Original Research Article

Assessment of haptoglobin gene polymorphism in cryptogenic ischemic stroke subjects

Abstract

Introduction: For a long time, it was assumed that stroke only affected people aged over 60, but recent studies have shown that it can occur in younger people. In the latter group, molecular factors and inter-individual differences in susceptibility are being increasingly incriminated. The aim of our study was therefore to evaluate the haptoglobin gene polymorphism in subjects with cryptogenic AVCI.

Methodology: This was a prospective case-control study and included subjects with cryptogenic ischemic stroke followed at the neurology department of FANN hospital. Healthy controls were recruited and matched with the cases according to sex and age ±2 years. The Hp gene polymorphism was determined using conventional PCR without enzymatic digestion and biochemical parameters were assayed using the Architect ci4100 system (Abott, USA).

Results: Our study included 35 patients with cryptogenic stroke. The mean age of the patients was 45±11 years and the sex ratio was 1:1. Assessment of cardiovascular risk factors showed a high frequency of hypertension (46.57%) followed by dyslipidemia (21.42%) and diabetes (10.71%). Drug use was found in 7.14% of subjects. With regard to haptoglobin genotypes, Hp2-2 was much more prevalent in stroke patients (21.42%) than in control subjects (14.28%). In contrast, the Hp1-1 genotype was more prevalent in control subjects, with a rate of 57.14%, compared with 39.28% for cryptogenic strokes.

Conclusion: Our results seem to show that the Hp2-2 genotype is involved in the occurrence of cryptogenic ischemic stroke. However, the impact of these parameters must be assessed in conjunction with other associated cardiovascular risk factors.

Key words: Cryptogenicstroke, Haptoglobin, genotyping, PCR

1. Introduction

Stroke is defined by the World Health Organisation (WHO) as <<The rapid development of localised or global clinical signs of cerebral dysfunction with symptoms lasting more than twenty-four hours, possibly leading to death, with no apparent cause other than vascular origin >> (1).

In 2016, the lifetime risk of stroke was 24.9% worldwide, with large regional and country differences (2). Ischemic stroke accounts for around 80% of strokes and is one of the main causes of death. It is a major public health problem. Most ischemic strokes occur in people over 70, rarely in those under 35. It is widely assumed that stroke increases significantly with age and is likely to affect older people.

A quarter of ischemic strokes occur in people of working age, and it is estimated that 3.6 million young people are affected each year (3). Around 10% of ischemic strokes occur in people under the age of 50. Ischemic stroke at a young age may still be increasing, as several recent studies have reported a rising incidence of stroke particularly at younger ages, since the 1980s, while incidence at older ages has fallen over the same period (3). In addition, 25-50% of ischemic strokes in young adults remain without a definite cause despite thorough investigation (4). In the United States, more than 750,000 people suffer strokes every year, a third of which are of cryptogenic origin, i.e. without an obvious cause.

Several studies have been carried out to assess the risk factors for stroke in young people, and the main factors identified are lifestyle-related, including a sedentary lifestyle, malnutrition, smoking and drug use. However, it is increasingly recognised that genetic factors play a very important role, and the polymorphism of certain genes is thought to lead to differences in susceptibility to strokes of undetermined cause, which are more common in young people (3, 5).
Conventional risk factors explain only a small proportion of all stroke risk (6). Evidence from studies of twins and family history suggests that genetic predisposition is important (7). Stroke is thus considered to be a complex multifactorial and polygenic disease, resulting from a large number of gene-gene and gene-environment interactions.
The general aim of our study was therefore to assess haptoglobin gene polymorphism in subjects with cryptogenic ischemic stroke.

2. Methodology

2.1. Design and Setting

This is a case-control study of subjects with cryptogenic ischemic stroke. Patients were recruited from the neurological clinic of the National University Hospital Centre of Fann (CHNU/FANN) in Dakar. Biochemical parameters were assayed in the biochemistry laboratory of the said structure.

2.2. Study Participants

our study involved subjects with cryptogenic stroke according to the TOAST classification (Trial of Org 10172 in Acute Stroke Treatment), called Embolic Stroke of Undetermined Source (ESUS) (8). Stroke was diagnosed on clinical grounds and confirmed by tomodensitometric data. The cryptogenetic origin of the strokes was confirmed by the absence of heart disease at high risk of embolism and stenosing atheroma (>50%) extra or intracranial. Subjects with stroke related to patent foramen ovale-interatrial septal aneurysm (PFO-ASIA), non-stenosing (<50%) potentially embolic atheroma, dissection, vasculitis and small cerebral artery disease (≤2.0 cm) were not included in the study.Healthy control subjects were recruited and matched with cases according to sex and age ± 2 years.

