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FACTOR V LEIDEN AND PROTHROMBIN GENE MUTATIONS IN PATIENTS WITH ARTERIAL THROMBOTIC DISEASES FROM KASHMIRI POPULATION

Genetics

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ABSTRACT

Aim: The role of FVL mutation and prothrombin gene mutation have been well established in venous thrombolism. The aim of this study was to investigate whether these mutations have a role in arterial thrombotic disease namely ischemic stroke and intracranial hemorrhagic in Kashmiri population.

Methodology: A case-control study was designed with 75 ischemic stroke patients, 75 intracranial hemorrhagic patients and 100 healthy controls. The mutations were analysed using ARMS- PCR and PCR-RFLP approach.

Result: The FVL GA genotype was found to be to be significantly associated with the increased risk of ischemic stroke (p=0.03) while no association was found with intracranial haemorrhage (p=0.07). The prothrombin 20210 G > A mutation was not found in any of the cases or controls.

Conclusion: The increased frequency of FVL mutation in ischemic stroke patients indicates a significant role of this mutation in the development of ischemic stroke in our population. We therefore suggest the routine screening of FVL mutation as a thrombophilic marker in Kashmiri patients with arterial thrombosis.

KEYWORDS

Kashmir, Ischemic stroke, Intracranial haemorrhage, Factor V Leiden, Prothrombin, Mutation

Introduction:

Stroke is one of the common neurological disorders. It is the third most common cause of mortality and the most common cause of disability worldwide **(WHO, 2002)**. Its prevalence amongst young adults ranges from 3 to 5% **(Hart R.G. et al. 1983; Adams H.P. et al. 1986).** Almost 85% of strokes are ischemic in origin and 15% are due to primary intracranial haemorrhage **(Warlow C.P. 1998; Goldstein L.B. et al. 2001; WHO, 2002).** Although the major risk factors of stroke are hypercholesterolemia, systolic and diastolic blood pressure, smoking, high alcohol consumption and oral contraceptives, yet prothrombotic genetic factors may also be important underlying factors for these infarcts **(Durai P.J. et al. 2007).** Recently, the most frequent genetic factors emerged to be associated with thrombophilia are the point mutations in the genes encoding coagulation factor V (FVL) and prothrombin gene. The FVL mutation (G to A substitution at position 1691 of the FV gene) prevents the inactivation of coagulation factor V by activated protein C, thereby leading to a state of hypercoagulability. FVL has been indicated as a major genetic risk factor for venous thromboembolism in adults and ischemic stroke in children across many studies. The prothrombin gene mutation (G to A substitution at position 20210 in the 3'-UTR of the prothrombin gene) is the second most common known inherited risk factor for thrombosis. This mutation increases the plasma prothrombin levels higher than normal without leading to any functional difference in prothrombin molecule. It almost increases the 2-fold risk of deep vein thrombosis (DVT) and pulmonary emboli (PE) **(Kim R.J. et al. 2003).**

Although these two mutations are well established as a risk factors for venous throm-bosis, but their role in arterial thrombotic disease like stroke is contentious. While many epidemio¬logical studies have shown their association with an increased risk of stroke and coronary artery disease **(Gómez Garcia E.B. et al 2002; Herrmann F.H. et al. 2001; Ye Z. et al. 2006)**, many other failed to find any significant association between these two mutations and stroke in adult populations **(Bertina R.M. et al. 1994; Kalafatis M. et al. 1994).**

To date no study has evaluated the effect of the FVL G1691 and Prothrombin G20210A mutations in patients with ischemic stroke and intracranial haemorrhage in Kashmiri population. In the present study, we therefore designed a case-control study to examine whether these mutations are genetic risk factors for ischemic or hemorrhagic stroke of Kashmiri population.

