The Role of ACE Gene Polymorphism on Pathogenesis of Ischemic Stroke

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ABSTRACT

Aim: *to describe the role of ACE gene polymorphism on pathogenesis of ischemic stroke in patients with the history of hypertension. The study was conducted in a population of Palembang city.*

Methods: *approximately 3 ml of peripheral blood samples were obtained by using venipuncture on antecubital vein. The samples were collected in tubes that contained ethylene diamine tetraacetic acid (EDTA) for DNA analysis. The DNA was extracted from leukocytes according to the standard DNAzol® Extraction Protocol. Samples were stored at -80°C until the analysis. Template DNA was then amplified by using a pair of sense oligonucleotide primer of 5'-CTGG AGACC ACTCCCATCCTTTCT-3' and antisense primer 5'-GATGGTGGCCATCAC ATTCGTC AGAT-3', 10 pmol of each primer. The PCR mixture contained 20 ng of genomic DNA, 3 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl pH 8.4, 5% dimethyl-sulphoxide (DMSO), each of 0,5 mM deoxyribonucleoside triphosphate (dNTPs) and 1 unit of Taq polymerase in a final volume of 50 µL. The DNA was amplified by 30 cycles; denaturation at 940C for 1 min, annealing at 580C for 1 min, and extension at 720C for 2 min, followed by a final extension at 720C for 4 min by using PCR Thermal (Icycler, Biorad, USA). PCR products were separated by electrophoresis on a 2% agarosa gel, and identified by ethidium bromide (0.1%) staining, and finally visualized by ultraviolet light. They were documented by using the geldoc (Biorad, USA). The PCR product is a 190 bp fragment in the absence of insertion (D) and a 490 bp fragment in the presence of insertion (I).*

Results: *ischemic stroke with hypertension or with the history of hypertension was found more frequently in male (70%) and ≥ 55 year old subjects (60.0%). The study showed that the frequency of II genotype was higher than DI and DD. Moreover, the frequency of I allele was higher than D allele. In healthy normotensive group, the results remained the same. However, different results were found in infarct-stroke group with hypertension history in which the frequency of DI genotype was higher than in II genotype and the DD. The study showed that there was no significant correlation (p=0.188) between ACE gene polymorphism and infarct stroke in subjects with the history of hypertension. ACE gene just has approximately 5% role in developing ischemic stroke.*

Conclusion: *there is no significant correlation between ACE gene polymorphism and the development of ischemic stroke in patients with history of hypertension of the population in Palembang. However, the study showed that there is a different pattern of genetic control on ACE compared to previous studies ever done in Caucasians.*

Key words: *ACE gene polymorphism, ischemic stroke.*

INTRODUCTION

Stroke as part of cerebrovascular disease is the third leading cause of death and the major cause of disability in the world. It is still a health problem worldwide.[1,2]

Indonesia has no complete epidemiological data, but the proportion of patients with stroke is likely to increase every year. M. Djamil Hospital in Padang reported 267 stroke patients in 2000, consisted of 48.6% hemorrhagic stroke and 51.4% ischemic stroke. The Department of Neurology, Faculty of Medicine, University of Sriwijaya/Mohammad Hoesin Hospital, Palembang reported similar result in 2005, i.e. 347 cases of strokes consisting of 192 (55.3%) patients with ischemic stroke and 155 (44.7%) patients with hemorrhagic stroke; mostly were of 40-70 years age group and the major risk factor of hypertension in 51.3% cases.[3-6]

Nowadays, Indonesia is a country with the highest number of stroke patients in Asia caused by various etiologies. If we provide no better stroke management, the prediction for rate of stroke may be doubled by the year 2020. The WHO reported that the contribution of hypertension to stroke is approximately 61.9% worldwide. Therefore, explorations on concept and preventive strategies at primary and secondary levels

of action in patients with hypertension are absolutely essential.[2,7,8]

