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EVALUATION OF *PROTHROMBIN* GENE POLYMORPHISM FREQUENCY IN PATIENTS WITH ISCHEMIC STROKE IN IRAQI POPULATION

Dhaneen Mahdi Abdul-Zahra and Rand Muhammed Abdul-Hussein Al-Husseini*

Faculty of Science, University of Kufa, Al-Najaf Governorate, Iraq.

Abstract

Stroke is one of the leading causes of death worldwide, and increasing evidence indicates that coagulation abnormalities cause stroke and the formation of a secondary ischemic stroke, either in the accompanying vessel or in the form of an embolus. Therefore, the balance between coagulation and fibrinolysis is a critical component of the ischemic stroke. The study population was included 75 ischemic stroke patients and 25 healthy subjects. The estimated incidence of ischemic stroke increased in the age group 65-74 years, with a significant difference ($p<0.05$) in comparison with the other groups. Results of coagulation tests indicated that no significant differences were found in the results of prothrombin time between the IS patients and control. A PCR-SSP technique was performed for detection prothrombin gene 20210 G/A (rs1799963) polymorphism, in ischemic stroke patients. The genotype distribution results of the 20210 G/A SNP of prothrombin gene were not showed significant differences ($p>0.05$) between controls (GG : n = 25, 100%; GA: n = 0; AA: n = 0) and ischemic stroke patients (GG : n = 66, 88%; GA: n = 8, 10.66%; AA : n = 1, 1.333%). The results of prothrombin time did not differ according to the prothrombin gene genotype and it statistically not significant ($p>0.05$) in ischemic stroke patients that carrier A allele or G allele than controls. Results showed that G allele of the 20210G/A and the mutant allele (A) was not statistically significantly linked with the ischemic stroke (OR = 0.251, 95% CI = 0.032-1.996, p = 0.16). However, the 20210 G/A mutant allelesare not entirely absent among Iraqi patients with ischemic stroke.

Key words: Stroke, *Prothrombin* gene, Iraq, 20210 G/A polymorphism

Introduction

Ischemic stroke (IS) is a neurological deficit attributed to an acute injury of the central nervous system by a vascular cause, it occurs as a result of obstruction by a blood clot (thrombus) or plugs within a blood vessel supplying blood to the brain- carotid or vertebral artery or less likely a cerebral vein (Al-Gazally *et al*., 2014; Hameed and Ahmed, 2018). This disease considered as the most common type of stroke in older adults with high mortality and morbidity rates (Al-Qaysi *et al*., 2015; Xu *et al*., 2018). Genetic factors that are associated with increased stroke risk are a family history of stroke and a number of single nucleotides polymorphisms SNPs (Baird, 2010). Hypercoagulability disorder is a hereditary or acquired condition that increases the risk of thrombosis (Linnemann & Hart, 2019). Hypercoagulability may have an important role in the pathogenesis of ischemic stroke (Carcaillon *et al*., 2011; Bona, 2016). Factor V Leiden (G1691A) and the prothrombin gene mutation (20210 G/A; rs1799963) comprise the most common genetic associations with thrombosis and thus comprise the most commonly requested genetic thrombophilia investigations (Favaloro, 2019). Prothrombin (coagulation Factor II) is a complex glycoprotein that plays a central role in blood coagulation. It is the zymogen precursor to the protease thrombin that catalyzes the formation of the fibrin clot and regulates a multitude of other cellular responses related to coagulation and hemostasis (Wendeler *et al*., 2014). Prothrombin is activated to thrombin by the prothrombinase complex through sequential cleavage at two distinct sites (Adams & Huntington, 2016). *Prothrombin* is encoded by the FII gene, which is located on the short arm of chromosome 11 (Degen & Davie, 1987). Previous data have shown that the 20210G > A polymorphism of the Factor II gene is related to an increased prothrombin level, which may in turn lead to a procoagulant state (Sarecka-Hujar *et al*., 2017). This

***Author for correspondence :** E-mail : rand.alhusseini@uokufa.edu.iq

study aimed to assess the diagnostic prevalence and prognostic significance of *prothrombin* polymorphism: G20210A in ischemic stroke patients, and determine whether prothrombin time is associated with genotypes.

Materials and Methods

Study group consists of 75 patients with confirmed ischemic stroke. They were admitted to Alsader Specialized Center of the Middle Euphrates Neurosciences and 25 healthy subjects as controls. Tests were performed on 2ml of venous blood, which was collected from ischemic stroke patients and control group. 1ml was collected in sodium citrate tubes centrifuged at 5000 rpm for 5 minutes; plasma was used freshly for the Prothrombin time (PT). 1 ml was collected in tubes with anticoagulant EDTA and used for PCR test.

