Original Article

Angiotensin converting enzyme polymorphism and ischemic stroke

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ABSTRACT

الأهداف: استكشاف العلاقة بين تعدد الأشكال الجيني لإنزيم المحول للأنجيوتنسين (ACE) والسكتة الدماغية في عينة مصرية.

المنهجية: شملت هذه الدراسة المقطعية 70 مريضا بالسكتة الدماغية المنومين بقسم الأمراض العصبية في مستشفى جامعة المنوفية ، و 30 من الأشخاص الأصحاء كعينة ضابطة. خضع المرضى للفحص العصبي الكامل ، مقياس السكتة الدماغية الصحية المعهد الوطني (NIHSS)، وتصنيف TOAST. تم التنميط الجيني لتعدد الأشكال الجيني ACE عن طريق تفاعل سلسلة البلمرة (PCR) لكل من المرضى و الأصحاء.

النتائج: كان تعدد أشكال النمط الوراثي لحذف الإدراج (I/D) من ACE أكثر (42.9% مقابل 33.3% في العينة الضابطة) وتواتر D أليل هو أكثر من أليل I في مرضى السكتة الدماغية ولكن هذا الاختلاف لم يصل إلى مستوى كبير ليكون عامل خطر. فيما يتعلق بالعلاقة بين مسببات السكتة الدماغية وتعدد الأشكال الجينية ACE ، كان تعدد الأشكال I/D هو النوع السائد في SVS و LVS ، بينما كان DD هو النوع السائد في الجلطات الناتجة عن انصمام مصدره القلب، لم تكن هناك فروق ذات دلالة إحصائية بين NIHSS في تعدد الأشكال الجينية ACE مختلفة.

الخلاصة: على الرغم من اختلافات التنميط الجيني ACE بين مرضى السكتة الدماغية المصرية، لا يمكننا اعتباره عاملاً مؤهلاً لحدوث السكتة الدماغية أو شدتها.

Objectives: To assess the polymorphism of angiotensin converting enzyme (ACE) in ischemic stroke in an Egyptian sample.

Methods: One hundred subjects (70 ischemic stroke patients, and 30 healthy controls) were included in case control cross sectional study during the period from January 2017 to January 2018, at Neurology Department, Menufia University Hospital, Shibin EL-Kom, Egypt. Patients underwent complete neurological assessment, national institute health stroke scale (NIHSS), and Toast classification. All subjects underwent genotyping of ACE gene polymorphism.

Results: There are 42.9% from the patients versus 33.3% from controls showed geno typing of Insertion/ Deletion (I/D) and Allele D is more frequent regarding I allele in ischemic stroke patients but this difference did not reach significant level to be risk factor. The I/D polymorphism was the predominant type in SVS and LVS while DD was the predominant type in cardio-embolic one. There were no significant differences between NIHSS in different ACE gene polymorphism.

Conclusion: In spite of ACE genotyping differences among Egyptian ischemic stroke patients, we cannot consider it a predisposing factor to stroke occurrence or severity.

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Stroke causes death and permanent disability worldwide.[1] The yearly stroke incidence in Egypt was estimated around 150,000 to 210,000 cases.[2] Ischemic stroke represents 87% of stroke patients as reported by the American Heart Association.[3] A focal retinal or spinal or cerebral ischemia causing acute neurological deficit is considered ischemic stroke.[4] Genetic factors have an important role in ischemic strokes.[5] Identification of stroke genes is needed for early suspicion, primary prevention and incidence reduction.[6] The Angiotensin Converting Enzyme (ACE) presents in a large amount in cerebral blood vessels producing Angiotensin II from angiotensin I,

which has an important effect on blood vessels smooth muscle tone and proliferation, endothelial function and aldosterone level affecting by this on blood pressure, vascular remodeling, atherosclerosis and ischemic stroke occurrence.[7,8] Angiotensin converting gene regulates activity of ACE where allelic differences represent up to 47% of the variation in ACE concentrations.