Rigat et al reported for the first time in 1990 regarding the ACE gene involving three types of polymorphism, i.e. homozygote deletion (DD) and insertion (II) as well as the heterozygote (ID). Subsequently, investigators have attempted to detect the possibility of other loci in ACE gene that contributes to ACE activity. Until now, 160 types of polymorphisms along the ACE gene itself have been reported.[9-10] Rigat et al., reported that DD genotype is a risk factor in the development of coronary arterial disease and myocardium infarction. The underlying mechanism for the fluctuated ACE activity has not been convinced. It is assumed that there is an influence or interaction between the ACE gene and other gene including the influence of environmental factor.[11]

Some studies revealed significant correlation between ACE gene polymorphism and the pathogenesis of primary hypertension, atherosclerosis and left ventricle hypertrophy (LVH); however, the correlation between ACE gene polymorphism and pathogenesis of ischemic stroke is still on debate due to some controversial findings.[9,10,12] A study about the role of ACE gene polymorphism on the pathogenesis of ischemic stroke in patients with or without history of hypertension has never been performed in Indonesia. Considering that there are controversial findings, high prevalence of ischemic stroke and hypertension in Indonesia, including Palembang city and the lack of evidence on the correlation between ACE gene polymorphism and ischemic stroke in patients with history of hypertension, therefore, a study investigating the role of ACE gene polymorphism in patients with ischemic stroke and history of hypertension in Palembang population is extremely necessary.

The present study was aimed to reveal a correlation between ACE gene polymorphism and ischemic stroke in patients with history of hypertension of a population in Palembang city. The study was expected to provide theoretical principles, as well as consideration and input for clinicians in practicing the prompt preventive and curative strategies for an individual with certain ACE gene polymorphism in the correlation with ischemic stroke and history of hypertension.

METHODS

The present study was a cross-sectional study. Study population included patients who had their first ischemic stroke attack and had history of hypertension treated at Muhamad Hoesin Hospital, Palembang. The control group included healthy normotensive subjects who had fulfilled the inclusion criteria. By using a formula of Madiyono et al (2008), the sample size of each group was determined, i.e. 30 subjects.[13]

The inclusion criteria of the study group were: (1) Patients who had first attack of ischemic stroke and had history of hypertension, both male and female, Indonesian citizens living in Palembang city; (2) Age 40–69 years, including female with transitional period to menopause; (3) Willing to participate in the study and had signed the informed consent form; (4) had not suffered from atrial fibrillation; (5) no diagnosis of mitral stenosis; (6) had no diabetes mellitus; (7) had not suffered from dyslipidemia; (8) had no diagnosis of chronic renal disease. Inclusion criteria for sex- and age-matched control with 5 years interval were healthy subjects, male or female, Indonesian citizens living in Palembang.[14]

Blood samples were collected through venipuncture on antecubital vein as much as 3 ml and were collected in tubes containing EDTA, which subsequently followed by DNA extraction immediately. The DNA was extracted from leukocytes according to the standard DNAzol® Extraction Protocol. Samples were then stored at 80°C until the analysis. Basic principle steps of the isolation were lysis of leukocytes, removing cellular protein by proteinase, removing RNA by adding an RNase and isolating DNA.

Template DNA was then amplified by using a pair of sense oligonucleotide primer of 5'-CTGGAGACCACTCCCATCCTTTCT-3'and antisense primer 5'-GATGGTGGC CATCACATTCGTCAGAT-3', 10 pmol of each primer. The PCR mixture contained 20 ng of genomic DNA, 3 mM MgCl2, 50 mM KCl, 10 mM Tris-HCl pH 8.4, 5% dimethyl-sulphoxide (DMSO), each of 0,5 mM deoxyribonucleoside triphosphate (dNTPs) and 1 unit of Taq polymerase (Pharmacia, Uppsala, Sweden) in a final volume of 50 µL. Amplification was performed by denaturation denaturation at 940C for 1 min, annealing (nucleotide primer attachment) at 58^0C for 1 min, and extension (DNA elongation) at 72^0C for 2 min as many as 30 cycles followed by a final extension at 72^0C for 4 min.

The instrument utilized in the study was PCR Thermal (Icycler, Biorad, USA). Results of PCR were separated by electrophoresis on a 2% agarosa gel, which had been enriched with ethidium bromide (0.1%), then visualized by ultraviolet light and were documented by using the geldoc (Biorad, USA). The PCR product is a 190 bp fragment in the absence of insertion (D) and a 490 bp fragment in the presence of

insertion (I).

