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Effect of homocysteine on ischemic stroke and myocardial infarction in Iraqi population

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Abstract: Objectives: Evaluate the effect of Plasminogen activator inhibitor on ischemic stroke and myocardial infarction in Iraqi population.
Design and Methods: The study was conducted on (60) patients, (30) patients with acute ischemic stroke and (30) patients with acute myocardial infarction, and (30) apparently healthy subjects were taken as control group.
The level of hmocysteine was determined using Enzyme linked immunosorbent assay.
The methylene tetra hydro folatereductase genotyping was performed using Restriction Fragment Length Polymorphism Polymerase Chain Reaction (RFLP-PCR) technique.
Results: Both ischemic stroke and myocardial groups had significantly higher level of homocysteine($P<0.01$).
According to methylene tetra hydro folatereductase gene, a C677T polymorphism was detected by using polymerase chain reaction restriction fragmentation length polymorphism. The alleles were designated as CC, CT and TT. There was statistically no significant difference in both the genotypic distribution and allelic frequency between both patient groups versus healthy controls. However the present study showed that subjects with TT genotype had the highest level of homocysteine in all study groups, while subjects with CC genotype had the lowest level of homocysteine in all study groups.
Conclusion: Homocysteine may consider as an independent risk factor for ischemic stroke and myocardial infarction.
Key words: Homocysteine, ischemic stroke, myocardial infarction, genetic polymorphism, plasminogen activator inhibitor, methylene tetra hydro folatereductase gene.

Introduction

Ischemic stroke is death of brain tissue due to interruption of blood flow to a region of the brain, caused by occlusion of a carotid or vertebral artery or, less likely, a cerebral vein[1].

Myocardial infarction (MI) means interruption of blood supply to a part of the heart, and it is almost always due to the formation of occlusive thrombus at the site of rupture or erosion of an atheromatous plague in coronary artery causing heart cell to die and without treatment the infarct related artery remains permanently occluded in 30% of patients[2].

Homocysteine is a sulphur containing amino acid formed from the metabolism of methionine, an essential amino acid derived from dietary protein[3]. Although homocysteine was first isolated by Butz and du

Vigneaud in 1932[4] it was not until 1964 that Gibson *et al*. reported that patients with homocystinuria had vascular anomalies and arterial thrombosis[5].

An elevated plasma homocysteine level can result from many different factors, including vitamin deficiencies, renal impairment, and inborn errors of homocysteine metabolism [6].

Homocystinuria, i.e., an abnormal elevation of homocysteine in the urine, is caused by several autosomal recessive disorders. People with these genetic variations have extremely high homocysteine levels. A deficiency in the enzyme cystathionine beta-synthase is quite rare, but leads to homocysteine levels greater than 100 µmol/L and often causes cardiovascular disease by the age of 30 years [7].

A deficiency in the enzyme methylene tetrahydrofolatereductase (MTHFR) is a more common cause of mildly to moderately elevated plasma homocysteine levels [8]. The MTHFR deficiency involves a variation at position 677 in the MTHFR gene in which cytosine is replaced by thymidine (thus called C677T or 677C>T). Ten percent of the population are homozygous for this variant (TT), 43% are heterozygous (CT), and 47% are unaffected (CC). Heterozygotes have slightly higher homocysteine levels than unaffected people, while people with the TT genotype have approximately 20% higher homocysteine levels[9].

Cohort and genetic polymorphism studies showed a quantitatively similar association between decreased Hcy concentrations and risk of heart disease and stroke, but there is heterogeneity between the results from different studies, possibly due to differences in folate and B-vitamin status among populations. Among the genetic polymorphism studies, those with the greatest difference in Hcy concentrations between the MTHFR T/T and C/C homozygotes showed the greatest difference in CVD risk[10].

Different heart associations in the US, Canada, and Europe all agreed that the evidence was not strong enough to recommend routine population screening for elevated Hcy concentrations[11,12].

However, many evidences suggest using Hcy concentrations as a prognostic factor for CVD events and mortality in patients with CVD or persons with high risk of CVD events[13].

Methods

The stroke group who subjected to this study were (30) persons in the age group ranging from 30 - 83 years , the mean ± standard deviation (SD) was (61.73 ± 14.1 years). This group comprised of males (53%), with their age ranging from 30 - 74 years old, the mean ± SD was (56.75 ± 14.9 years) , and females (47%) with age ranging from 48 - 83 years, and mean ± SD was (67.4 ± 10.9 years).