2.3. Sampling and data collection

Samples were collected in two tubes. The dry tube was used to measure uric acid and lipid levels. The EDTA tube was used for haptoglobin gene genotyping. Biochemical parameters were assayed using enzymatic techniques with the Architect ci4100 system (Abott, USA).
DNA was extracted from whole blood collected in an EDTA tube. We performed a manual extraction (saline method) using QIAmp® genomic DNA and RNA kits (Paris, QIAGEN). After extraction, purity and concentration were determined using the NanoDropTM One.Amplification by conventional PCR was carried out in a medium containing MgCl2, Green Taq polymerase, dNTP (dATP, dCTP, dGTP, dTTP), DNA template and the following primers:

- Hp A: 5’GAGGGGAGCTTGCCTTTCCATTG3’
- Hp B: 5’GAGATTTTTGAGCCCTGGCTGGT3’

Primers A and B were used to amplify sequences specific to the 1757 bp Hp1 allele and a sequence specific to the 3481 bp Hp2 allele.

- Hp C: 5’CCTGCCTCGTATTAACTGCACCAT3’
- Hp D: 5’CCGAGTGCTCCACATAGCCATGT3’

Primers C and D were used to amplify a 349 bp sequence specific for the Hp2 allele.

The 4 primers were all synthesised by Applied Biosystems (9). The mix consisted of 12.5 µL of go taq, 6.5 µL of nuclease-free water and 1 µL of the 4 Hp gene primers multiplied by the number of samples. The Hp genes were amplified by conventional PCR using the Proflex system (Biosystems, Spain) according to the following program:

- 95°C to preheat the machine for 5 minutes
- 95°C for 1 minute to denature the DNA strands
- 69°C for 2 minutes for hybridisation in the presence of primers A and B or primers A, B, C, D or in the presence of primers C and D only, repeated for 35 cycles
- 72°C for 10 minutes for elongation

The PCR product was visualised by agarose gel electrophoresis in the presence of ethydium bromide (BET) and a molecular weight marker. Haptoglobin genotyping was determined by observing the amplified DNA fragments with the Azure C200 instrument (Biosystèmes, Spain) (figure 1).

