ORIGINAL ARTICLE

Association of endothelial nitric oxide synthase gene (G894T) polymorphism and hypertension in diabetic Bataknese patients

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ABSTRACT

Aim To assess endothelial nitric oxide synthase (eNOS) gene (G894T) polymorphism and nitric oxide (NO) level in hypertensive diabetic Bataknese patients.

Methods A hospital-based, case control study (hypertensive and normotensive diabetic patient) was conducted. Genotyping of eNOS gene (G894T) was done using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP). Nitric oxide was quantified by sandwich *enzyme*-linked immunosorbent assay (Sandwich ELISA).

Results GT polymorphism and T allele were higher in the hypertensive diabetic patients, 37.5% (p=0.6) and 54% (p=0.39), respectively. Nitric oxide level tended to be lower in the hypertensive diabetic patients (88.87μmol/L) comparing to the normotensive (95.42 μmol/L (p=0.54), as well as GT and TT polymorphism type (p=0.75).

Conclusion eNOS gene (G894T) polymorphism is not associated with NO level and hypertension in the diabetic Bataknese patients.

Key words: diabetes mellitus, hypertension, nitric oxide, nitric oxide synthase type III, polymorphism

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INTRODUCTION

Hypertension is one of the major public health problems in the world. It is called "silent killer" since it may have no warning signs and symptoms (1). Around 31% of all adults worldwide suffer from hypertension. While prevalence decreases in high-income countries, hypertension increases in low/middle-income countries (2). In 2015, prevalence of hypertension in Indonesia was 33.4% (3). Hypertension could cause numerous health problems to human body. One of the most deadly complications of hypertension is cardiovascular disease, which accounts for 35% of deaths in Indonesia (4).

Hypertension is associated with several risk factors. Type 2 diabetes mellitus (T2DM) is one of the common underlying diseases of hypertension. When hypertension and T2DM co-exist, the risk of stroke or coronary arterial disease (CAD) will increase 2-4 times (5,6). Pathogenesis of hypertension in T2DM is a complex process. Endothelial dysfunction is the main cause of cardiovascular complication in T2DM (7,8). Hyperglycaemia, free fatty acid, and insulin resistance reduce nitric oxide (NO) and increase endothelin-1 that cause prolonged vasoconstriction leading to hypertension (9).

Nitric oxide synthesis is catalysed by nitric oxide synthases (NOS). Endothelial NOS (eNOS) is an NOS isoform responsible for catalysing the formation of NO from l-arginine in endothelial cells, and therefore plays a central role in vascular function (10). The eNOS is expressed by the eNOS gene. Glu298Asp (aspartate instead of glutamate in codon 298) as a result of G894T (substitution of guanine by tymin in position 894 at exon 7) is an eNOS gene polymorphism known to be responsible for altered NO production and to cause some cardiovascular problems, such as hypertension and CAD (11-14) and in some theories also causes T2DM (15-16).

A study on eNOS gene polymorphism in hypertensive diabetic patients has never been conducted on Bataknese ethnicity, Indonesia. Knowledge in this field is important to determine better management for this population.

The aim of this study was to assess eNOS gene (G894T) polymorphism and NO level in hypertensive and normotensive diabetic Bataknese patients in Indonesia.

PATIENTS AND METHODS

Patients and study design

This was a hospital-based, case control study of eNOS gene polymorphism in T2DM patients. Samples were all Bataknese T2DM patients who visited Internal Medicine Department of Universitas Sumatera Utara Hospital, Medan, Indonesia from June to July 2019.

Patients with T2DM and hypertension were incorporated in a case group, while patients with T2DM but without hypertension (normotensive) were included in a control group. Hypertension was defined as having systolic blood pressure ≥140 mmHg or diastolic blood pressure ≥90 mmHg (17). Cases and controls were matched on sex, age, HbA1C and plasma glucose level. Inclusion criteria were T2DM patients and Bataknese ethnicity (pure ethnic for three generation). T2DM was defined as having fasting plasma glucose (FPG) ≥126 mg/dL and 2 hours post-prandial plasma glucose (2h PP) ≥200 mg/dL (18). Bataknese is an ethnic group originating from Medan (19). In this study, we included only pure descendants of Batak tribe whose parents and grandparents are of pure Bataknese ethnicity. Patients with secondary hyperglycaemia (e.g. corticosteroid medication) and kidney complications were excluded.

This research was approved by the Ethical Committee of School of Medicine, Universitas Sumatera Utara.