PT determination was done by kit, which is BIOLABO REAGENT products (02160, Maizy, France/ REF: 13560). The clotting time was measured at 37ºC in the presence of tissular thromboplastin and calcium, the PT (in sec) so measured and converted into PT(%) or INR.

Genomic DNA was isolated by using protocol of DNA Mini Kit, which designed for purifying DNA from Geneaid Biotech. Ltd., Taiwan Company (Cat. No. GS100, LOT. No. FE31708-B). Amplification the of SNPs fragments in *prothrombin* gene was done by using a conventional PCR thermocycler (Labnet/USA). The amplification steps consisted of a first denaturation step where DNA was initially denatured for 10 minutes at 95ºC and then 10 cycles were performed as follows: 94ºC for 30 seconds, 60ºC for 30 seconds and 72ºC for 1 minute. Then, 25 cycles were performed as follows: 94^0C for 30 seconds, 55ºC for 30 seconds and 72ºC for 1 minute. The PCR amplification was completed by a final extension at 72ºC for 7 minutes. Amplification of the prothrombin genes resulted in 340-bp products. DNA was amplified with primers specific for prothrombin 20210 G/A polymorphism for each sample. The primer-pairs which listed in table 1, were synthesized by Accu Oligo® Bioneer Corporation, USA. The primers sequences were published by Ranguelov *et al*., (2002) and Othman *et al*., (2010). PCR sequence-specific primers (PCR-SSP) technique was performed for detection and genotyping *prothrombin* single-nucleotide polymorphism in blood samples. The basis of this method is that a perfectly matched primer is more efficient in a PCR reaction than mismatched primers. So, specificality is determined by the use of sequence specific primers (SSP) in two reactions (in two tubes) with two sets of primers: one primer is specific for each allele (allele specific primer)

Table 1: Primers sequences for prothrombin 20210 G/A polymorphism.

Gene	Primers sequences		PCR product
Prothrombin (PT) (20210 G/A)	PT	52 -TCTAGAAACAG	340bp
	common	TTGCCTGGCAG-32	
	PT Wild	52 -GCACTGGGAGC	
	(WT)	ATTGAGGATC-32	
	PT mutant	52 -GCACTGGGAGC	
	(MT)	ATTGAGGATT-32	

and is paired with a second common primer. There is a reduction in the efficiency of Taq polymerase to amplify DNA when there is a 3' single-base (3' terminal nucleotide) mismatch inhibits the initiation of a non-specific reaction between the target DNA and the allele-specific primer so even primer pairs do not specifically anneal and are not efficiently amplified (Sadler *et al*., 1994; Ayyadevara *et al*., 2000). Therefore, only the required allele will be amplified. Then the amplified product in the two tubes can be detected by agarose gel electrophoresis for wild type (WT) and the mutant type (MT) separately. This method is a relatively simple and inexpensive procedure for prothrombin genotyping. The gel electrophoresis method, which included preparing the gel loading and running the gel, was done according to Sambrook and Russell (2001).

Statistical analysis

Statistical analyses of all results were carried out by the help of Statistical Package for the Social Sciences (SPSS) version 23 software statistical package using t-test and Chi-square test (with P value at level of significance less than 0.05) to compare values of results between groups. Result values were expressed as mean ± SE, number of patients, or percentages.

Results and Discussion

The clinical assessment revealed that the frequency of distribution of patients according to gender were 39 (52%) females and 36 (48%) males. The differences of gender were statistically not significant.

These results agree with Sohrabji *et al*., (2017) and Chauhan *et al*., (2017) studies that showed Women are affected by stroke, having a higher incidence and worse outcomes than men, also more women than men have strokes each year, in part because women live longer.

These findings are consistent with those of Dehlendorff *et al*., (2015) study that showed after the age of 60 years, women had more severe strokes than men.

These results are disagreement with Zafar *et al*.,

(2016) study that showed IS to be significantly more common in males than females (p = 0.021). On the other hand, these results were in disagreement with palm *et al*., (2012) study which reported that etiology of ischemic stroke differs between sexes, with large-artery atherosclerotic stroke and associated diseases (coronary artery disease and peripheral artery disease) being more common in men.

These results show that women diagnosed with ischemic stroke are more than men, largely due to increase in stroke incidence in older postmenopausal women, also lower stroke incidence observed in pre-menopausal women and robust preclinical evidence of neuroprotective and anti-inflammatory properties of estrogen (Koellhoffer & McCullough, 2013; Spychala *et al*., 2017).