Statistical Analysis

Statistical analysis was performed to provide answer for the main objective of our study, i.e. to reveal the correlation between ACE gene polymorphism and ischemic stroke in patients with history of hypertension treated at Moh. Hoesin Hospital, Palembang.

The statistical analysis was continued to find any correlation between ACE gene allele (D or I allele) and ischemic stroke in subjects with history of hypertension by using the stata software.

RESULTS

A total 629 stroke patients were treated at The Department of Neurology, M. Hoesin Hospital in Palembang between October 2006 and May 2007. There were 351 (55.8%) ischemic stroke patients and 278 (44.2%) hemorrhagic stroke patients.

Of 351 ischemic stroke patients, a screening was performed by consecutive sampling method if the patients had fulfilled the inclusion criteria resulting 30 subjects in the study group. The study group was then categorized based on age (of 5-year interval) and sex. Moreover, by similar method and matching, we found 30 age- and sex-matched control subjects, were obtained from 115 normotensive healthy subjects who had responded to our request for participating in the study and who had fulfilled the inclusion criteria.

The collected data were then processed by statistical analysis to recognize general characteristics of subjects and the result of analysis is presented on **Table 1**.

Table 1. General characteristics of subjects

Characteristics	Group		Total n = 60
	Ischemic Stroke n = 30	Healthy n = 30	
Sex :			
- Male	21 (35.0%)	21 (35.0%)	42 (70.0%)
- Female	9 (15.0%)	9 (15.0%)	18 (30.0%)
Age (years) :			
- 40 – 44	4 (6.7%)	4 (6.7%)	8 (13.4%)
- 45 – 49	3 (5.0%)	3 (5.0%)	6 (10.0%)
- 50 – 54	5 (8.3%)	5 (8.3%)	10 (16.6%)
- 55 – 59	11 (18.3%)	11 (18.3%)	22 (36.6%)
- 60 – 64	6 (10.0%)	7 (11.7%)	13 (21.7%)
- 65 – 69	1 (1.7%)	0 (0.0%)	1 (1.7%)

Table 1 shows that ischemic stroke with hypertension or history of hypertension was more frequently found in male subjects (70%) than in female and most of them were in the 55–59 years age group (36.6%). About 36 subjects (60.0%) of ischemic stroke patients with history of hypertension were found in the similar age group or over 55 years old.

DNA genotyping was performed by PCR of all subjects who had ischemic stroke and history of hypertension as well as on normotensive healthy subjects. The results of amplification were visualized by electrophoresis.

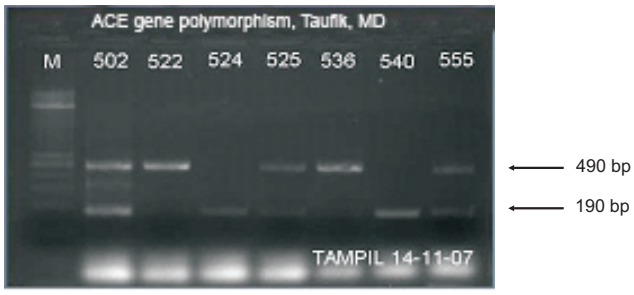

Figure 1. Results on visualization of PCR product in our study. The figure shows that in the studied population, there were three ACE genotypes providing evidence on ACE gene insertion/ deletion polymorphism. Lane 502 (DI), 522 (II), 524 (DD), 525 (DI), 536 (II), 540 (DD) and 555 (DI). M=marker ladder 100 bp.

Biomolecular laboratory evaluation performed in all subjects resulted in data about distribution of ACE genotype and allele frequency as presented on the **Table 2**.