There are more than 160 ACE gene polymorphisms, mainly, single nucleotide one (SNPs). The insertion deletion determiner of ACE gene is alu repeat sequence 287 bp at intron 16 of 17q23 chromosome (dbSNP ID: rs1799752).[9] Wide spread attention were paid to ACE gene among ischemic stroke patients, many researchers have reported the association.[10] Our objective is to assess the polymorphism of ACE in ischemic stroke in an Egyptian sample.

Methods. A Cross sectional case-control study was carried out from January 2017 to January 2018. Out of 113 stroke patients referred to the study at Department of Neurology, Menoufia University Hospital, Shibin EL-Kom, Egypt, only 70 patients were included. Patients excluded were mainly those with hemorrhagic stroke, impaired renal, or hepatic functions, or non-consenting patients. Thirty healthy controls, matching patients in age and sex were taken from workers at the Faculty of Medicine, Menoufia University hospital, Shibin EL-Kom, Egypt.

Ethics Approval. This study was approval by Faculty of Medicine, Menoufia University Research and Ethics Committee. An informed consent was taken from all participants. The study was performed according to principles of Helsinki Declaration.

Inclusion criteria. Age more than 18 years, both gender, acute first and recurrent ischemic strokes.

Exclusion criteria. hemorrhagic stroke, patients with severe hepatic or renal impairment or thrombophilias and non consenting subjects. Patients diagnosis depends on clinical background and brain imaging (CT, MRI). Patients were asked for clinical data (smoking, obesity, DM, HTN, hyperlipidemia, cardiac history as AF, myocardial infarction, cardiomyopathy, and neurological problems (migraine, TIA, recurrent stroke).

The NIHSS involves 11 items that assess stroke outcome and severity. A number between 0 and 4 is given to each item, 0 being normal functioning and 4 being completely impaired, some elements only have a scale from 0 to 2 and 42 is the highest score possible. The higher the score, the more impaired a stroke patient is. Score of (1-4) represents mild one, (5-15) moderate, (16-20) moderate to severe and (21-42) severe.[11]

Ischemic stroke patients in this study underwent MRI brain echocardiograghy, duplex on carotid and vertebral arteries, lab tests for vasculitis. Then, patients were divided into large, small vessel and cardiac strokes regarding Trial of ORG 10172 in Acute Stroke Treatment (TOAST) classification, which is an Etiological classification of Ischemic strokes (from clinical and radiology), large artery atherosclerosis means >50% stenosis in branch cortical artery or major brain artery, small artery stroke means a lesion less than 1.5 cm in brain imaging plus lacunar syndrome and cardiac one means major cardiac cause (one or more) for embolism and no other stroke causes.[12]

The EDTA tubes were used to collect venous blood samples from patients and controls for DNA extraction. Spin column DNA kits were used for DNA extraction from whole blood (Vivantis, SNF Medical, GF-1 blood DNA extraction kits, lot: 12354c, Berlin, Germany). Preparation of genotyping PCR master mix by kits obtained from (Thermo Fisher Scientific, USA) including Dream Tag Green PCR Master Mix (2x): contains dream tag polymerase supplied in 2x, dream tag green buffer with dATP, dCTP, dGTP, dTTP 0.4 mmol each and 4 mmol MgCl2, template DNA, distilled water and the following primers were obtained from (Analysis AB, Invitrogen, USA)

- Forward Primer 1 (5 -CTG GAG ACC ACT CCC ATC CTT TCT-3)
- Reverse Primer1(5-GAT GTG GCC ATC ACA TTC GTC AGA T-3)

The master mix was mixed thoroughly and dispensed appropriate volumes were dispensed into PCR tubes. The PCR amplification using T professional thermo cycler (Biometra, An Analytik Jena Company, Germany) included initial denaturation at 95º for 5min then (30 denaturation cycles at 95º for 50 sec, annealing at 62º for 50 sec and extension at 72º for 50 sec) and final extension at 72º for 10 min. The PCR products were visualized as follow: Insertion (I) allele was showed in a 490- bp product and Deletion (D) allele was showed in a 190-bp fragment. Genotype II: a 490-bp band, DD genotype: a 190-bp band and ID genotype: a 490-bp and a 190-bp band

Statistics. Results were collected, tabulated, statistically analyzed by IBM personal computer and

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Table 1 - Shows stroke risk factors, severity and etiological classification.