The MI group who subjected to this study were (30) persons in the age group ranging from 38 – 90 years , the mean ± standard deviation (SD) was (60 ± 12.2 years). This group comprised of males (60%), with their age ranging from 38 - 70 years old, the mean ± SD was (55.5 ±10.4 years), and females (40%) with age ranging from 50 - 90 years, and mean ± SD was (66.7 ± 11.9 years).

Thirty apparently healthy individuals were taken as a control group of the age ranging from 38 – 79 years, the mean ± standard deviation (SD) was (57.87 ± 13.68years). This group comprised of males (57%) their age ranging from 38 - 80 years , mean ± SD was (61.76 ± 13.57 years), and females (43%) their age ranging from 39 - 79 years , mean ± SD was (52.8 ± 12.5 years) .

The age and sex of this group were matched to age and sex of patient groups, where statistical analysis showed nonsignificant differences in the age and sex between patient and control groups ($p > 0.05$).

A permission was taken from all subjects to contribute tothis study after they were told about the aim and advantages of this study.

The level of serum homocysteine was determined by using enzyme linked immunosorbent assay technique with a procedure presented by the manufacturer[14].

For MTHFR genotyping, a set of primers including (forward primer: 5′-TGAAGGAGAAGGT GTCTGCGGGA-3′ and thereverse primer was 5′ -AGGACGGTGCGGTGAGAGTG-3′. were used to amplify 198-bpproducts[15].

A master premix of GoTaq® Green Master Mix (Promega-Green Master Mix) was used.

PCR optimization was done as a first step by using a gradient temperature ranging from 63.4^{0}Cto 72.6 ^{0}Cwith 0.3 step differences in PCR wells. This was highly important to determine the optimum annealing temperature. After the determination of optimum annealing temperature (68.6^{o}C), the PCR reaction mixture consisted of 20-50 ng template DNA, 200 mM of each dNTP, 5µl buffer, 1 U Taq DNA polymerase, 10pmol of each primer and 1.5 mM $MgCl_2$ in 20 µl of total reaction volume.

Amplification reactions were carried out by using GTC Series thermocycler (Cleaver Scientific /UK) apparatus.

After determination of the optimum annealing temperature the following program was set in the thermocycler to amplify the target DNA fragments as shown in table (1).

Table (1) Amplification conditions of MTHFR genotyping

Cycles	Function	Time(min)	Temp.(C^O)	Stage
	Initial denaturation	5	94	1
30	DNA denaturation	0.5	94	2
	Primer annealing	0.5	68.6	
	Template elongation	0.5	72	
	Final elongation	5	72	3
Hold	Incubation	-	4	4

The 198-bp PCR product (10 ul) was digested with therestriction enzyme HinfI at 37°C for 3-4 hours in thebuffer recommended by the manufacturer. HinfIcanrecognize the C-to-T substitution in the fragments. Thisone nucleotide substitute corresponds to a conversionof Ala-to-Val residue in the MTHFR encoding region.

Results

Plasma concentration of (Hcy) wasincreased significantly in patients with stroke and patients with MI when compared with control group (P < 0.01). As illustrated in figure (1) .

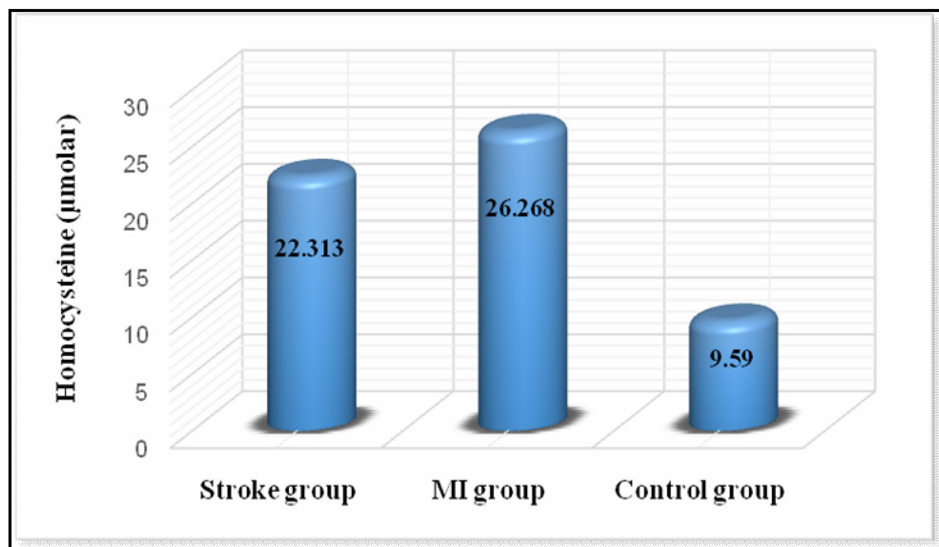

Figure (1) Plasma Hcy concentration (µmolar) in studygroups.