Methods

Demographic data, glucose level, NO level, eNOS gene polymorphism were collected in this study. Sex and age were obtained from the medical record. HbA1C and blood plasma level were examined by Automatic Cobas® 6000 C (Roche Diagnostics, Mannheim, Germany) according to manufacturer's instruction. Nitric oxide was quantified by sandwich *enzyme*-linked immunosorbent assay (Sandwich ELISA) on serum sample. Six ml blood was taken in non-ethylenediaminetetraacetic acid (*EDTA*) vacutainer and processed to obtain serum. Endothelial NOS gene (G894T) polymorphism was examined by deoxyribonucleic acid (DNA) isolation, continued by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP).

Isolation of DNA was conducted by using *Wizard Genomic DNA Purification Kit* (Promega, Madison, WI). PCR was done in 30 µl PCR mixture consisting of 15 µL *GoTaq Green Master Mix* (Promega), 11 µL ddH_2O, 1 µl forward primer (5'-AACCCCCTCTGGCCCACTCCC-3') 10 µM and 1µL reverse primer (5'-TCCATCCCACCCAGTCAAT-3') 10 µM and 2 **µL** DNA (15). The mixture was incubated initially at 95 °C for 8 minutes, followed by 40 cycles of denaturation at 94 °C for 1 minute, annealing at 63 °C for 45 seconds, extension at 72 °C for 1 minute and final extension at 72 °C for 7 minutes. After that, RFLP was carried out by incubating 5 **µL** PCR product, 0.5 **µ**L 10× Buffer R (with BSA) (Thermoscientific), 3.5 µl H_2O and 0.5 **µL** Mbol restriction enzyme (Thermoscientific) in 37 ^oC for 1 hour (15). The electrophoresis of PCR-RFLP products was performed in 3% agarose gel containing ethidium bromide (0.5 µg/mL) and photographed using the gel documentation system. Results were categorized into homozygote GG, which showed single 206 bp band, homozygote TT 119 bp and 87 bp bands, and heterozygote 206 bp, 119 bp and 87 bp bands.

Statistical analysis

Bivariate analysis between age, HbA1C and plasma glucose level in case and control group were done using independent t-test. Analysis of nitric oxide level and eNOS gene polymorphism between case and controls were done using independent t-test and chi-square test, respectively. eNOS gene polymorphism was analysed with nitric oxide level by using ANOVA. Hardy-Weinberg equilibrium was performed in both case and control group for the analysed polymorphism.

RESULTS

A total of 112 patients were included in this study with 56 patients in both case and control group. The case group consisted of 23 male and 33 female patients, while the control group had 24 male and 32 female patients. All patients' characteristics were not different statistically (Table 1) (p>0.05).

No significant differences between the groups were observed both in NO level (p=0.536) and eNOS gene polymorphism (p=0.601). In the case group, NO level (mean 88.87 µmol/L) tended to be lower than the control group (mean 95.42 µmol/L) (Table 1). More GT polymorphism (37.5%) was seen in the case group than the control group (29.57%), while more GG polymorphism (60.71%) was seen in the control group than the case group (60.71%). Moreover, there was no association between polymorphism type and NO level (GG mean 95.02µmol/L, GT 87.09 µmol/L, TT 80.80 µmol/L, p=0.75), although the level tended to be lower in GT and TT polymorphism. Genotypic distribution of the G894T polymorphism was in Hardy-Weinberg Equilibrium (HWE) among both case and control group (p = 0.568 and p=0.265, respectively) (Table 2).

Table 1. Patient characteristic and nitric oxide (NO) level in the case and control group

Variable	Case group (Mean±SD)	Control group (Mean±SD)	p
Age (years)	59.32 ± 8.562	57.71 ± 10.302	0.371
HbA1C (%)	8.305 ± 1.875	7.659± 1.725	0.060
FPG (mg/dL)	184.93 ± 73.322	165.29 ± 71.126	0.153
2hPP (mg/dL)	247.38 ± 92.994	228.41 ± 77.670	0.244
NO (µmol/L)	88.87±54.359	95.42±57.133	0.536

FBG, fasting plasma glucose; 2hPP, 2 hours post-prandial plasma glucose

Table 2. Polymorphism type in the case and control group

eNOS gene polymorphism		No (%) of patients in the group		Total	p
		Case	Control		
Genotype	GG	34 (60.71)	39 (69.64)	73	
	GT	21 (37.50)	16 (28.57)	37	0.601
	TT	1 (1.79)	1 (1.79)	2	
Allele	G	89 (79.46)	94 (83.93)	183	0.388
	T	23 (20.54)	18 (16.07)	41	

eNOS, endothelial nitric oxide synthase

DISCUSSION

To our knowledge, this is the first study assessing genetic background of hypertension in T2DM patients in Indonesia. Moreover, a study of eNOS gene polymorphism has never been performed in a Bataknese patient who resides in the western part of Indonesia. Only diabetic patients were included because the combination of T2DM and hypertension could lead to worse complications. Special management is needed for this population.