Items	Patients n=70	Controls n=30	χ^2	*P*-value
	n (%)			
Age (years)				
Mean± SD	56.17±9.61	53.13±8.80	1.49*	0.141
Gender				
Male	48 (68.6)	16 (53.3)	2.12	0.146
Females	22 (31.4)	14 (46.7)		
DM	38 (54.3)	0	26.27	<0.001
HTN	26 (40.0)	0	16.67	<0.001
Obesity	16 (22.9)	0	8.16	0.004
Cardiac risk				
No	54 (77.1)	30 (100)	8.06	0.043
AF	4 (5.7)	0		
Others	6 (8.6)	0		
AF& others	6 (8.6)	0		
Hyperlipidemia	30 (42.9)	0	18.37	<0.001
Smoking	10 (14.3)	0	4.76	0.029
Migraine	4 (5.7)	0	1.79	0.181
TIAS	8 (11.4)	0	3.73	0.054
Recurrent attach	14 (20.0)	0	6.98	0.008
Toast classifications				
attack	36 (51.5)			
1 (small vessel stenosis)	24 (34.2)			
2 (large vessel stenosis)	10 (14.3)			
3 (cardioembolism)				
NIHSS range	2-12			
Mean±SD	6.89±2.56			
Minor stroke less than5	12 patients			
Moderate (5-15)	58 patients			

*student t-test was used, DM - diabetes mellitus, HTN - hypertension, NIHSS - National Institute Health Stroke Scale, SD - standard deviation, TIA - transient ischemic attack, AF - atrial fibrilation

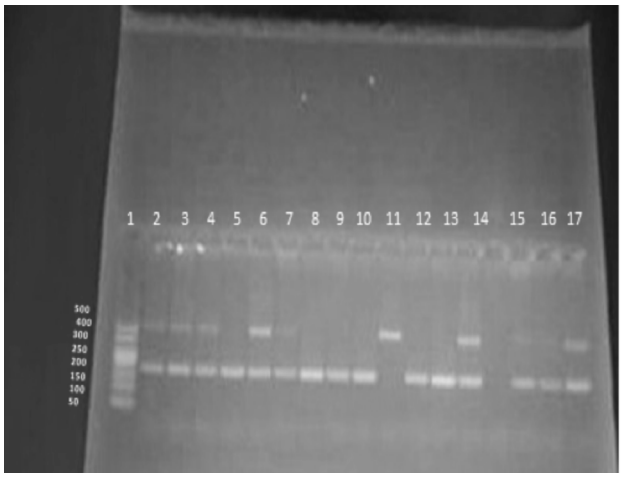

Figure 1 - Shows ACE I/D genotypes: Lane 1 shows 50bp ladder, lane 2,3,4,6,7,15,17 show ID genotype at 190,490bp,lane5,8,9,10,12,13 show DD genotype at 190bp while lane 11 shows II genotype at 490bp.

statistical package SPSS version 22 (Armonk, NY: IBM Corp, 2013). Data were presented as descriptive statistics including percentage (%), mean (x), standard deviation (SD) and range. Statistical evaluation of data was carried out using Chi-square test ($\chi 2$), Students t-test, ANOVA test and regression analysis. A *p*-value of <0.05 was considered statistically significant.

Results. Table 1 shows stroke risk factors, severity and aetiological classification. The most predominant polymorphism in ischemic stroke patients was Insertion/Deletion (ID) genotype but the most predominant ACE gene polymorphism in control group was Deltion/Deletion (DD) and both groups difference as regards ACE polymorphism did not reach the significant level (wasn't a risk factor) (p=0.316). Also, as regards the prevalence of Deletion (D) or Insertion (I) alleles of ACE gene, both groups had no significant difference (p=0.127). (**Table 2**)

A significant difference was found among ACE gene polymorphisms in ischemic stroke patients as regards TOAST classification where ID polymorphism was the predominant type in SVS and LVS while DD was the predominant type in cadioembolic class (p=0.031).