Materials and Methods:

Subjects:

We included a total of 150 stroke patients among which 75 cases were those with intra-cranial haemorrhagic (ICH) and 75 cases with ischemic stroke (ISC). Patients were recruited from the Department of Neurology, Sher-i-Kashmir Institute of Medical Sciences hospital. A total of 100 age and sex matched healthy controls were also included in the study. Both the patients and controls gave informed consent to participate in the study. The study was approved by ethics committee of the institute. A pretested, semi-structured questionnaire was used to collect the information on clinical and laboratory parameters. Data from all the patients was obtained from personal interviews with patients and/ or guardians, and based on prior medical records or standard clinical examination and tests. The data collected included age, gender, smoking status, Diabetes, Hypertenion, Dyslipidemia, ICH Score, Location of ICH, Vascular Type of Ischemic Stroke, NIHSS Severity Grading. The exclusion criteria for selecting the patients was ischemic stroke patients with atrial fibrillation on ECG or a known valvular heart disease predisposing to cardiac emboli or documentation of L.V. clot or wall motion abnormalities on precordial echocardiography and haemorrhagic stroke patients with history of trauma, SAH, suspected or documented aneurysm or ICSOL.

Sample collection and DNA extraction:

About 2-3 mL of peripheral blood was collected from stroke patients in tubes containing ethylenediamine tetraacetic acid (EDTA) and DNA was isolated using a Zymogen (Irvine, CA, USA) DNA extraction kit. The quality of the DNA was checked by agarose gel electrophoresis. The extracted DNA was stored at -20°C for further use.

Mutation Detection:
Amplification refractory mutation system (ARMS) PCR assay was established for the molecular analysis of Factor V (Leiden) G1691A- and Prothrombin G20210A-mutations. The PCR primers were designed based to amplify a 150bp fragment form exon 10 of Factor V gene surrounding nucleotide 1691 and 350bp fragment from PT gene surrounding nucleotide 20210 was amplified. The primers used in the PCR amplification reactions included a common primer and normal allele specific primer or a mutation specific primer. The primer sequences are given in **Table 1.** The PCR reaction was set in a final volume of 25ul mixture containing 1X PCR buffer (Biotools), 0.2 mM dNTP mixture (biotools), 150 ng each primer (Sigma), 1U Taq DNA polymerase (Biotools 5U/µl), and 200 ng genomic DNA (0.2 µg/µl). Amplification was done at 94 for 7 min, 30 cycles of 94 °C for 30 s, X °C for 30 sec min, 72 °C for 30sec min followed by extension at 72 °C for 7 min. The primer sequences and annealing temperatures are given in **Table 2**. The presence or absence of PCR products were visualized under UV light after electrophoresis using 2% agarose gel (Genie, Bangalore, India).

Statistical analysis:
Statistical analysis was performed by using Chi-square-testing and Fisher's exact test. A value of $p < 0.05$ was considered significant.

Result:
A total of 150 patients suffering from stroke, among them 75 subjects with ISC and 75 subjects with ICH were evaluated for factor V (FV) G1691A- and prothrombin (PT) G20210A-mutations in an age and gender matched case-control study with 100 healthy controls. Among 75 ISC cases, 43 patients were females (F) and 32 were males (M) (F/M ratio = 1.34). 45 patients were ≤55 of age and 30 patients were >55 of age. The mean age of ISC patients was 55.69 years. 54 patients were hypertensive and 21 were non-hypertensive. Dyslipidaemia was present in 34 patients and absent in 41 patients, 18 patients had diabetes and 57 were non-diabetic, 46 patients were smokers and 29 patients were non-smokers. According to NHISS Grading System, 12 patients had mild form of disease, 26 patients had moderate disease, 15 patients had severe disease and 4 patients had very severe form of disease. Among the ICH patients, 41 patients were males and 21 patients were females. 27 patients were ≤55 of age and 35 patients were >55 of age. The mean age of ICH patients was 58.29 years. 50 ICH patients were hypertensive and 12 were non-hypertensive. The location of ICH was lobar in 17 ICH cases and non-lobar in 45 ICH cases. The ICH Score was 0 in 10 patients, 1 in 16 patients, 2 in 24 patients, 3 in 11 patients and 4 in one patient. The frequency distribution analysis of selected demographic and risk factors in ISC and ICH cases and controls is presented in **Table 2.**

In our study there was no case of a homozygous FVL mutation in both patients and controls. However, 4 ISC and 3 ICH patients were found to be heterozygous for FVL mutation. The allelic and genotypic frequencies indicated GA genotype to be significantly associated with the risk of ISC in our population **(p=0.03)** while no association was found with the risk of ICH **(p=0.07) Table 5**. As for prothrombin 20210 G > A mutation, all of the cases and controls were wild for this mutation. The absence of prothrombin 20210 G > A gene variants in our study population limits the power to de¬tect a significant association with stroke. The frequencies (patients vs. controls), odds ratio (OR), 95% confidence interval (95% CI), and P value obtained from the comparison between stroke patients and controls with FVL and PT20210A versus the control group are summarized in Table II.

