Medica Hospitalia
Journal of Clinical Medicine
Med Hosp 2023; vol 10 (1) : 63–68

Original Article

Analysis of Genetic Variation of Angiotensinogen M235T Gene in Ischemic Stroke Patients treated at Dr. Kariadi General Hospital, Semarang using Polymerase Chain Reaction Restriction Fragment Length Polymorphism (PCR-RFLP) Method

Dodik Tugasworo, Retnaningsih, Aditya Kurnianto, Suryadi, Dani Rahmawati, Rahmayanti, Jethro Budiman

Department of Neurology, Dr. Kariadi Hospital / Faculty of Medicine Diponegoro University, Semarang, Indonesia

p-ISSN: 2301-4369 e-ISSN: 2685-7898
https://doi.org/10.36408/mhjcm.v10i1.833

Accepted: September 27th, 2022
Approved: January 16th, 2023

Author Affiliation:
Department of Neurology,
Dr. Kariadi Hospital/Faculty of Medicine
Diponegoro University, Semarang,
Indonesia

Author Correspondence:
Dodik Tugasworo
Dr. Sutomo Street No. 16 Semarang,
Central Java 50244, Indonesia

E-mail:
dodiktugasworo2020@gmail.com

Abstract

Background : Stroke is the leading cause of death and disability in the world. The incidence of ischemic stroke is influenced by genetic factors, environmental factors and their interactions. Genetic variation of the Angiotensinogen (AGT) M235T gene is associated with hypertension and diabetes mellitus, which are risk factors for stroke. The objectives of this study was to examine the genetic variation of the Angiotensinogen M235T gene in patients with Ischemic Stroke treated at Dr. Kariadi General hospital, Semarang.
Methods : The subjects of the study were 72 ischemic stroke patients who were treated at the outpatient clinic of the Neurology Department Dr. Kariadi Semarang in January – December 2013. DNA extraction of research subjects was performed at the CEBIOR laboratory, Diponegoro National Hospital from January to March 2020. Amplification was performed with Polymerase Chain Reaction (PCR). Digestion of PCR products was using Restriction Fragment Length Polymorphism (RFLP) method.
Results : Out of 72 samples, the AGT M235T CT were found in 37 samples (51.4%), the AGT M235T TT gene was found in 35 samples (48.6%) and no samples showed the AGT M235T CC.
Conclusion : There are 3 types of genetic variants of the AGT M235T gene, including the AGT M235T CT, the AGT M235T TT and the AGT M235T CC. Among the three types of variants, the variant of the AGT M235T CT gene is the most common variant found in ischemic stroke patients treated at the Dr. Kariadi General Hospital Semarang.

Keywords : AGT M235T, Angiotensinogen, hypertension, ischemic stroke

INTRODUCTION

Stroke is the main cause of morbidity and mortality in the world.[1,2] There was around 25,7 juta million stroke cases in the world in 2013, which approximately 80% of stroke cases are ischemic strokes. Ischemic stroke is a multifactorial disease with a strong genetic component.[2,3] Ischemic stroke is influenced by genetic factors, environmental factors and the interaction of both factors.[1]

Data from various studies conducted mostly on Caucasian ethnicity showed that there were 23 variants of the AGT gene that had been cloned and sequenced, genetic variations were found in the promoter (g.-217G> A, g.-20A> C, g.-6G> A) and in exon 2 (p.T174M and M235T) which are reported to be associated with essential hypertension and cardiovascular disease and stroke. Polymerase chain reaction restriction fragment length polymorphism (PCR-RFLP) is a technique for analyzing DNA fragments using restriction enzymes with endonucleases.[4–6]

The lack of data regarding the genetic contribution of patients with cardiovascular disease such as ischemic stroke in Asian populations, especially Indonesia, has encouraged researchers to identify the genetic variant of angiotensinogen in post-ischemic stroke patients. Angiotensinogen was chosen because in various studies it was stated that angiotensinogen is associated with hypertension and diabetes mellitus which are risk factors for stroke.