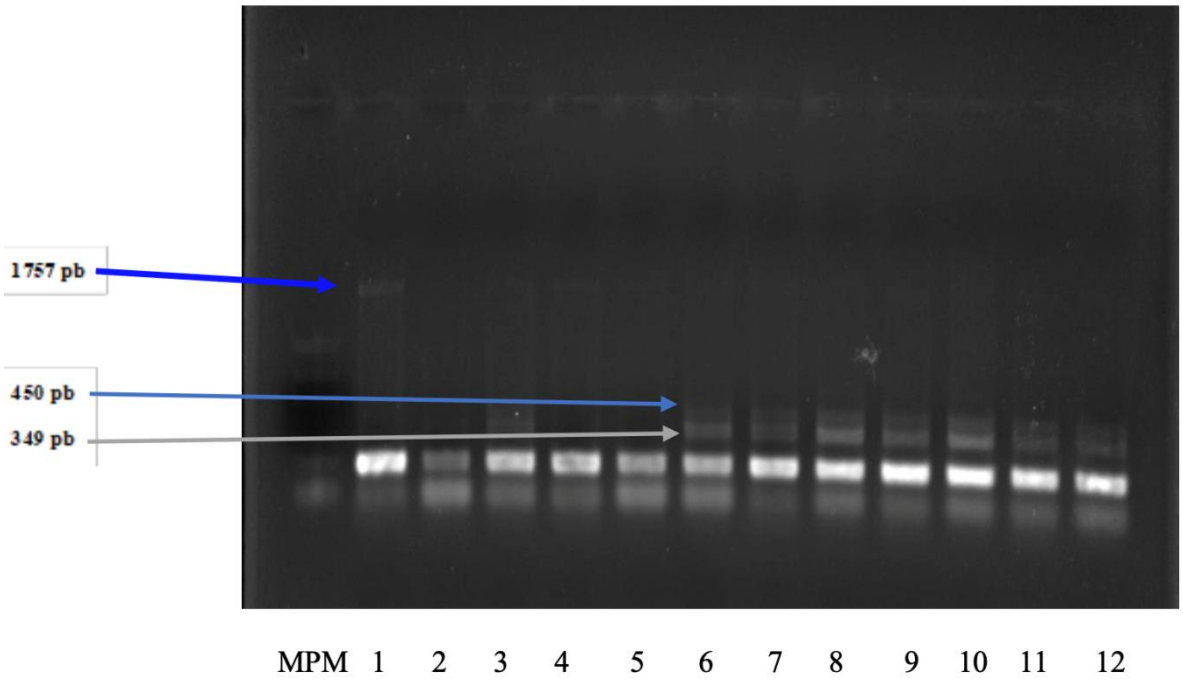

Figure 1:Visualization of DNA bands on agarose gel. (10)
1= Hp1-1; 2= Hp2-2; 3= Hp2-2; 4= Hp1-1; 5= Hp1-1; 6= Hp2-1; 7= Hp2-2; 8= Hp2-2; 9= Hp2-2; 10= Hp2-2; 11= Hp2-2; 12= Hp2-2; MPM= Molecular weight marker

2.4. Data Analysis

Our data were collected using Microsoft Excel 2016. XLSTAT 2018 was used to process the data. The Wilcoxon-Mann-Whitney test and the Kruskal-Wallis test were used to compare means and the Chi2 test to compare frequencies. A p-value of less than 0.05 was considered a statistically significant difference.

2.5. Ethical Considerations

This study was approved by the Research Ethics Committee ("Comité d 'éthique de la recherche – CER") of Cheikh Anta Diop University (UCAD) in accordance with the rules laid down by Senegal's National Health Research Ethics Committee under number: 0412/2019/CER/UCAD.

3. Results

Our study population consisted of 40 patients with cryptogenic stroke. The mean age of the patients was 46 ± 4 years, with extremes of 21 and 70 years (see Table 1). The sex ratio of the study population was 1:1. Determination of the cardiovascular risk factors in our cohort showed that hypertension was the most frequently found abnormality, with a rate of 46.57%. Diabetes was found in 10.71% of subjects, after dyslipidemia, sedentary lifestyle and smoking. Evaluation of biological parameters in the subjects included in the study showed higher plasma values in stroke subjects than in control subjects for all parameters except HDL-cholesterol. Comparison of mean values between cases and controls showed a statistically significant difference for HDL-cholesterol and uric acid (see Table 1).

Table 1: Epidemiological, clinical and biological characteristics of the study population

	Cryptogenic stroke	**Controls**	***P***
Mean age (years)	45 ± 11	46±12	**<0.001**
Sex ratio	1	1	-
Total cholesterol (g/l)	2,00±0,78	1,89±0,42	0.844
HDL-c (g/l)	0,45±0,13	0,59±0,23	0.028
LDL-c (g/l)	1,04±0,53	1,14±0,40	0.154
Triglycerides (g/l)	0,90±0,44	0,79±0,30	0.363
Blood glucose (g/l)	0,97±0,21	0,97±0,05	0.072
Uric acid (mg/l)	53,51±11,65	35,53±6,29	<0.0001
CRP (mg/l)	3,64±2,91	3,95±1,66	0.081
HTA (%)	46.57	-	-
Diabetes (%)	10.71	-	-
Dyslipidemia (%)	21.42	-	-
Sedentary lifestyle (%)	21.42	-	-
Smoking (%)	21.42	-	-
Alcoholism (%)	14.28	-	-
Drugs (%)	7.14	-	-

Statistical analysis of the results showed a higher frequency of Hp1-1 and Hp2-1 genotypes in cryptogenic strokes and controls, with frequencies of 39.28% and 57.14% respectively. The Hp2-2 genotype was found in 21.42% of cryptogenic strokes and in 14.28% of controls.
Our results showed a different distribution of Hp alleles between cases and controls. In cryptogenic stroke patients, the Hp2 allele was found in more than 60% of the population. In control subjects, however, the Hp1 allele was much more prevalent, with a frequency of over 80%. Comparison of genotype frequencies between cryptogenic stroke and controls did not reveal any statistically significant differences (Figure 2).