The major risk factor of ischemic stroke in both genders was Hypertension, because its effect on alterations in cerebral artery structure and function that can impair blood flow (Pires *et al*., 2013), however, males were more likely to have a history of smoking, heart disease and dyslipidemia (Memis *et al*., 2016), (Wu *et al*., 2014), while in women more likely to have a past medical history of atrial fibrillation or hypertension and less likely to have a history of heart disease, dyslipidemia, or smoking (Reeves *et al*., 2009).

These results are disagreement with study done by Mohamed and Alshekhani (2016) that found Stroke incidence more common in male (59%) than female (41%).

These results are disagreement with Zafar *et al*., (2016) study showed IS to be significantly more common in males than females (p = 0.021). Women have more stroke events than men because of their longer life expectancy and much higher incidence at older ages (Reeves *et al*., 2008).

Assessment of age presentation of patients at diagnosis revealed that 7(9.3333%) in age group (35-44), 8 (10.666%) in age group (45-54), 9 (12%) in age group (55-64), 29 (38.666%) in age group (65-74) and 22(29.333%) in age group (75-84) Fig. 1. Their ages ranged from 28 to 84 years, with a mean age of 66.9333333 years.

The estimated incidence of ischemic stroke increased in the fourth age group (65-74), with a significant difference ($p < 0.05$) in comparison with the other groups.

These results are agreed with Ahangar *et al*., (2018) study that showed about 84.3% of stroke occurred in patients aged > 50 years.

These findings were comparable to the findings of Jung *et al.,* (2012) study where the 46.098 patients included in their study with mean ± SD age was 66.1 ± 12.3 years and the increases in ischemic stroke disease prevalence were largest for persons aged 65 years or older.

These results showed that patients aged 65 years or older are more at risk than younger for ischemic stroke, maybe aging and growth of the population that led to an increase in the absolute number of strokes, especially in the elderly, that is expected to increase dramatically in the coming years (Béjot *et al*., 2019).

These results are agreed with Abd *et al*., (2017) study documented in Iraq, which showed the rate of stroke in Iraqi patients significantly associated with advance age. Soriano-Tárraga *et al*., A study (2016) showed that stroke is related to aging and corresponds to current findings.

These findings are consistent with the study of Abdul-Razak and Shati (2008) that showed that age is significantly related to the incidence of stroke, and advancing age (age more than 60 years) showed increased incidence of stroke. Advanced age is the most important predictor of poor outcome (Aksoy *et al*., 2013).

Several aging-related changes in the brain have been identified that are associated with an increase in vulnerability to ischemic stroke in the elderly (Chen *et al*., 2010).

Results of coagulation tests represented by prothrombin time and INR for IS patients were showed in table 2. No significant differences were found in the results of PT and INR between the IS patients and control.

The present study showed there is no significant difference in prothrombin time level between the IS patients and control group.

This finding was comparable to previous findings of Kravchenko and his colleagues (2018) who reported that

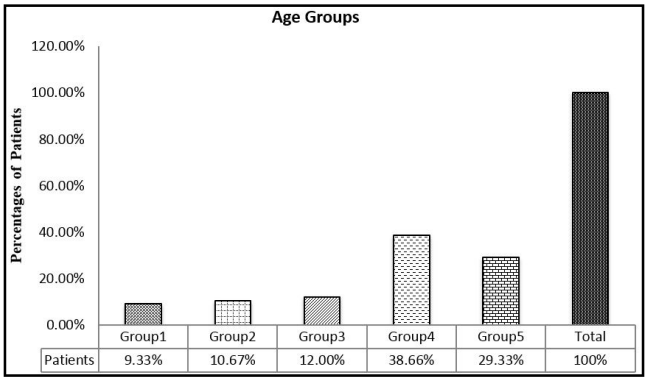

Fig. 1: Age distribution of patients presented with ischemic stroke (Group1: 35-44 years, Group2: 45-54 years, Group3: 55-64 years, Group4: 65-74 years, Group5: 75-84 years).

Table 2: Coagulation tests (prothrombin time and INR) of ischemic stroke patients and control group.

Parameter	Ischemic stroke patients (n=75)	Control (n=25)	P-Values
Prothrombin time (sec)	15.612 ±2.913	15.61538462 ±1.514	0.9
INR	1.15 ±0.312	1.156153846 ±0.118	0.9

Results values were expressed as mean± SE, *: p< 0.05 or significant differences between mean values. Abbreviations: PTT= partial thromboplastin time, INR= international normalized ratio.

no significant differences in prothrombin levels were detected in the patients compared with the control. While these current results were not consistent with the study of Antovic *et al*., (2002) which found a significant decrease in prothrombin time in ischemic stroke patients compared with the control group.