MATERIALS AND METHODS

The sample in this study was DNA storage from 72 subjects of a study entitled "Pengaruh Faktor Risiko terhadap Progresivitas Aterosklerosis Penderita Pasca Stroke Non Hemoragik" (main researcher: Dr. dr. Dodik Tugasworo, Sp.S(K)) at the outpatient clinic of the Neurology Department of Dr. Kariadi General Hospital Semarang in January – December 2013". The study subjects had inclusion criteria including acute ischemic stroke patients for the first time, 48 hours of stroke onset which had been proven by head CT scan without contrast and willing to participate in the study. Exclusion criteria for the subjects of this study included patients with haemorrhagic stroke, as evidenced by a non-contrast CT scan of the head; patients with severe systemic diseases, including: Chronic Kidney Disease, as evidenced by measuring the Glomerular Filtration Rate (GFR) and examination of serum urea and creatinine, chronic liver disease, proven by laboratory tests of liver function (such as: SGOT, SGPT, Gamma GT, and Alkali Phosphatase), malignancy, proven by a complete history of malignancy in the patient and family and physical examination, Congestive Heart Failure, proven by physical examination and examination electrocardiography; acute ischemic stroke patients who died or were discharged before the 7th day of onset; acute ischemic stroke patients who died before the 14th day of onset; patients died; and patients withdrew.

Analysis of genetic variation

Analysis of genetic variation of the AGT M235T gene by PCR-RFLP was carried out from January to March 2020 at the CEBIOR Laboratory, Diponegoro National Hospital. Amplification with Polymerase Chain Reaction was carried out using a Forward primer with a DNA sequence of 5'-CAGGGTGCTGTCCACACTGGACCCC-3' and Reverse with a DNA sequence of 5'-CCGTTTGTGCAGGGCCTGGCTCTCT-3'. The total volume of each PCR reaction was 24 µl containing 400 ng of genomic DNA, 1.0 µl of primer F (Sigma, city, country), 1.0 µl of primer R (Sigma), 1.0 µl of deoxynucleotide triphosphates (dNTP), 2.5 µl of MgCl2 and 0.25 µl of Taq DNA polymerase (MRC Holland lot.D56). The PCR reaction was carried out using a thermocycler with the program: initial duplex DNA denaturation 10 minutes at 95°C, followed by 35 cycles consisting of: 1 minute denaturation at 94°C, 1 minute primers annealing at 59°C and 1 minute 30 seconds primers extension at 72°C. Final elongation for 10 minutes at 72°C.

AGT M235T gene variant was detected by Restriction Fragment Length Polymorphism (RFLP) technique using restriction endonuclease Tth111I enzyme. Product PCR was carried out in a 100 µl reaction mixture and contained: 85 µl H2O, 10 µl buffer and 5U Tth111I. The restriction mixture was ingested for >3 hours at 55°C (37°C overnight). DNA fragments were visualized in 3% Agarose gel stained with 8µl 10mg/mlethidium bromide. For normal (wild type) genotype (CC) is a PCR product at 165bp, with no truncation site for Tth111I. M235T homozygous subject (TT genotype) after enzymatic destruction with Tth111I on two fragments of 141 bp and 24 bp. Heterozygous subjects (CT genotype) at 165 bp, 141 bp and 24 bp.

RESULTS

In this study, the number of samples analyzed was 72 samples from 72 subjects who had completed all research procedures in the preliminary study. The mean age of the subjects was 61.64 ± 7.995 years, most of the subjects were male (61.1%). The average BMI of the study subjects was normal, 22.27 ± 2.138 kg/m^2 (Table 1).

The results of the analysis of genetic variation of the AGT M235T gene by PCR-RFLP showed that the AGT M235T CT gene variant was the most variant, 37 (51.4%) samples, while the AGT M235T TT variant was found in 35 (48.6%). In this study, there were no samples showing AGT M235T CC variants (Table 2).

TABLE 1
Subject characteristics

Variable (n=72)		F	%	Mean±SD
Age (year)	1–60	30	41.7	61.64 ± 7.995
	>60	42	58.3	
Gender	Male	44	61.1	
	Female	28	38.9	
BMI (kg/m^2)				22.27 ± 2.138
Smoking history	Yes	31	43.1	
	No	41	56.9	
Diabetes Mellitus	Yes	6	8.3	
	No	66	91.7	
Dyslipidemia	Yes	62	86.1	24 (33.3%)
	No	10	13.9	
Obesity	Yes	10	13.9	
	No	62	86.1	
Hypertension	Yes	24	33.3	
	No	48	66.7	

