International Conference on Sustainable Biotechnology (ICoSBi)
Available on-line at: https://sicash.sliit.lk/

The Prevalence of β-Fibrinogen -455G/A Variant among Young Stroke Patients in Sri Lanka

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Abstract

Stroke in young adults (< 50 years) is an emerging public health concern, with both environmental and genetic factors contributing to its pathogenesis. Stroke is the second most common cause of death in the world. A stroke can be caused by a blood clot in the brain, causing brain damage that leads to disabilities, diminishing the patient's quality of life. The incidence of stroke in young patients is increasing in global and Sri Lankan populations, making young stroke a common diagnosis. A patient's genetic makeup can make them more susceptible to stroke, as the level of fibrinogen in the blood rises, increasing the risk of ischemic stroke. The β-fibrinogen gene (FGB), particularly the -455G/A promoter polymorphism, has been implicated in increased plasma fibrinogen levels, thereby enhancing thrombotic risk. This study investigates the prevalence of the β-fibrinogen -455G/A variant among young stroke patients in Sri Lanka and its potential association with ischemic stroke. Genomic DNA was extracted from peripheral blood samples using the Qiagen QIAamp DNA Blood Mini Kit. The DNA was amplified using PCR then Restriction Fragment Length Polymorphism (RFLP) was carried out using Hae III restriction enzyme. The digested products were analysed using gel electrophoresis. Allele frequencies were calculated; the Wildtype allele (p) was 0.82, and the Mutant allele (q) was 0.18, and aligned with the Hardy-Weinberg equilibrium. These allele frequencies were compared to similar studies to conclude that there was a significant prevalence of the mutant allele in young stroke patients in the Sri Lankan population. The mutant allele in this polymorphism has been associated with an increased plasma fibrinogen level. This variation can be identified as a potential risk factor for stroke in young patients in Sri Lanka.

Keywords*:* β-Fibrinogen, gene polymorphism, young stroke patients

https://doi.org/10.54389/HXEY2724

Introduction

The second most prominent cause of death in the world is strokes; there has been a 70% increase in incident strokes from 1990 to 2019, according to data published by the World Stroke Organisation. A stroke or a brain attack can be of two types, ischemic and hemorrhagic. When a blood clot blocks a blood vessel carrying blood to the brain, an ischemic stroke may occur. However, if a blood vessel in the brain ruptures and blood leaks into the brain, a hemorrhagic stroke will occur, leading to internal bleeding in the brain. Ischemic strokes are more common than hemorrhagic strokes. (Feigin et al., 2022) Strokes can lead to long-term brain damage, which can lead to severe disability or death. The most common risk factors are high blood pressure, obesity, diabetes, air pollution, and smoking. The incidence of stroke is much higher in patients over 50 years and continues to increase with age.

Each year, over 16% of all strokes affect young people aged 15 to 49 years. (Feigin et al., 2022). The incidence of strokes in young patients is high in the Sri Lankan population and is expected to increase further as certain risk factors, including high blood glucose and high blood pressure, are increasing among the youth (Mahesh et al., 2020). However, most young stroke patients do not show any traditional risk factors, and the possibility of death of younger stroke patients is higher (Smajlović., 2015 & Putaala et al., 2009). Strokes can decrease patients' quality of life by leading to disability and adversely affecting their socioeconomic standing.

The gene that encodes the Beta chain of fibrinogen has a single nucleotide polymorphism at the 455th nucleotide in the promoter region, where bases can be either adenine (A/H2) or guanine (G/H1). The genotype of patients can be identified by analysing their alleles. The goal of this research is to determine if a particular genotype or variant is more prevalent among young stroke patients within the Sri Lankan population, potentially providing insights into genetic predisposition to stroke. The HGVS nomenclature of the mutation is NM_005141.5: c.-463G>A (Gene-based) and NC_000004.12:g.154562556G>A (Genome-based).

Several studies have suggested that the A allele is associated with elevated plasma fibrinogen levels, making it more prevalent in stroke patients. However, conflicting evidence exists, as some studies found no significant correlation between this allele and an increased risk of stroke, highlighting the need for further investigation. (Lee et al., 2008, Gu et al., 2014 & Prasad et al., 2023). However, these studies have not specifically focused on young patients or the Sri Lankan population. Notably, two studies conducted in Japan (Nishiuma et al., 1998) and China (Liu et al., 2002) both concluded that the A or H2 allele is associated with increased fibrinogen levels in the blood, potentially influencing stroke risk.

Materials and Methods

In this study, 50 samples were selected from patients referred to the Centre for Genetics and Genomics with the clinical diagnosis of young stroke. The control group (10 samples) were selected from patients who showed no symptoms of young stroke. These patients were all between 5 to 49 years of age as this study defines young stroke patients as below the age of 50 years old. Ethical approval was granted by the Sri Lanka Institute of Information Technology (SLIIT) ethics review committee to conduct this study. Informed, written consent was obtained from participating patients.