The result of this study showed there is no significant difference in international normalized ratio (INR) level in ischemic stroke patients and control group.

These findings are in accordance with results reported by Green *et al*., (2008) which found that there was no significant differences were found in INR between control group and ischemic stroke patients. While these current results were not consistent with the study of Amiri *et al*., (2016) which found a significant increases in mean INR in stroke patients compared to control group (P = 0.001) (2.43 ± 0.9) (2.1 ± 0.93) respectively. The incidence rate of ischemic stroke events increased sharply in INR <1.59 (Yasaka *et al*., 2001). Low INR values were attributable to low-dose warfarin (Nakamura *et al*., 2013). Patients with a low international normalized ratio (INR) should be at greater risk for ischemic stroke (Cao *et al*., 2017). High INR predisposes a patient to a high risk of bleeding, while INR below the therapeutic target indicates that the dose of warfarin is insufficient to offer protection against thromboembolic events (Çoban *et al*., 2017).

The *prothrombin* gene SNP (20210 G/A) and ischemic stroke results showed that: all the 25 healthy subjects (25, 100%) had found as homozygous GG alleles; (GG: n = 25, 100%; GA: n = 0; AA : n = 0) table 3 and Fig. 2.

b) Ischemic stroke patients: Among the 75 ischemic stroke patients; 66 (88%) had found as homozygous GG alleles, 8(10.666%) found as heterozygous genotype (with the G and A alleles (GA) and 1(1.333%) had found as homozygous genotype AA alleles; (GG : n = 66, 88%; GA: n = 8, 10.66%; AA: n = 1, 1.333%) table 3 and Fig. 2.

Table 3: The results of genotypic frequencies of 20210 G/A at *prothrombin* gene in patients and controls.

Genotypes	Healthy controls (N = 25)	Ischemic stroke patients (N = 75)
GG	25 (100%)	66 (88%)
GA	0	8(10.666%)
AA	0	1(1.333%)
P-value	0.192*	
Alleles frequency	N(%)	N(%)
G allele	50(100%)	140(%92.666)
A allele	0	10(%7.333)
X^2	1.976	
P-value	0.16*	
OR (95%CI)	0.251(0.032-1.996)	

Data were expressed as number and a percentage (N%). *P >0.05 not significant. Abbreviations: X^2 = chi-square, OR = odds ratio, CI = confidence interval.

That means the frequencies of 20210 G/A of *prothrombin* gene in the 75 Iraqi ischemic stroke patients in Al-Najaf province were not with significant differences with that of the 25 healthy controls group (p>0.05).

This result showed that G allele of the 20210G/A and the mutant allele (A) was not statistically significantly linked with the ischemic stroke (OR = 0.251, 95% CI = 0.032-1.996, p = 0.16).

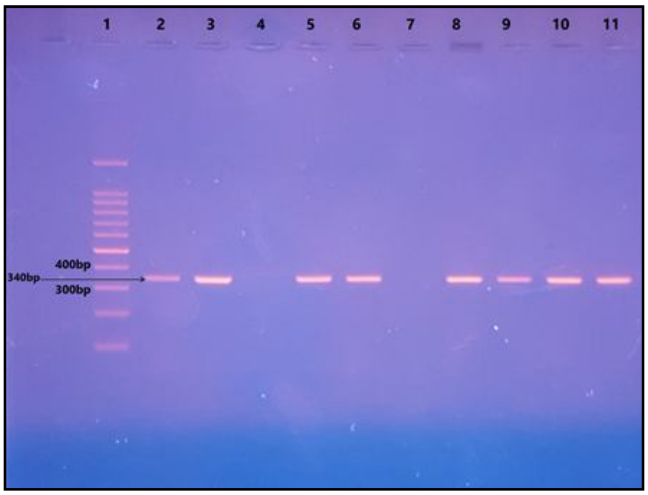

Fig. 2: The electrophoresis image of PCR-SSP analysis of *prothrombin* geneSNP(20210G/A) (Lane 1: 100 bp DNA Ladder; Lane 2&3 (sample 1): Heterozygous genotype (GA; 340-bp in each tube); Lane 4&5(sample 2): homozygous mutant (AA) genotype (tube 1 for wild type there was no band, tube 2 for mutant type 340-bp); Lane 6&7(sample 3): homozygous wild (GG) genotype (tube 1 for wild type 340-bp, tube 2 for mutant type there was no band); Lane 8&9(sample 4): Heterozygous genotype (GA); and Lane 10&11(sample 5): Heterozygous genotype (GA).

This conclusion indicates the 20210 G/A SNP did not show associations with ischemic stroke risk.