TABLE 2
Subject characteristic based on gene variant AGT M235T

Variable (n=72)	Gene variant AGT M235T		
	CC	CT	TT
Gene variant AGT M235T(%)	0	51.4	48.6
Age (year)	0	60.54±8.27	62.80±7.63
Gender, male (%)	0	26.38	34.72
BMI (kg/m^2)	0	22.25±2.20	22.30±2.09
Smoking history (%)	0	19.44	23.61
Diabetes Mellitus (%)	0	4.16	4.16
Dyslipidemia (%)	0	43.05	43.05
Obesity (%)	0	6.94	6.94
Hypertension (%)	0	20.83	12.5

DISCUSSION

The human AGT gene is a member of the serpin gene superfamily. The human AGT Complimentary DNA (cDNA) is 1,455 nucleotides long and codes for 485 amino acids in proteins. The AGT gene contains five exons and four introns, spanning 13 kb (Figure 1).[7,8]

The AGT gene provides instructions for making a protein called angiotensinogen. This protein is part of the renin-angiotensin system, which regulates blood pressure and the balance of fluids and salts in the body. In the first step of the blood pressure regulation process, angiotensinogen is converted to angiotensin I. Through an additional step, angiotensin I is converted to

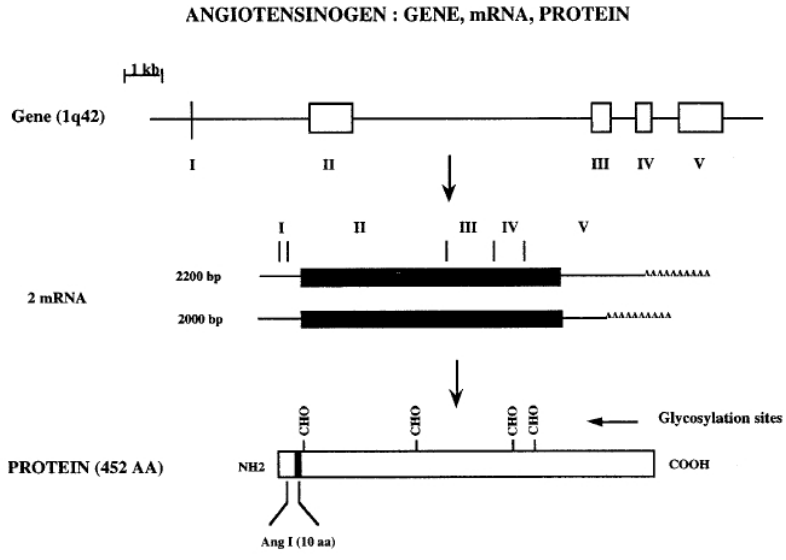

Figure 1. Schematic structure of AGT gene, mRNA, and protein[9]

angiotensin II. Angiotensin II causes blood vessels to narrow (constrict), which results in an increase in blood pressure. Angiotensin II also stimulates the production of the hormone aldosterone, which triggers the absorption of salt and water by the kidneys. Increasing the amount of fluid in the body also increases blood pressure. Proper blood pressure during fetal growth delivers oxygen to developing tissues, necessary for the normal development of the kidneys, especially for structures called the proximal tubules, and other tissues. In addition, angiotensin II may play a more direct role in kidney development, by influencing growth factors involved in the development of kidney structures.[10,11]

The genetic variation of the AGT gene that occurs in exon 2 involves a transition from thymine to cytosine which results in the replacement of methionine by threonine at amino acid position 235 to form the AGT gene variant M235T.[12-14] In this study, AGT gene mutations were found in 100% of the study subjects. AGT M235T CT gene variant was found in 37 subjects (51.38%), 19 subjects were male, 18 subjects were female. AGT M235T TT gene variant was found in 35 subjects (48.61%), 25 male subjects and 10 female subjects. This is contrary to the results of research from Mondry *et al*. (2005) which stated that the AGT M235T TT gene variant was found mostly in female subjects.[15]