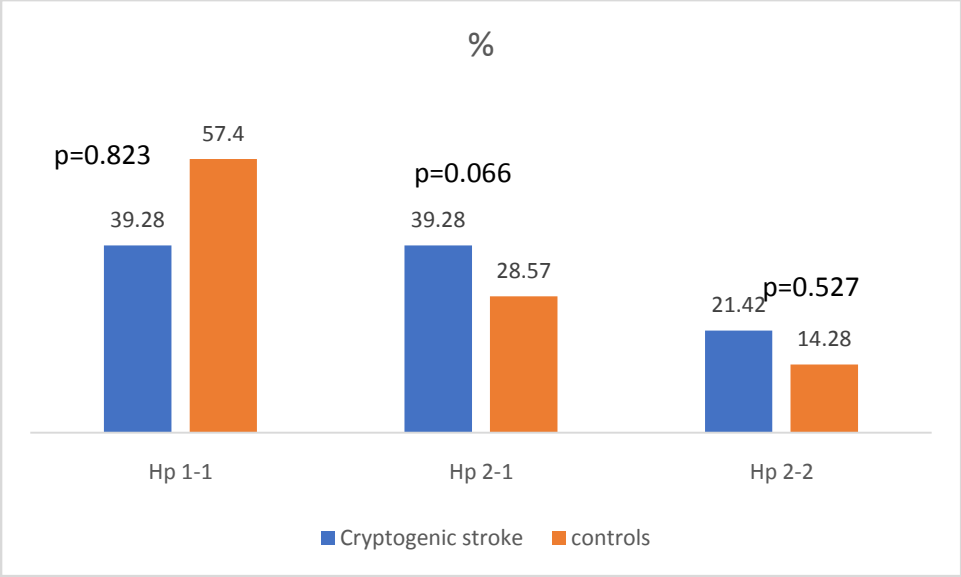

Figure 2: Hp genotype frequencies in stroke and control subjects

When the population was divided according to Hp genotype, the results showed differences more for clinical parameters than for biological parameters. Hypertension and lifestyle parameters such as smoking, alcoholism and drug use were more frequently found in subjects with the Hp2-2 genotype.
With regard to biological parameters, similar concentrations were found in the three Hp genotype groups. The comparison did not reveal any statistically significant differences (see table 2).

Table 2: Assessment of clinical, biological and lifestyle parameters according to haptoglobin genotype

	Hp1-1	**Hp2-1**	**Hp2-2**	**p**
HTA (%)	45.45	45.45	50.00	0.981
Diabetes (%)	9.09	18.18	-	0,499
Dyslipidemia (%)	27.27	18.18	16.66	0,830
Sedentary	18.18	27.27	16.66	0,830

lifestyle (%)				
Smoking (%)	18.18	9.09	50.00	0,137
Alcoholism (%)	18.18	-	33.33	0,154
Drugs (%)	9.09	-	16.66	0,421
Cholesterol Total (g/l)	2,01	2,00	1,98	0,643
HDL-c(g/l)	0,46	0,44	0,46	0,774
LDL-c (g/l)	0,96	1,10	1,09	0,578
Triglycerides (g/l)	1,05	0,79	0,82	0,642
CRP us (mg/l)	4,27	3,42	2,86	0,545
Uric acid (mg/l)	57,74	48,87	54,28	0,271
Blood glucose (g/l)	1,01	0,95	0,95	0,453

4. Discussion

This work is part of the search for biomarkers of predisposition to cryptogenic stoke, which are now a major public health problem worldwide and more specifically in our region. The aim of our work was therefore to evaluate the genetic polymorphism of haptoglobin in subjects with cryptogenic stroke. In our study population, the mean age was 46 years, with a sex ratio of 1:1. This reveals a young study population, especially as the minimum age of the subjects was 21 years. Similar results were found in studies conducted by Bejot, Dalpont and col (11) where the mean age of their study population was less than 30 years. In the study conducted by Bhat, Khanna et al (12), the mean age was less than 60, with a mean age of 41. Stroke usually occur in subjects over 60, but are now increasingly found in younger subjects (13). This change in epidemiological profile can be explained by the growing increase in known risk factors for stroke, such as the increase in physical inactivity, obesity, type 2 diabetes, and the growing consumption of alcohol and illicit drugs (13). In addition, we found no difference according to gender. This is consistent with the results published by Feigin and col (2), where the risk of stroke in men (24.7%) was not significantly different from that in women (25.1%). However, studies have shown a male predominance (60%) in Senegal (14). Other studies, such as those conducted by Antonio Gonzalez-Hermosillo and col(15), showed a predominance of women, with a higher risk of stroke in women (93%). This result may be explained by the high frequency of cardiovascular risk factors such as a sedentary lifestyle, obesity, hypertension and diabetes.