Discussion:
Considering the known effects of Factor V and PT genetic variants in hypercoagulability and predisposition to venous thromboembolism events like pulmonary emboli (PE) and deep venous thromboses (DVT), the present study focussed to evaluate the role of these mutations as a risk factor for arterial thrombotic diseases like ischemic stroke and intracranial haemorrhage in Kashmiri population.

The overall frequency of FVL in our healthy population was zero. Earlier also FVL has been least found in East Asian, African, and Australian populations. It is more commonly found in European populations about 5.2% in Caucasian Americans and 1.2% in African Americans **(Ridker P.M. et al. 1997).** In current study no homozygous FVL mutation was found in any of the stroke patients, however, the heterozygous FVL mutation was found in 5.33% of ISC cases and 4% of ICH cases. A statistically significant association was found between FVL and ISC but no statistical significance with ICH was observed. The potential mechanism for the association of FVL as a relevant risk for the development of cerebral infarction has been suggested to be paradoxical venous-to-arterial embolism via a patent foramen ovale (PFO) possibly due to venous occlusive disease. In many earlier studies, FVL has been associated with ischemic stroke in children **(Rosendaal F.R. et al. 1995, Barnes C. et al. 2006),** though no associated with ischemic stroke has been found in the general adult population **(Kim R.J. et al. 2003).** Although there have been some reports of arterial stroke associated with thrombophilia, a large study failed to confirm any significant association in adults **(Ridker P.M. et al. 1997)**. However, in a recent meta-analysis, FVL demonstrated a significant association with ischemic stroke in young adults. Although studies from Austria, Germany, Israel, and Turkey found FVL as risk factor for AIS, two studies from UK and our study from Argentina did not find this association **(Zenz W. et al. 1998; Ganesan V. et al. 1998; Akar N. et al. 1999; McColl M.D. et al. 1999; Nowak-Go¨ttl U. et al. 1999; Kenet G. et al. 2000).** The prevalence of thrombophilia in different geographic regions of the world may be different because of genetic racial differences or because the phenotypic expression of the disease is altered by environmental factors **(Lane D.A. et al. 1996).**

In our study the prothrombin mutation was not found among any of the ICH and ISC cases or healthy controls indicating a zero frequency of this mutation in our population and no role in arterial thrombosis. A lower prevalence of this mutation has also been reported earlier in many Asian countries like Thialand, Korea, China, Africa and Japan **(Branson H.E. et al. 1983, Heckmann J.G. et al. 2001, Behjati R. et al. 2006, Rosendaal F.R. et al. 1995)**. A variable prevalence has been reported for this mutation in earlier studies with about 3.1% in Sweden, 1.8% in Germany to 0.5% in Serbia and 2.6% in Turkey **(Sanson B.J et al. 1999; Seligsohn U. et al. 2001; Bauduer F. et al. 2005)**. Several studies assessing the role of PT (G20210A) in patients with myocardial infarction and ischemic stroke in comparison with control group **(Spina V. et al. 2000; Burke A.P. et al. 2002; Caplan L.R. et al. 1993; Sanson B.J. et al. 1999; Seligsohn U. et al. 2001)** have found no role in increasing the risk of arterial thrombosis **(Behjati R. et al. 2006; Rosendaal F.R. et al. 1995; Saadatnia M. et al. 2012)**. However, in one case-control study including 20 American female youths, a 25 fold increased risk of myocardial infarction was observed in the carries of prothrombin gene mutation (G20210A) who were smokers. No higher risk was found in those females who were carriers of this mutation but did not smoke.**(Simioni P. et al. 1999).** In another case-control study, 5.1% of young women with myocardial infarction were heterozygous for PT mutation as compared with 1.6% of control population with an age-adjusted odds-ratio of 4.0. The association was also particularly high in terms of smoking status **(Rosendaal F.R. et al. 1997).** Several studies have suggested that PT mutation cannot be a potential risk factor by itself but may perhaps increase the risk for stroke only in terms in terms of hypertension, diabetes mellitus or other stroke related risk factors **(Simioni P. et al. 1999; Mateo J. et al. 1997).** In another study, the prothrombin mutation was found in 5.1% of patients with coronary heart disease as compared with 1.96% healthy newborns **(Watzke H.H. et al. 1997).** On the other hand, three studies did not find a significantly increased prevalence of the mutation in patients with cerebrovascular disease (Corral A. et al. 1997; Bentolila S. et al. 1997; Martinelli I. et al. 19970. In studies from Germany and Turkey found this mutation as a risk factor for AIS, studies from Austria, Israel, UK did not find an association between and AIS in children **(Zenz W. et al. 1998; Ganesan V. et al. 1998; Akar N. et al. 1999; McColl M.D. et al. 1999; Nowak-Go¨ttl U. et al. 1999; Kenet G. et al. 2000].**