The pathogenesis of essential hypertension is influenced by genetic and environmental factors. Mutations in genes related to hypertension can affect blood pressure through changes in salt and water reabsorption by nephrons. Genes of the renin-angiotensin system (RAS) have been studied extensively for their role in blood pressure control. Angiotensinogen (AGT) gene variants may be associated with an increased risk of essential hypertension. Case Control Study by Kooffreh *et al*. (2012) in a population involving 1308 subjects (612 hypertensive patients and 696 controls), ethnic Calabar and Uyo, Nigeria showed that the prevalence of homozygous AGT mutations was 88.4% in subjects with hypertension and 92.2% in subjects without hypertension, whereas for heterozygous mutations, the prevalence was 10.9% in subjects with hypertension and 7.5% in controls. In contrast to the results of Raharjo *et al*. (2013) who stated that the genetic variation of M235T angiotensinogen had no association with the incidence of essential hypertension in ethnic Southeast Sulawesi.[16] Study by Shamaa *et al*. (2015) who aimed to evaluate the frequency of the AGT (M235T) variant in relation to essential hypertension in a group of Egyptian residents found that there is a positive risk of developing essential hypertension if you have the T allele in both homozygous and heterozygous conditions. Out of a total of 24 subjects with hypertension in this study, 15 (62.5%) subjects had the AGT M235T CT gene variant, 9 other subjects had the AGT M235T TT gene variant.[17,18]

Subjects with wild type were not found in this study, this is because the prevalence of wild type is generally 0.3% in subjects with hypertension and 0.7% in subjects without hypertension (control). In this study the number of subjects was small, 72 people consisting of 24 subjects with hypertension and 48 subjects with normotension causing no wild type found.[19]

Angiotensinogen (AGT) is a central component of the renin-angiotensin system that controls systemic blood pressure and several other cardiovascular functions and may play an important role in the atherosclerotic pathway. In a study conducted by Al Najai et al in the Saudi population, the role of 8 AGT gene

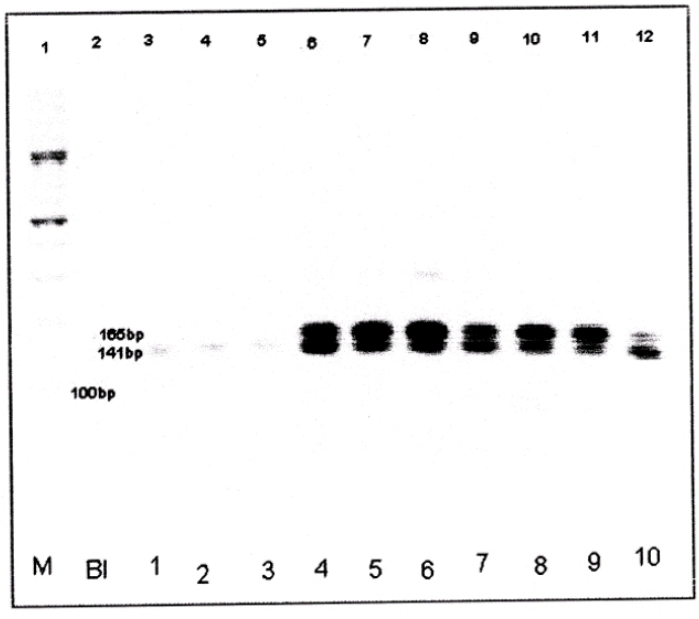

Figure 2. Analysis of gene variant AGT M235T

variants was evaluated in primary hypertension, type 2 diabetes mellitus (T2DM), and obesity. In this study, it was found that AGT is an independent risk gene for HTN, obesity and through the potential pleiotropic effect of AGT on the disease pathway leading to atherosclerosis.[18]

This study discusses the Angiotensinogen M235T gene in ischemic stroke patients in a population in Indonesia that has never been reported. A wider sample size and demographic variations of research subjects can be a development for this research topic.

CONCLUSION

Of the 72 samples analyzed, 3 variants of the Angiotensinogen M235T gene were found, namely CT, TT and CC variants. The AGT M235T CT gene variant was the most common variant, 37 (51.4%) samples.