The SVS and LVS have no difference in the prevalence ACE genotypes ($p1$=0.605) but SVS significantly differs from cardioembolic ($p2$=0.009) and LVS vs cadioembolic ($p3$=0.039). Also, the 3 classes significantly differs as regards ACE gene alleles (p=0.006) where cadioembolic had more predominance of D alleles than SVS and LVS. (**Table 3**)

The NIHSS mean values do not significantly differ among different ACE gene polymorphism in ischemic stroke patients (0.226) otherwise, DD had significantly lower mean values of NIHSS than II ($p2$<0.001) (**Table 4**).

Discussion. Studying of gene variants may play role in prognosis, treatment, and prevention of ischemic stroke especially that the thrombotic risk in patients with arterial thrombosis could not be totally explained by known risk factors as hypercholesterolemia, obesity, smoking, diabetes, and hypertension.[12]

The ACE produces angiotensin II that causes vasoconstriction, vascular hypertrophy, and inhibition of bradykinin and thus elevates blood vessels stiffness promoting hypertension and atherosclerosis. Also, it decreases tissue perfusion and activates plasminogen-activator inhibitor type I. So, ACE is considered an essential cerebrovascular risk factor.[13] The ACE gene polymorphism determines ACE activity. Serum

Table 2 - Genotypic variations of ACE among Egyptian ischemic stroke patients.

Items	Patients n=70 N (%)	Controls n=30	χ^2	*P*-value	OR (95% CI)
Gene polymorphism of ACE:				0.316	
DD	26 (37.1)	16 (53.3)	2.31	P1=0.203	0.54 (0.21-1.40)
ID	30 (42.9)	10 (33.3)		P2=0.232	0.46 (0.13-1.66)
II	14 (20.0)	4 (13.3)		P3=0.819	1.17 (0.31-4.37)
ID+II vs DD			2.26	0.133	0.82 (0.62-1.08)
ID+DD vs II			0.63	0.426	1.63 (0.49-5.42)
ACE gene alleles:	n=140	n=60			
D alleles	82 (58.6)	42 (70.0)	2.33	0.127	1.65 (0.86-3.15)
I alleles	58 (41.4)	18 (30.0)			

OR - Odd's ratio; CI - Confidence interval, P1 - DD vs ID; P2 - DD vs II, P3 - II vs ID, ACE - Angiotensin Converting Enzyme, DD - Deltion/Deltion genotype, ID - Insertion/Deletion genotype, II - Insertion/iInsertion genotype

Table 3 - Angiotensin converting enzyme gene polymorphism (ACE) as regards TOASTclassification in ischemic stroke patients.

Items	TOAST classification SVS n=36	LVS n=24 N (%)	Cardiac n=10	χ^2	*P*-value
ACE gene polymorphism					0.031
DD	10 (27.8)	8 (33.3)	8 (80.0)	10.64	*p*1=0.605
ID	16 (44.4)	12 (50.0)	2 (20.0)		*p*2=0.009
II	10 (27.8)	4 (16.7)	0		*p*3=0.039
	n=72	n=48	n=20		
ACE gene alleles					
D alleles	36 (50.0)	28 (58.3)	18 (90.0)	10.32	0.006
I alleles	36 (50.0)	20 (40.7)	2 (10.0)		*p*=10.370
					*p*2=0.001
					*p*3=0.011

P1 - SVS vs LVS, p2 - SVS vs cadiac, p3 - LVS vs cardiac, SVS - small vessel stroke, LVS - large vessel stroke, DD - Deltion /Deletion, ID - Insertion/Deletion, II - Insertion/insertion gentypes, TOAST - Trial of ORG 10172 in acute stroke treatment

Table 4 - Angiotensin converting enzyme gene polymorphism (ACE) as regards NIHSS in ischemic stroke patients.