Ridker *et al*., (1999) study found that the G20210A *prothrombin* gene variant was not associated with increased risk of stroke. This study is consistent with the current study.

The study done by Chang and Zhang (2000) found that Factor II gene G20210A mutant allele is absent in Chinese patients with ischemic stroke and normal subjects and this mutation may not be a major risk factor for thrombogenesis in Chinese people. This study is compatible with the present study.

Lopaciuk *et al*., (2001) study indicated that *prothrombin* G20210A genotype, was not associated with an increased risk for ischemic stroke in young adults. This study is consistent with the current study.

These results are consistent with Weischer *et al*., (2010) study which showed that there was no association between G20210A *prothrombin* heterogeneity and IS risk.

These findings are in accordance with results reported by Bondarenko *et al*., (2011) who showed that there were no association between *prothrombin* gene polymorphism and the risk of ischemic stroke development.

The study done by Içli *et al*., (2013) that showed there was no statistically significant difference in *prothrombin* G20210A variant among stroke patients and control groups. This study corresponds to the current study.

In the study conducted by Jiang *et al*., (2014) observed that the association of the prothrombin G20210A mutation with ischemic stroke did not achieve statistical significance (OR = 2.5, 95% CI = 0.9-6.5, p = 0.07). This study is consistent with the current study.

These findings consistent with Pirhoushiaran *et al*., (2014) study conducted in Iran which observed that FII G20210A SNP was not significant differences between IS patient and control groups.

The study of Erten *et al*., (2015) observed that mutation of *prothrombin* gene G20210A is not associated with stroke and is not important in the case of ischemic stroke. This study agrees with the current study.

Recently the study by Ayas *et al*., (2019) showed that no significant difference was found in *Prothrombin* 20210 G/A polymorphism with ischemic stroke risk. This result close to the present study.

The present study inconsistent with the study of De Stefano *et al*., (1998) which found that *prothrombin* mutation G20210A was significantly higher in stroke patients than in the control group.

Lalouschek *et al.,* 's study (2005) that found the frequency of the FII G20210A mutation was significantly higher in patients compared with controls (6% versus 1%; adjusted OR, 6.1; 95% CI, 1.3 to 28.3; P = 0.021). These results disagreement with the current study.

A study by Eterovic and his colleagues (2007) showed that G20210A mutations in *prothrombin* gene were significantly higher in IS patients than controls. This study is incompatible with the current study.

The results of prothrombin time did not differ according to the *prothrombin* gene genotype and it statistically not significant ($p > 0.05$) in ischemic stroke patients that carrier A allele or G allele than controls table 4.

Bauer, (1993) reported that the elevated levels of plasma prothrombin could lead to a higher occurrence of thrombin formation and subsequently more incidence of fibrin clotting.

The G20210A point mutation in factor II or *prothrombin* is associated with increased plasma levels of prothrombin and increased risk of venous thrombosis in its heterozygous form (Poort *et al*., 1996; Van Cott and Laposata, 1998).

Soria *et al*., study (2000) demonstrated that the polymorphism in *prothrombin* gene G20210A associated with increased prothrombin levels as well as increased susceptibility to thrombosis.

In a study conducted by Jadaon (2011) mentioned that the mutation of *Prothrombin* G20210A causes higher levels of the clotting factor prothrombin in the blood and creating a higher tendency towards blood clotting (hypercoagulability).

The mutation in the *prothrombin* gene may enhance the thrombogenic potential (Dobrynina *et al*., 2012).

Table 4: The results of prothrombin time (sec.) according to genotype of gene polymorphism (20210 G/ A) in ischemic stroke patients and controls.

Genotype	Prothrombin time (sec.)		P values
	Healthy controls(N=25)	Ischemic stroke patients(N=75)	
GG	15.615 ± 1.514	15.522 ± 2.993	0.273*
GA	0	16.38±2.4	
AA	0	15.3	

Results values were expressed as mean± SE, *: p> 0.05 or no significant differences between mean values.

Ghatak *et al*., (2013) study demonstrated that this mutation in FII 20210 G/A is associated with an increased plasma concentration of prothrombin, which leads to an increased potential for thrombin generation.

Sarecka-Hujar *et al*., study (2017) showed that the 20210G > A polymorphism of the *prothrombin* gene is related to an increased prothrombin level, which may in turn lead to a procoagulant state in ischemic stroke patients.

Conclusions

The genotype distribution results of the 20210 G/A SNP of prothrombin gene were not showed significant differences between controls and ischemic stroke patients. However, the 20210 G/A mutant alleles are not entirely absent among Iraqi patients with ischemic stroke.

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