REFERENCES

1. Zhong L-L, Ding L-S, He W, Tian X-Y, Cao H, Song Y-Q, *et al*. Systolic hypertension related single nucleotide polymorphism is associated with susceptibility of ischemic stroke. European Review for Medical and Pharmacological Sciences. 2017; 21: 2901–6.
2. Rasyid A, Hidayat R, Harris S, Kurniawan M, Mesiano T. Stroke iskemik. In: Aninditha T, Wiratman W, editors. Buku ajar neurologi. Tangerang: Penerbit Kedokteran Indonesia; 2017. p. 452–75.
3. Wang X, Cheng S, Brophy VH, Erlich HA, Mannhalter C, Berger K, *et al*. A meta-analysis of candidate gene polymorphism and ischemic stroke in six study populations: association of lymphotoxin-alpha in non-hypertensive patients. Stroke. 2009 March; 40(3): 683-95.
4. Al-Najai M, Muiya P, Tahir AI, Elhawari S, Gueco D, Andres E, *et al*. Association of the angiotensinogen gene polymorphism with atherosclerosis and its risk traits in the Saudi population. BMC Cardiovasc Disord. 2013;13(1):1.
5. Padma G, Charita B, Swapna N, Mamata M, Padma T. Novel variants detected in AGT gene among patients with essential hypertension. 2015;1–5.
6. Shaikh R, Shahid SM, Mansoor Q, Ismail M, Azhar A. Genetic variants of ACE (Insertion/Deletion) and AGT (M268T) genes in patients with diabetes and nephropathy. 2014.
7. Lu H, Cassis LA, Kooi CW Vander, Daugherty A. Structure and functions of angiotensinogen. 2016;39(7):492–500.
8. Dickson ME, Sigmund CD. Genetic basis of hypertension: Revisiting angiotensinogen. Hypertension. 2006;48(1):14–20.
9. Corvol P, Inserm U. Molecular Genetics of Human Hypertension : Role of. 1997;18(5):662–77.
10. PubChem [Internet]. Bethesda (MD): National Library of Medicine (US), National Center for Biotechnology Information; 2004-. PubChem Gene Summary for Gene 183, AGT - angiotensinogen (human); [cited 2022 Oct. 29]. Available from: https://pubchem.ncbi.nlm.nih.gov/gene/AGT/human
11. Procopciuc L, Popescu T, Jebeleanu G, Pop D, Zdrenghea D. Essential arterial hypertension and polymorphism of angiotensinogen M235T gene. J Cell Mol Med. 2002;6(2):245–50.
12. Ehret GB, Caulfield MJ. Genes for blood pressure: An opportunity to understand hypertension. Eur Heart J. 2013;34(13):951–61.
13. Yamasan BE, Gulyasar T, Sipahi T, Sivri N, Palabiyik O. Investigation of Angiotensinogen M235T and T174M Gene Polymorphisms in Coronary Artery Disease. Erciyes Med J. 2021;43(5):475–81.
14. Elalfy MS, Soliman F, Ebeid E, Kamal TM, Eissa DS, Abdel E, *et al*. Angiotensinogen M235T Gene Polymorphism is a Genetic Determinant of Cerebrovascular and Cardiopulmonary Morbidity in Adolescents with Sickle Cell Disease. J Stroke Cerebrovasc Dis. 2019;28(2):4419.
15. Mondry A, Loh M, Liu P, Zhu A-L, Nagel M. Polymorphisms of the insertion / deletion ACE and M235T AGT genes and hypertension: surprising new findings and meta-analysis of data. 2005;11:1–11.

16. Raharjo S, Chahyadi A, Fadhilah A, Kholidha AN. Hubungan polimorfisme gen angiotensinogen m235t dengan hipertensi esensial pada etnis sulawesi tenggara. Semin Nas Ris Kuantitatif Terap 2017. 2017;(April):16–20.
17. Shamaa MM, Fouad H, Haroun M, Hassanein M, Ayman M, Hay A. Association between the Angiotensinogen (AGT) gene (M235T) polymorphism and Essential Hypertension in Egyptian patients. Egypt Heart J. 2015;67(1):1–5.
18. Gao T, Huang L, Fu Q, Bai Y. Association of polymorphisms in the AGT gene(M235T, T174M) with ischemic stroke in the Chinese population. JRAAS - J Renin-Angiotensin-Aldosterone Syst. 2015;16(3):681–6.
19. Kooffreh ME, Kumar PL. A study of the M235T variant of the angiotensinogen gene and hypertension in a sample population of Calabar and Uyo, Nigeria. Egypt J Med Hum Genet. 2013;14(1):13–9.