Table 2. Distribution of ACE genotype and allele frequency

ACE Genotype and allele frequency	Group		Total
	and allele frequency	Healthy	
Distribution of ACE Genotype :	n = 30	n = 30	n = 60
- DD genotype	7 (11.7%)	5 (8.3%)	12 (20.0%)
- DI genotype	12 (20.0%)	7 (11.7%)	19 (31.7%)
- II genotype	11 (18.3%)	18 (30.0%)	29 (48.3%)
ACE gene allele frequency:	n = 60	n = 60	n = 120
- D allele	26 (21.67%)	17 (14.17%)	43 (35.84%)
- I allele	34 (28.33%)	43 (35.83%)	77 (64.16%)

The present study has successfully provided evidence on the occurrence of ACE gene polymorphism by detecting 3 ACE genotypes, i.e. the DD homozygote, II and DI heterozygote. The total ACE genotype distribution in the population of our study was II > DI > DD and total allele frequency of I > D. Among the group of ischemic stroke with history of hypertension, there was different distribution of ACE genotype, i.e.

DI > II > DD with similar allele frequency, namely I > D. It appeared that there was dominant contribution of DI genotype and allele I in association with ischemic stroke with history of hypertension. Results in normotensive healthy subjects were similar to the population, i.e. distribution of genotype was II > DI > DD and the allele frequency was I > D. In this case, we could see the dominant contribution in normotensive healthy subjects as shown by II genotype and I allele.

Table 3. Correlation between ACE gene polymorphism and ischemic stroke in subjects with history of hypertension

Characteristics	Group		*p*
	Ischemic stroke (n=30)	Healthy (n=30)	
ACE gene polymorphism:			
- DD genotype	7 (23.0%)	5 (17.0%)	0.188
- DI genotype	12 (40.0%)	7 (23.0%)	
- II genotype	11 (37.0%)	18 (60.0%)	

Note: Chi-square; Contigency coefficient (C) = 0.230 and C2 = 0.0529 (5%).

Table 3 shows that there was no significant correlation (p=0.188) between ACE gene polymorphism and ischemic stroke in subjects with history of hypertension in our study. Further statistical analysis only demonstrated contingency value of 5% (C2=0.0529). The correlation between ACE gene allele (D or I allele) and ischemic stroke in subjects with history of hypertension was found as seen on **Table 4**.

Table 4. Correlation between distribution of ACE genotype and allele frequency in subjects with ischemic stroke and history of hypertension

Characteristics		ACE Gene Polymorphism				ACE Gene Alleles	
		DD	DI	II	DD+DI	D	I
Ischemic Stroke Subjects	Number	7	12	11	19	26	34
	Percentage (%)	23	40	37	63	43	57
Normotensive Health Subjects	Number	5	7	18	12	17	43
	Percentage (%)	17	23	60	40	28	72
P		0.78	0.81	-	0.84	0.04*	-

Note: using Stata software, II genotype and I allele as the references

Table 4 compares directly of each ACE genotype and alleles among both groups, there was significant difference (p=0.04) of statistical analysis against D alleles among both groups.

DISCUSSION

Various epidemiological studies show that genetic factors do then roles as risks of ischemic stroke. A study has been conducted to recognize the prevalence of 60 types polymorphism located in 35 genes that had the potency as risk factors of ischemic stroke including angiotensin (AGT) and angiotensin receptor AT-1 (AGTR-1). The study demonstrated that some polymorphism had significant correlation to the occurrence of ischemic stroke, e.g. Varg506gln factor, MTHFR, factor II 20210 G/A and ACE I/D.[15,16]

Two candidate genes, ACE and MTHFR genes, have been mostly studied in non-European (non-Caucasian) group. Polymorphism of MTHFR genes has appeared to be associated with the occurrence of thrombosis which may cause damage to vascular wall and coagulation system. Hence, ACE gene had been selected as the candidate gene in our study.[17,18]

The present study has been conducted in subjects with ischemic stroke due to or associated with hypertension. It has been explained that the main patophysiology of hypertension includes RAAS with ACE as the central role. Therefore, it is logical for ACE gene to be the candidate genes and it has become another reason to get ACE gene as the candidate genes. NCBI has recorded approximately 160 polymorphism along ACE gene itself, but the most famous and the mostly studied for its association to cardiovascular event is insertion/deletion polymorphism of ACE gene (I/D polymorphism ACE gene).[17]