Nitric oxide is well-known for its capability to reduce the risk of cardiovascular disease in humans, including hypertension. T2DM is a risk factor of low NO level in humans. Low NO level in T2DM could cause vasoconstriction resulting in hypertension (9). In this study, we found no difference of NO level in hypertensive and normotensive diabetic patients. However, mean NO level tended to decrease in hypertensive diabetic

patients. Although NO is usually higher in both diseases when they occur separately (20), the study on NO level in co-existence of T2DM and hypertension is scarce and conflicting. Manju et al. stated that NO level was lower in diabetic patients as compared to non-diabetic patients, but failed to demonstrate significant difference when comparing hypertensive and normotensive diabetic patients (21). Shahid et al. found lower NO level in hypertensive diabetic patients than in normotensive diabetic patients (22). Given the fact that our study and published studies are case control studies with a low number of patients and a high number confounding factors, an exact causality could not be made. A cohort study should be conducted in the future to assess this problem.

The effect of eNOS gene polymorphism on hypertensive and normotensive diabetic patients was also assessed. We found more GT polymorphism types than GG polymorphism types in hypertensive diabetic patients, although the difference was not significant. T allele also tended to be higher in hypertensive diabetic patients. G894T polymorphism was associated with hypertension in some ethnicities. Asian, especially Chinese and Indian ethnicities were associated with a higher risk for hypertension caused by this genetic predisposition (23,24). In some areas outside Asia, such as Egypt, the association was also observed (13). Allele T was associated with the higher risk for hypertension, especially in females (25). However, in several studies, for example in Sudan (26), among East Asian (27), and among Whites (23), no association was found. In Indonesia, one study showed no association between eNOS gene (G894T) polymorphism and essential hypertension in Javanese patients, who live on a separated island with Bataknese. We have concluded that the effect of G894T polymorphism is ethnicity specific, therefore, the management should be made based on the ethnic group. This study indicates that the management of hypertension might not have to consider G894T polymorphism in Bataknese diabetic patients.

Furthermore, the effect of G894T polymorphism on NO level was studied. In this study no association was found. Single nucleotide polymorphism (from guanin to tymin) happening in eNOS gene was known to cause reduction of NO level in humans. However, it was not observed in Asian ethnicity and CAD (28). In this study, patients included also had T2DM in addition to hypertension. T2DM as an underlying disease could contribute to lower NO level in both groups. Also, other factors affecting NO level were not assessed. Causal relationship could not be drawn in this study.

This study has several limitations to be considered for interpretation. Several confounding factors were not assessed. Since hypertension and T2DM are of multifactorial etiology, the cause of NO elevation may not be only a result of gene polymorphism. For example, as the mean age of patients in both groups was above 50 years, we could suspect that the environmental effect could mask the effect of genetic background. In a next study, younger patients and several confounding factors should be addressed to produce better results. Also, the case-control nature of this study prevents us from making a conclusion on causal relationship of G894T polymorphism and hypertension in diabetic patients.

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TRANSPARENCY DECLARATION

Conflicts of interest: None to declare.

REFERENCES

1. World Health Organization. Cardiovascular Diseases. 2019. https://www.who.int/news-room/fact-sheets/detail/hypertension (01 July 2019)
2. Bloch MJ. Worldwide prevalence of hypertension exceeds 1.3 billion. J Am Soc Hypertens 2016; 10:753-4.
3. Peltzer K, Pengpid S. The prevalence and social determinants of hypertension among adults in Indonesia: a cross-sectional population-based national survey. Int J Hypertens 2018; 5610725.
4. World Health Organization. Indonesia. 2019. https://www.who.int/nmh/countries/idn_en.pdf (01 July 2019)
5. Lonati C, Morganti A, Comarella L, Mancia G, Zanchetti A, IPERDIA Study Group. Prevalence of type 2 diabetes among patients with hypertension under the care of 30 Italian clinics of hypertension: results of the (Iper)tensione and (dia)bete study. J Hypertens 2008; 26:1801-8.