Whether PT mutation is associated with stroke remains controversial considering the different ethnic background and geographical distribution of this mutation. Despite the known effect of PT mutation on the incidence of venous thrombosis, the role of this mutation in arterial thrombotic events is still debatable.

Although the ischemic stroke is associated with the combination of multiple risk factors, the high prevalence of FVL mutation in ISC patients as compared to zero frequency in healthy controls in our population points out the significant role of this mutation in the development of ISC in our Kashmiri population. Therefore, current study suggests the routine screening of FVL mutation as a thrombophilic marker in Kashmiri patients with ISC.

Conflict of interest: None declared.

Table 1: Frequency distribution analysis of selected demographic and risk factors in stroke cases and controls.

Variables	ICH Cases N=75(%)	Controls N=100(%)	p-value	ISC Cases N=75(%)	Controls N=100(%)	p-value
Age (in years) ≤55 >55	31 (41.33%) 44 (58.66%)	49(49%) 51(51%)	0.35	45 (60%) 30 (40%)	49(49%) 51(51%)	0.16
Gender Female Male	25 (33.33%) 50 (66.66%)	44 (44%) 56(56%)	0.16	43(57.33%) 32 (42.66%)	44(44%) 56(56%)	0.09

Table 2: Various clinical characteristics of ISC and ISC patients.

Parameters	ICH Cases	ISC cases
Age ≤55years >55years	31 44	45 30
Gender Females Males	25 50	43 32
Hypertension Hypertensive Non- Hypertensive	62 13	54 21
Diabetes Diabetic Non-diabetic		18 57
Smoking Smoker Non-Smoker		46 29
Dyslipidaemia Present Absent		34 41
Vascular Type Large Vessel Small Vessel	----	55 20
NHISS Grading Mild Moderate Severe Very Severe	---	15 36 20 4
Location of ICH Lobar Non-Lobar	18 57	-----
ICH Score 0 1 2 3 4	14 18 28 13 2	-----

Table 3: Primer sequences and annealing temperatures of *Factor V Leiden and prothrombin* genes for mutation analysis.

Mutation	Primer sequence	Annealing temp.	Amplicon size (bp)
FVL G1691A	(C):5'GGACTACTTGACAATTACTGTTCTCTTG-3' (N):5'-GCAGATCCCTGGACAGACG-3' (M):5'-GCAGATCCCTGGACAGACA-3'	560C	150bp
PT G20210A	(C):5'TCTAGAAACAGTTGCCTGGCAG-3' (N):5'-GCACTGGGAGCATTGAGGATC-3' (M):5'-GCACTGGGAGCATTGAGGATT-3'	580C	340bp
F= forward primer; R= reverse primer; bp= base pair			

Table 4: The allelic and genotypic frequency of Factor V Leiden mutation in Stroke cases and controls:z

Group	No	Factor V Leiden c.1691 G > A genotype			*P value*	Allele Frequency		*P value*
		GG	GA	AA		G	A	
Control	100	100	0	0		200	0	
ISC	75	71	4	0	**0.03**	146	4	**0.03**
ICH	75	72	3	0	0.07	147	3	0.07

Table 5: The allelic and genotypic frequency of prothrombin gene mutation in Stroke cases and controls:

Group	No	Prothrombin g.20210 G > A genotype			*P value*	Allele Frequency		*P value*
		GG	GA	AA		G	A	
Control	100	100	0	0		200	0	
ISC	75	75	0	0	1	150	0	1
ICH	75	75	0	0	1	150	0	1

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