The figure (1) also showed that homocysteine level differed significantly between patients with stroke and patients with MI.

The process of amplification of MTHFR gene by PCR resulted in a product with 198 bp , as shown in figure (2).

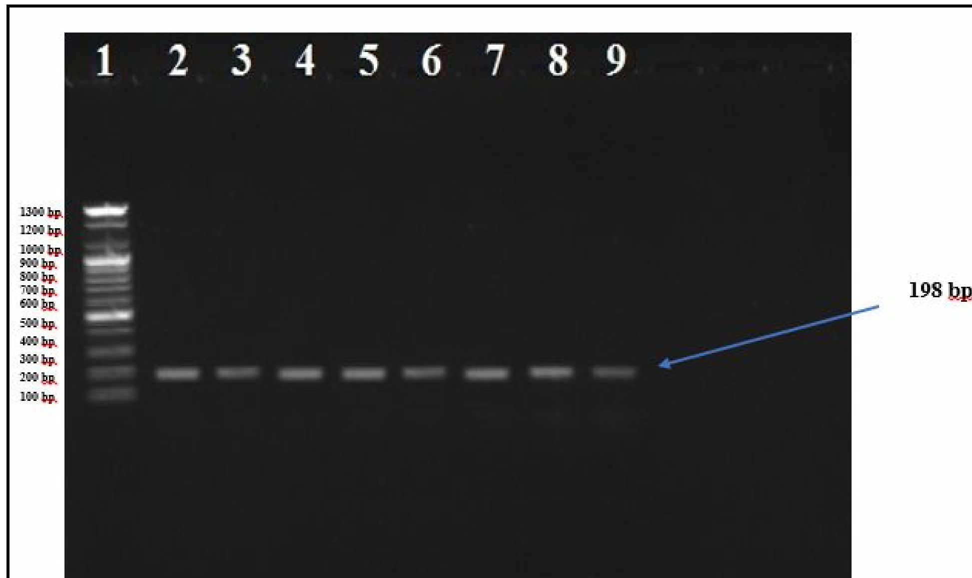

Figure (2) MTHFR gene amplification on agarose gel (2%).

To detect the C677T polymorphism in the MTHFR gene, a polymerase chain reaction restriction fragmentation length polymorphism (PCR – RFLP) was applied by using a specific restriction enzyme (HinfI), HinfI can recognize the C-to-T substitution in the fragments. This one nucleotide substitute corresponds to a conversion of Ala-to-Val residue in the MTHFR encoding region.

The two different alleles were designated T (Val) and C (Ala), the 198-bp fragment derived from the C allele is not digested by HinfI, whereas the fragments of the same length from the T allele is digested by HinfI into 175 and 23-bp fragments. Subjects homozygous for the mutation showed two DNA fragments of 175-bp and 23-bp, whereas homozygous subjects without it showed a DNA fragment of 198-bp. Heterozygous subjects showed three DNA fragments of 198-bp, 175-bp and 23-bp., as shown in figure (3).

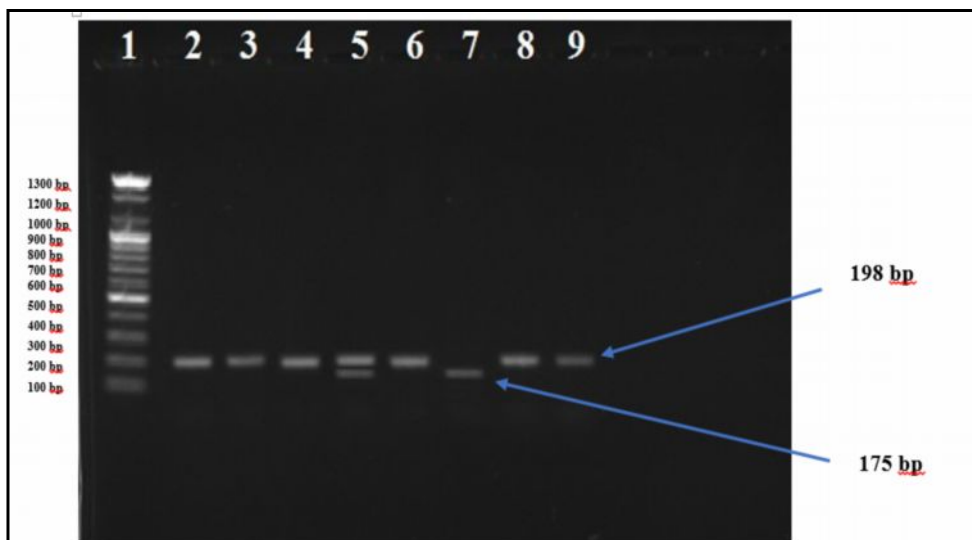

Figure (3): Electrophoretic pattern represents the MTHFR genotyping, where lane 1 is 100 bp DNA ladders,

- Lanes (2,3,4,6,8 and 9) representing homozygous CC genotype.
- Lane 5 representing heterozygous CT genotype,
- Lane 7 representing homozygous TT genotype.