Items	NIHSS in patients (n=70) Mean ±SD	Test of significance	*P*-value
ACE gene polymorphism:	6.23±2.80	ANOVA=	0.226
DD	7.13±2.75	1.52	P1=0.057
ID	7.57±1.09		P2<0.001
II			P3=0.215
ACE gene alleles:			
D alleles	6.23±2.80	t=1.71	0.095
I alleles	7.57±1.09		

P1 - DD vs ID, p2 - DD vs II, p3 - II vs ID, ACE - Angiotensin Converting Enzyme, DD - Deltion/Deltion genotype, ID - Insertion/ Deletion genotype, II - Insertion/iInsertion genotype

concentration and activity of ACE increases with D/D polymorphism than homozygotes of I/I polymorphism and intermediate level in I/D heterozygous polymorphism.[14]

Different studies on different ethnic population showed a significant relation between ACE polymorphism and ischemic stroke[10] but others studies did not observe this association.[15] In our study we tried to assess ACE polymorphism among Egyptian ischemic strokes and if there is association between etiological types of ischemic stroke and this polymorphism.

In our study, the most predominant polymorphism in ischemic stroke patients was insertion deletion genotype ID (42.9%) against (33.3%) in the healthy controls but *p*-value was not significant (p=0.3), it was followed by deletion-deletion genotyping (37.1% against (53.3%) in healthy control and followed by insertion-insertion genotype (20% against 13.3% in controls) and allae D more than allae I. Also, on assessing this polymorphism according to type of stroke, we found that ID polymorphism was the predominant type in SVS and LVS while DD was the predominant type in cardio embolic stroke. Also, our study showed no significant correlation between ACE polymorphism and resulting deficit from stroke as assessed by NIHSS.

There are results partially similar to our results, that occurrence of stroke do not significantly relate to ACE polymorphism but among his ischemic stroke patients

there was dominant ID polymorphism and I allele as polymorphism were ID> II > DD with I > D.[16]

The ACE polymorphism among young Mexican ischemic stroke patients were reported and I/D genotype was 47.3% in patients against 46% in controls. A higher percent of I/I genotype was found (35.3.9%) compared with us (20%).[17]

Stankovic et al[18] found that ACE genotype or allele frequency does not significantly differ between ischemic stroke and healthy subjects but D allele patients had higher risk for large vessel ischemic stroke.

Murali et al[19] revealed a significant association of I/I polymorphism and allele I with ischemic stroke male subjects more than fifty age in south indian sample. But Markoula et al[20] found I/D ACE polymorphisms was related to stroke onset in females.

According to Kalita J et al,[10] the genotype D/D was more risky among Asian patients with small vessels disease. Also, Das, et al[21] showed that genotype DD and allele D predispose to stroke especially hemorrhagic one. In contrast, in Turkish population, there is no association between ischemic stroke or its subtypes and genotype DD and/or allele D of ACE polymorphism.[22] Dominguez et al[14] and Karagiannis et al[23] have found similar findings in both Spanish population and Greek population respectively.

Mostafa et al[24] 2016 studied ACE polymorphism in Egyptian ischemic stroke patients then reported DD as more one in patients group with no specificity to large nor small vessels disease. Regarding polish population Pera et al[15] 2006 did not report arelation between ischemic stroke etiological types and ACE polymorphism. A previous study in Egyptian patients gave similar results to our study where ID genotype was more abundant in this study but this study differs from us in number of the sample (30 patients versus 17 controls).[25] Frequency of alleles of ACE polymorphism differs among different populations, D allele is more in Caucasian but I allele is more in South American and Polynesian populations.[26]

Regarding to normal Egyptians, Hamdy et al[27] found that each of ID and DD genotypes had similar percentage (45.5%). Genetic factors of stroke may cause stroke by risk factors predisposition or modulation or, both, by affection directly on risk, evolution and outcome of stroke.[28] Ethnic population different results may be due to variable distribution frequencies of ACE polymorphism, stroke type, different matching criteria and selection bias.

In conclusion, in spite of ACE genotypic variation among Egyptian ischemic stroke patients with abundance of I/D one especially in large and small vessel strokes, we cannot consider it a predisposing factor to stroke occurrence nor severity.

Limitations of our study involved small number of studied sample, involvement of classical risk factors as hypertension and diabetes in our sample, small number of cardiac strokes; also, study does not involve ACE serum concentration.

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