An experienced phlebotomist drew 2.5 mL of peripheral blood from each patient into Ethylenediaminetetraacetic acid (EDTA) tubes. Genomic DNA was extracted using the Qiagen QIAamp DNA Blood Mini kit according to the manufacturer's instructions. The primers used in this study were

designed based on a previous study conducted in China. (Zhang *et al*., 2012). Forward primer – 5′ GAA CAT TTT ACC TTA TGT GAA TTA AGG 3′ Reverse primer – 5′ GAA GCT CCA AGA AAC CAT CC 3′ put together by integrated DNA technologies (IDT).

The PCR mix contained 1x PCR buffer, 1.5 mM Magnesium Chloride ($MgCl_2$), 0.25 mM deoxynucleotide triphosphates (dNTP) mix, 2 mM Forward primer, 2 mM Reverse primer and 1 U/µL Taq polymerase. 60 – 100 ng/µL of high-quality DNA from each sample was added. The total reaction volume was adjusted to 25 µL. The PCR cycle consisted of the initial denaturation at 95 ℃ for 5 minutes, followed by denaturation at 95 ℃ for 50 seconds, annealing at 58.2 ℃ for 45 seconds, and extension at 72 ℃ for 1 minute for 35 cycles, then a final extension at 72℃ for 7 minutes. This PCR amplifies a fragment in the β fibrinogen gene promoter region. This produces a 669 bp PCR product.

The RFLP was then carried out using a restriction mix consisting of 0.1 µL of Hae III, 1.5 µL of Cutsmart buffer, 10 µL of amplified DNA, and the total reaction volume was adjusted to 15 µl using distilled water. The digestion cycle consisted of the digestion at 37 ℃ for 16 hours and inactivation at 80 ℃ for 20 minutes. The Hae III restriction enzyme can differentiate between the mutant allele and wildtype allele as it digests the DNA fragment at a certain restriction site to produce two fragments if the mutation is absent. If the patient is Homozygous and has the wild-type allele, the DNA fragment will be digested into 2 fragments. If the patient is Homozygous and has the mutant allele, the DNA fragment will not be digested as the restriction site will be mutated. If the patient is heterozygous some of the DNA will be digested into two fragments, and some DNA will remain undigested. Figure 1 below explains the digestion process in detail.

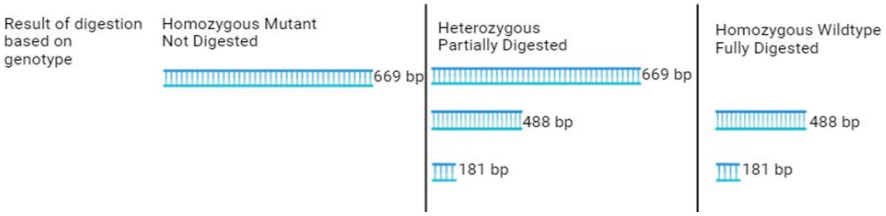

Figure 1: *The digestion process based on the genotype. If the patient is Homozygous mutant, the fragment will not be digested. If the patient is Heterozygous, the fragment will be partially digested. If the patient is Homozygous wild-type, the fragment will be fully digested.*

After digestion, the samples were electrophorized using a 2% agarose gel. The Gel was visualised using the UV trans illuminator documentation system.

Results

The resulting DNA bands observed after gel electrophoresis were used to determine the genotype of the patient sample. The possible banding patterns that can be observed are shown in Figure 2.

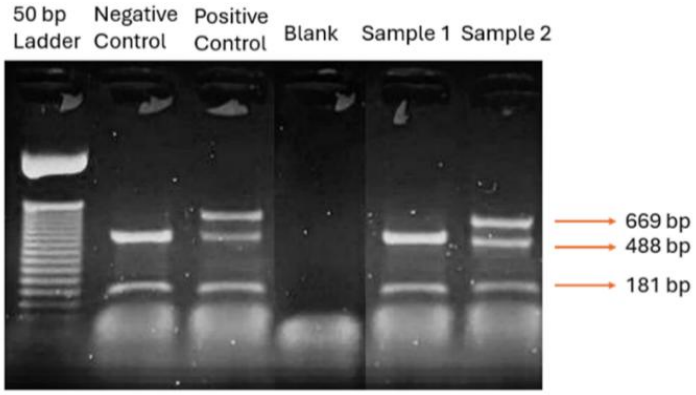

Figure 2: *Gel image of digested PCR Products. The first well shows the 50 bp ladder, the second well shows the negative control that is homozygous for the wild type allele and two bands are observed at 488 bp and 181 bp, the third well shows the positive control that is heterozygous (GA) and three bands are observed at 669 bp, 488 bp and 181 bp. Sample one run in the fifth well is negative and sample two run in the sixth well is positive.*

The genotype frequencies were calculated of both the control and sample groups. Mentioned in Table 1.

Table 1: *Genotype frequency of the control and sample groups*

Genotype	Number of Control Samples	Number of Patient Samples
GG	10	32
GA	0	18
AA	0	0
Total	10	50

The allele frequencies of the Sri Lankan population can be compared with other Asian young stroke populations. Mentioned in Table 2.

