al. [26] reported that blood pressure of the inpatients with hyperhomocysteinemia (HHcy) was significantly higher than that of the inpatient without HHcy, and that blood pressure of the Wistar rats was significantly increased with the increases of serum Hcy levels. Therefore, NNMT might affect blood pressure through regulating Hcy synthesis. Souto et al. [5] conducted a genomewide linkage scan for genes affecting variation in plasma Hcy levels, and found one haplotype (including rs694539) in *NNMT* gene significantly associated with plasma Hcy levels in Spanish population, which indicates that *NNMT* gene could be a major genetic determinant of plasma Hcy levels in Spanish families. Zhang et al. [28] further tested the association between the A/G polymorphism (rs694539) of *NNMT* gene and plasma Hcy concentration in Japanese population and reported: 1) *NNMT* genotype may affect the Hcy levels in subjects with

HHcy (Hcy ≥ 13.7 umol/L), but not in subjects with normal Hcy concentration; 2) In subjects aged ≥ 40 years, the *NNMT* GG genotype had a higher plasma Hcy concentration than AA + AG genotype under low plasma folate level (<10.9 nmol/L). Although the direct association between rs1941404 variation and plasma Hcy concentration has not been reported yet, rs1941404 variation and several related haplotypes that include rs1941404 are significantly associated with a hyperhomocysteinemia-related disorder, schizophrenia [15,29]. These reports indicate that rs1941404 variation might increase the risk of hypertension through augmenting plasma Hcy concentration.

NNMT enzyme activity has a significant variability among individual, and this difference was attributed not to the structure of the enzyme but to the expression levels of mRNA and protein [30,31]. Earlier studies carried out in human liver biopsies detected no SNPs or insertion/deletion events within either the exons or the 5'-flanking region of *NNMT* [30,31]. Smith et al. [31] found no cDNA transcript differences among subjects with low, intermediate and high NNMT activity, which indicates that the activity differences stem from differences in mRNA steady-state levels and protein expression levels. As Figure 1 shows, the 4 SNPs studied in this investigation are all in introns. These noncoding SNPs are not able to change the structure of NNMT, but they might be related to the regulation of transcription, thus changing the phenotype. As mentioned above, Bromberg et al. [15] found that rs1941404 variation and several related haplotypes that include rs1941404 are significantly associated with a hyperhomocysteinemia-related disorder, schizophrenia. Meantime, they assessed *NNMT* mRNA levels in post-mortem frontal cortex of schizophrenia patients [15]. The results show that *NNMT* mRNA levels were about 35% lower in schizophrenia patients compared to those in control subjects, and that one intronic SNP (rs949374) in *NNMT* gene was significantly associated with *NNMT* mRNA levels before correction for multiple testing (P = 0.047) [15]. Therefore, the intronic SNP (rs1941404) may also play a regulatory role in the transcription of *NNMT* gene.

5. Conclusions

For the first time we found that a SNP (rs1941404) in the DNA sequence of *NNMT* gene is significantly associated with primary hypertension under a recessive inheritance mode. At this locus, the minor homozygote CC carriers are the susceptible population and the CT and TT carriers are the non-susceptible population. Although precise mechanism of the regulation process is still unclear, this finding suggests that the variant of rs1941404 is involved in the etiopathology of primary hypertension in Chinese Han population.

Acknowledgments

This investigation was supported by the National Science Foundation of China (21365013) , the higher education reform research project of Jiangxi Province (JXJG-18-2-41) and the Key Lab of Aquatic Training Monitoring and Intervention of General Administration of Sport of China (201901).

Conflict of interest

The authors declare no conflict of interest.

Author contributions

Xiang-Xiang Guan: investigation, evaluation, and writing; Xiao-Juan Zhu: investigation; Zhao-Hui Deng: investigation; Yu-Rong Zeng: investigation; Jie-Ru Liu: investigation; Jiang-Hua Li: investigation, evaluation, conceptualization, and writing.

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