6. Cheung B. 2010. The hypertension-diabetes continuum. J Cardiovasc Pharmacol 2010; 55:333-9.
7. Avogaro A, Albiero M, Menegazzo L, de Kreutzenberg S, Fadini GP. Endothelial dysfunction in diabetes: the role of reparatory mechanisms. Diabetes care 2011; 34(Supplement 2):S285-90.
8. Tan KC, Chow WS, Ai VH, Lam KS. Effects of angiotensin II receptor antagonist on endothelial vasomotor function and urinary albumin excretion in type 2 diabetic patients with microalbuminuria. Diabetes Metab Res Rev 2002; 18:71-6.
9. Lüscher TF, Creager MA, Beckman JA, Cosentino F. Diabetes and vascular disease: pathophysiology, clinical consequences, and medical therapy: Part I. Circulation 2003; 108:1527-32.
10. Leineweber K, Moosmang S, Paulson D. Genetics of NO deficiency. Am J Cardiol 2017; 120(8S):S80-8.
11. Men C, Tang K, Lin G, Li J, Zhan Y. ENOS-G894T polymorphism is a risk factor for essential hypertension in China. Indian J Biochem Biophys 2011; 48:154-7.
12. Moraes MP, eSilva KSF, Lagares MH, Barbosa AM, Martins JVM, Campedelli FI, da Costa IR, Rodrigues DA, Moura KKVO. Polymorphism of the genes eNOS, GSTT1 and GSTM1 are significantly associated with atherosclerotic disease in hypertensive patient. Genet Mol Res 18:GMR18089.
13. Arafa S, Abdelsalam S, El-Gilany AH, Mosaad YM, Abdel-Ghaffar A. Endothelial nitric oxide synthase Glu 298 Asp (G894T) and apolipoprotein E gene polymorphism as possible risk factors for coronary heart disease among Egyptians. Egypt Heart J 2018; 70:393-401.
14. Zhu B, Si X, Gong Y, Yan G, Wang D, Qiao Y, Liu B, Hou J, Tang C. An association between the endothelial nitric oxide synthase gene G894T polymorphism and premature coronary artery disease: a meta-analysis. Oncotarget 2017; 8:77990-8.
15. Moguib O, Raslan HM, Rasheed IA, Effat L, Mohamed N, El Serougy S, Hussein G, Tawfeek S, AbdelRahman AH, Omar K. Endothelial nitric oxide synthase gene (T786C and G894T) polymorphisms in Egyptian patients with type 2 diabetes. J Genet Eng Biotechnol 2017; 15:431-6.
16. Angeline T, Krithiga HR, Isabel W, Asirvatham AJ, Poornima A. Endothelial nitric oxide synthase gene polymorphism (G894T) and diabetes mellitus (type II) among South Indians. Oxid Med Cell Longev 2011; 2011:462607.
17. Chobanian AV, Bakris GL, Black HR, Cushman WC, Green LA, Izzo Jr JL, Jones DW, Materson BJ, Oparil S, Wright Jr JT, Roccella EJ. The seventh report of the joint national committee on prevention, detection, evaluation, and treatment of high blood pressure: the JNC 7 report. J Am Med Assoc 2003; 289:2560-71.
18. American Diabetes Association. Classification and diagnosis of diabetes. Diabetes Care 2015; 38:8-16.
19. Simanjuntak BA. Struktur sosial dan sistem politik Batak Toba hingga 1945 (Toba Batak social structure and political system until 1945) [Indonesian]. Jakarta: Yayasan Obor Indonesia, 2006.
20. Ayub T, Khan SN, Ayub SG, Dar R, Andrabi KI. Reduced nitrate level in individuals with hypertension and diabetes. J. Cardiovasc Dis Res 2011; 2:172-6.
21. Manju M, Mishra S, Toora BD. Relationship between glycosylated hemoglobin, serum nitric oxide and mean arterial blood pressure. Int J Biomed Sci 2014; 10:252-7.
22. Shahid SM, Mahboob T. Diabetes and hypertension: correlation between glycosylated hemoglobin (HbA1c) and serum nitric oxide (NO). Aust J Basic Appl Sci 2009; 3:1323-7.
23. Niu W, Qi Y. An updated meta-analysis of endothelial nitric oxide synthase gene: three well-characterized polymorphisms with hypertension. PloS One 2011; 6:e24266.
24. Shankarishan P, Borah PK, Ahmed G, Mahanta J. Endothelial nitric oxide synthase gene polymorphisms and the risk of hypertension in an Indian population. Biomed Res Int 2014; 2014:793040
25. Li J, Cun Y, Tang WR, Wang Y, Li SN, Ouyang HR, Wu YR, Yu HJ, Xiao CJ. Association of eNOS gene polymorphisms with essential hypertension in the Han population in southwestern China. Genet Mol Res 2011; 10:2202-12.
26. Gamil S, Erdmann J, Abdalrahman IB, Mohamed AO. Association of NOS3 gene polymorphisms with essential hypertension in Sudanese patients: a case control study. BMC Med Genet 2017; 18:128.
27. Zintzaras E, Kitsios G, Stefanidis I. Endothelial NO synthase gene polymorphisms and hypertension: a meta-analysis. Hypertension 2006; 48:700-10.
28. Luo Z, Jia A, Lu Z, Muhammad I, Adenrele A, Song Y. Associations of the NOS3 rs1799983 polymorphism with circulating nitric oxide and lipid levels: a systematic review and meta-analysis. Postgrad Med J 2019; 865:361-71.