Table 2: *Allele frequencies compared to other Asian young stroke populations.*

Gene, Variant (SNV ID)	Population	Sample Size (n)	Wildtype Allele Frequency	Mutant Allele Frequency	References
Fibrinogen	Sri Lanka	50	0.82	0.18	-
β	China	91	0.814	0.186	Liu et al., 2002
455 G/A	South Korea	267	0.833	0.167	Lee et al., 2008
(rs 1800790)	Japan	84	0.85	0.15	Nishiuma et al., 1998

Discussion

This study was carried out using 60 blood samples, where 50 samples were patient samples of patients with a clinical diagnosis of young stroke, and 10 samples were used as the control group of patients who showed no symptoms of young stroke. All patients were in the age range of 5 to 49 years to be considered as young stroke patients. Out of the 50 patient samples, 32 patients were identified as Homozygous for the wildtype allele (GG), 18 patients were Heterozygous (GA), and no patients were identified as Homozygous for the mutant allele (AA). From the control samples, all 10 samples were identified as Homozygous for the wild-type allele (GG). This shows that there is a significant difference between the patient sample group and the control group. The prevalence of the mutant allele is higher than in the control group. However, no significant difference was observed between female and male populations as the number of Heterozygous patients was the same in both groups. Furthermore, the allele frequencies of the patient group were calculated. Allele frequency of Wildtype allele (p) was 0.82, and the Allele frequency of Mutant allele (q) was 0.18. These values were confirmed to be in line with Hardy-Weinberg equilibrium by calculating the chi-squared value. These allele frequencies can be compared to similar studies, as shown in Table 2.

The interaction between β-fibrinogen gene -455 G/A polymorphism and plasma fibrinogen level was observed in ischemic stroke patients in South Korea (Lee et al., 2008). The stroke patients were separated into two groups of non-cardioembolic stroke, as large-artery atherosclerosis and small-vessel occlusion (Lee et al., 2008) to observe if there was a difference between the prevalence of this variant in the two groups. This study concluded that this variant was linked to a high plasma fibrinogen level. The allele frequency of the mutant allele in this population was 0.167 and is lower than the Sri Lankan population at 0.18 so the prevalence of this variant is higher in Sri Lankan stroke patients. However, no difference was observed in the allele frequencies of the two subtypes of stroke (Lee et al., 2008). Moreover, there is a possibility that this variant is linked to a high plasma fibrinogen level in Sri Lankan populations.

The relationship between ischemic stroke, plasma fibrinogen level, and the β-fibrinogen gene -455 G/A variant in Japan (Nishiuma et al., 1998). These factors were analyzed in three patient groups as hypertensive with ischemic stroke, hypertensive without ischemic stroke, and normotensive in the same age group. This study concluded that there is a positive correlation between this variant and ischemic stroke in hypertensive patients. The plasma fibrinogen level was reported to be significantly higher in the stroke group. They also suggested that the mutant allele can be an independent risk factor for ischemic stroke (Nishiuma et al., 1998). The allele frequency of the mutant allele in this population was 0.15 and is lower than the Sri Lankan population at 0.18, so the prevalence of this variant is higher in Sri Lankan stroke patients. Furthermore, there is a possibility that this variant is linked to a high plasma fibrinogen level and can be a risk factor of stroke in Sri Lankan populations as well.

The relationship between the β-fibrinogen gene -455A/G variant and plasma fibrinogen level was investigated to determine if the variant influences ischemic stroke patients in China (Liu et al., 2002). This study shows that male stroke patients had a higher mutant allele frequency. The plasma fibrinogen concentration was greater in the Heterozygous and Homozygous mutant patients than in the Homozygous wildtype patients. The allele frequency of the mutant allele in this population was 0.186 and is slightly higher than the Sri Lankan population at 0.18. This study concluded that the expression of plasma fibrinogen is affected by this variant; the mutant allele can be a risk for ischemic stroke (Liu et al., 2002). The same can be assumed for this Sri Lankan population, as the allele frequency of the two populations is similar.

Conclusion

The results observed through this study show a significant difference between the control group and the patient sample, which proves the prevalence of the mutant allele among young stroke patients in the Sri Lankan population. When the data was analysed in comparison to similar studies, the link between the studied polymorphism (β-fibrinogen -455A/G) and an increased level of fibrinogen in blood plasma was suggested. This variation was also identified as a possible risk factor for stroke. To improve the reliability of these results, further research should be carried out to analyse the level of plasma fibrinogen in each sample, which can then be used to explain the association between the presence of the mutant allele and the fibrinogen concentration.

Acknowledgment

I extend my gratitude to the Centre for Genetics and Genomics at the Faculty of Medicine in the University of Colombo for my research. This paper is based on research originally conducted for my undergraduate thesis submitted to the Sri Lanka Institute of Information Technology (SLIIT) for the fulfillment of the BSc Honours in Biotechnology degree.

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