The assessment of cardiovascular risk factors (RF) in our study population showed a significant frequency of diabetic subjects, with a rate of 10.71%. Diabetes is now considered to be the most common RF, and studies have shown that it can lead to serious complications if not properly managed. These complications include retinopathy, chronic kidney disease, limb amputation and cardiovascular disease, the most important of which is stroke. There are several possible mechanisms by which diabetes leads to stroke. These include vascular endothelial dysfunction, arterial stiffness, systemic inflammation and capillary membrane thickening (16). In our study, hypertension was found in 46.57% of subjects, followed by smoking, dyslipidemia and sedentary lifestyle. Similar results were found by Boehme and col (17) where hypertension was found in 54% of strokes. High blood pressure is a major risk factor for ischemic and hemorrhagic stroke. A sedentary lifestyle was also found in our study, with a frequency of 21.42%, confirming previous studies which have shown that physically active people have a lower risk of stroke and mortality than inactive people (17).

Biochemical parameters between cases and controls showed statistically significant differences for HDL-cholesterol ($p=0.028$) and uric acid ($p<0.0001$). Similar results have been found in several studies (19-20). In addition, other studies have shown that high levels of triglycerides (TG) and low levels of HDL-cholesterol were considered risk factors for coronary heart disease and ischemic stroke (4). As for uric acid, it has been shown that hyperuricemia is linked to obesity, high blood pressure, reduced HDL-cholesterol and sensitivity to insulin reduction. In fact, the combination and presence of multiple risk factors could explain part of the increased risk of stroke (20).

Analysis of the haptoglobin polymorphism showed a higher frequency of the Hp1-1 genotype in control subjects, with a frequency of 57.14%, followed by Hp2-1 with a frequency of 39.28%. In contrast, in subjects with cryptogenic stroke, the Hp1-1 genotype was found at a lower frequency of 39.28%. In addition, the Hp2-2 genotype was found in 21.42% of cryptogenic strokes, i.e. more frequent than in control subjects (14.28%).

Similar results were found in several studies carried out in the region. In 2021, Sagne and col. found the same results in the general population and also demonstrated a link between cardiovascular risk and the Hp2-2 genotype (10).In addition, a Senegalese study showed the predominance of the Hp2-2 genotype in subjects with stroke of known or unknown cause, with a frequency of 36.96% (21). Other similar results have been found in the literature, further confirming our findings (21-22).

Haptoglobin is a protein in the blood that binds to free hemoglobin, preventing its loss from the kidneys and protecting it from oxidative damage. There are two common alleles for the haptoglobin gene and individuals can have one of three possible genotypes (Hp1-1, Hp2-1 and Hp2-2). Individuals who are homozygous for the haptoglobin 2 allele (Hp 2-2), i.e. they have two copies of the haptoglobin 2 allele. It is associated with the lowest levels of haptoglobin protein in the blood. In people with the Hp 2-2 genotype, who have lower levels of haptoglobin, elimination of free hemoglobin may be less efficient, which may lead to increased oxidative stress. This can lead to cell damage and inflammation. Strokes, particularly ischemic strokes, are often associated with oxidative stress and inflammation of brain tissue. People with the Hp2-2 genotype may have less protection against oxidative damage, which could potentially contribute to a higher risk of stroke. Thus, the Hp2-2 genotype is associated with cardiovascular disease, including cryptogenic stroke, due to its potential to increase oxidative damage caused by the Hp2-2-hemoglobin complex (23).

According to MacKellar and Vigerust, people with the Hp2-2 genotype have a much higher risk of being exposed to neurological, infectious and renal pathologies (24), diabetes and also cardiovascular complications such as myocardial infarction and stroke (25). Hp2-2 has been implicated in the development of diabetes, with a risk of increased inflammation, oxidative stress and atherosclerotic plaque instability (26). Studies have shown that the Hp1-1 protein

eliminates free hemoglobin more efficiently than the Hp2-2 protein. There are more Hp-Hb molecules in the plasma of individuals with the Hp2-2 genotype. This mechanism would be all the more important in subjects already exposed to significant oxidative stress (26-27).
However, this association is not universal, and other factors such as age, sex and other genetic and environmental influences also play a role in the risk of stroke generally or when it is of cryptogenic origin.

5. Conclusion

Stroke is a multifactorial disease in which molecular risk factors play an increasingly important role. Our results suggest that the Hp2-2 genotype is involved in the onset of cryptogenic ischemic stroke. However, an assessment of the expression of these genes or a metabolomic study would give us a better understanding of the role of these biomarkers in the pathogenesis of cryptogenic strokes.

Limitations of Study

The main limitation of the study was the size of the sample, due in part to the nature of the population (cryptogenic stroke). problems of optimal patient follow-up made our recruitment difficult.

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