The present study has successfully provided evidence on the occurrence of ACE gene polymorphism in a population of Palembang city by revealing findings of 3 ACE genotypes, i.e. DD, DI and II. Such findings become important since serum ACE activity and plasma ACE level are determined by those three genotypes. Rigat et al., who had reported about ACE genes polymorphism for the first time, suggested that the gene is responsible for 47% phenotypic interindividual variation of ACE gene in plasma ACE level.[11]

The statistical analysis showed that in the population of our study, the distribution of ACE genotype reflecting II > DI > DD and total allele frequency of I > D . Among the group of ischemic stroke with history of hypertension, we found that the distribution of ACE genotype was DI > II > DD with allele frequency of I > D. It appears that the control of ACE activity and plasma level is regulated due to dominant contribution of DI genotype and allele I in association with ischemic stroke in subjects with history of hypertension. On the contrary, the distribution of genotype in the normotensive healthy group was II > DI > DD with

allele frequency of I > D. Here, we could see that the control is regulated by dominant contribution of II genotype and allele I, which is similar to the population.[12]

Results of statistical analysis comparing ACE gene alleles of both groups demonstrated that allele D has a significant correlation to ischemic stroke with history of hypertension. Allele D has important role since if allele D occurs together with allele I in the same locus, they will develop the DI genotype, which is similar to the ACE genotype controlling plasma ACE activity in ischemic stroke group of the present study.

A study by Rigat et al., who performed analysis on ACE genes in 80 healthy Caucasian subjects, found a genotype distribution of DI > DD > II. Barcelo et al., who studied plasma ACE activity and ACE gene polymorphism in patients with obstructive sleep apnoe syndrome (OSAS) and healthy subjects reported a genotype distribution of DI > DD > II in both groups. Our study results, particularly in ischemic stroke group with history of hypertension, the control of plasma ACE activity and level both were dominated by DI genotype, but the DD genotype has the weakest contribution.11,14

Sinarja et al., studied a correlation between ACE gene polymorphism and body mass index (BMI) in 115 hypertensive patients who were treated at Faculty of Medicine, University of Indonesia, Jantung Harapan Kita Hospital in Jakarta. They found that the distributions of DD, DI, II ACE genotype in 71 non-obese hypertensive subjects were 4.2%, 40.9%, and 54.9%, respectively. Moreover, the distributions of such genotype in 44 obese hypertensive subjects were 2.3%, 22.7% and 75%, respectively. It seems that genetic control on plasma ACE level and activity in the study conducted by Sinarja et al was determined by II genotype (II>DI>DD), which is similar result to the normotensive healthy subjects in our study.[18]

The similar pathophysiology of myocardium and cerebral infarction has made many researchers try to study about the role of ACE gene polymorphism on stroke; however, we have failed to demonstrate any significant correlation (p=0.188) between ACE gene polymorphism and ischemic stroke in subjects with history of hypertension of a population in Palembang city. The result of contigency analysis only demonstrated 5% of value, which means that there were only 5% ischemic stroke cases in subjects with history of hypertension of a population in Palembang city that had a correlation to ACE gene polymorphism.[19]

Some studies supported that there is a correlation between ACE gene polymorphism and ischemic stroke, but many of others denied. Napoli and Papa, provided evidences that ACE inhibitor treatment may reduce the risk of stroke in patients with hypertension and if the stroke had occurred, it might have minimized the effect. Such findings have been supported by Gorelick who demonstrated that ACE inhibitor may prevent the development of stroke. Both findings indicate that ACE gene polymorphism may probably affect the pathogenesis of stroke.[20,21]

In contrast, Mannami et al,. suggested that there was no correlation between ACE gene polymorphism and the development of atherosclerosis in carotid artery. Zee et al., also suggested that the incidence of stroke had no correlation with ACE gene polymorphism. Sethi et al., stated that there was clinically no significant correlation between ACE gene polymorphism and stroke, but statistically such correlation was not significant.[25,26]

CONCLUSION

The present study found no significant correlation between I/D ACE gene polymorphism and ischemic stroke in subjects with history of hypertension; however, the statistical analysis found a correlation between ACE gene allele D and ischemic stroke in subjects with history of hypertension. Nevertheless, the present study showed that there is a different pattern of domination in controlling ACE gene compared to the previous studies conducted among Caucasians.

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