All subjects enrolled in the present study were categorized depending on the process of fragmentation of MTHFR gene being (CC) for homozygous wild genotype, (CT) for heterozygous polymorphism, and (TT) for homozygous polymorphism, as summarized in table (2).

Table (2) MTHFR gene polymorphism with allele frequency

Group	Genotype			Total	Allele frequency	
	CC	CT	TT		C	T
Patients with stroke	17 (57%)	7 (23%)	6 (20%)	30	68%	32%
Patients with MI	16 (53%)	8 (27%)	6 (20%)	30	67%	33%
Control	19 (63%)	6 (20%)	5 (17%)	30	73%	37%
Total	52	21	17	90		

From the table (2), homozygous wild genotype (CC) is the abundant genotype in all study groups.

In order to evaluate the significance of these results, Chi square test was used to investigate the odds ratio and significance of genotyping and allele frequencies, as shown in table (3).

Table (3) MTHFR gene polymorphism characterization in patient groups and control group.

Genotype	Patients with stroke	Control	Odds ratio	Patients with MI	Control	Odds ratio	Patients with stroke	Patients with MI	Odds ratio
CC	**17**	**19**	**1.34**	**16**	**19**	**1.4**	**17**	**16**	**1.1**
CT	**7**	**6**	**0.97**	**8**	**6**	**1.1**	**7**	**8**	**1.14**
TT	**6**	**5**	**Reference group**	**6**	**5**	**Reference group**	**6**	**6**	**Reference group**
C	**68%**	**73%**	**0.79**	**67%**	**73%**		**68%**	**67%**	**1.1**
T	**32%**	**27%**		**33%**	**27%**		**32%**	**33%**	

*** Significant difference (P < 0.05).**
**** CI $_{95\%}$: confidence interval at 95 % level.**

To demonstrate the effect of C677T polymorphism in MTHFR gene on homocysteine level in study groups, an ANOVA test was conducted, as shown in table (4).

Table (4): Plasma homocysteine level (µmolar) in regard to genotypes of MTHFR gene in control and patient groups.

Genotype	Hcy level in stroke group (Mean ± SD)	Hcy level in MI group (Mean ± SD)	Hcy level in control group (Mean ± SD)
CC	19.9± 3	22.1 ± 6.5	8.1 ± 1.1
CT	23.5 ± 4.9	28.5 ± 1.2	10.8 ± 1
TT	27.9 ± 4.6	34.3 ± 1.2	13.7 ± 2.4

*** Significant difference (P < 0.05)**

Discussion

It is generally accepted that plasma homocysteine has pro-coagulative effects and induces endothelial damage, which may lead to thrombotic vascular disease [16]. Hofmann *et al.*had shown that the induction of high homocysteine levels enhances the expression and activity of key participants in vascular inflammation,

atherogenesis, hypercoagulation status and vulnerability of the established atherosclerotic plaque. The level and activity of tissue-destructive enzymes have been shown to be increased with hyperhomocysteinaemia[17].

These enzymes, present in the atherosclerotic plaque, may promote lesion instability and rupture. Thus higher homocysteine levels could cause ischaemic stroke or cardiovascular ischemia through its hypercoagulative effect[18].

A common missense mutation in the MTHFR gene, a C-to-T substitution at nucleotide 677, is responsible for reduced MTHFR activity and is associated with moderate increase in plasma homocysteine concentrations. C677T homozygous genes have significantly elevated plasma homocysteine levels[19].

The results of this study suggested no association between C677T polymorphism in the MTHFR gene and the incidence of neither stroke nor myocardial infarction.

Methylenetetrahydrofolate reductase plays a relevant role in Hcy metabolism, and the T allele of the C677T polymorphism is known to induce an impaired MTHFR *in vitro* activity, ranging from 30% to 70%, due to the increased enzyme thermo-liability [20].

The significant difference ($P < 0.05$) in the level of homocysteine between MTHFR genotypes can be contributed to the effect of C677T polymorphism of MTHFR gene which reduces the activity of MTHFR, thereby lowering its role in the metabolism of homocysteine into methionine[21,22].

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