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HindIII and MseI in the Biomolecular Profile of Hemophilia in Cameroon: A Primer Study

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Abstract

Numerous studies are being carried out on polymorphisms within the genes coding for factors VIII and IX due to their clinical relevance in the context of hereditary disorders. The quests for polymorphism can be used to screen for haemophilia through an affected family. In Cameroon, very few studies on factors VIII and IX gene polymorphisms have been conducted. Thus, this study was aimed at detecting HindIII SNP of the F8 gene and MseI SNP of the F9 gene by the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RLFP) method and determining their involvement in the severity and complications of haemophilia. Fifty-five consented haemophilia patients from the *Centre Hospitalier et Universitaire de Yaoundé* (CHUY) were recruited for this study on a convenience sampling basis. Finger-prick blood was collected and spotted on filter papers from which DNA was extracted and then subjected to the PCR-RFLP. We were able to recruit 55 (37.16%) patients out of the 148 haemophilia patients registered at the CHUY until December 2018. The average age was 15 years. Of the 55 haemophilia patients, 42 (76.36%) had type A haemophilia and 13 (23.64%) had type B haemophilia, 41 patients (74.55%) had a severe form, 10 (18.18%) had a moderate form and 4 (7.27%) a mild form). Of the 55 patients recruited, 38 (69.09%) already had an osteoarticular complication and the remaining 17 (30.91%) had no complication. Fifty-three (96.36%) participants had HindIII SNP and a significant association was found with the severity of hemophilia (P = 0.00). No association (P = 0.56) was found with osteoarticular complications of haemophilia. On the other hand, 15 (27.27%) participants were diagnosed with

MseI SNPs and no association was found with the severity (P = 0.89) or complications (P = 0.68) of haemophilia.

Keywords

Haemophilia, SNP HindIII, SNP MseI, PCR-RFLP, Cameroon

1. Introduction

Life is not possible without blood. Blood loss is not just accidental; there are those related to blood abnormalities, among which hemophilia which is a constitutional hemorrhagic disease, is seriously at risk of fatal hemorrhage, linked to the X chromosome and transmitted genetically according to a recessive mode [1]. This genetic anomaly is characterized by a deficiency of factor VIII (haemophilia A) with an occurrence rate of around 1 in 5000 male births and factor IX (haemophilia B) with an occurrence rate of around 1 in 30,000 male births [2]. Its prevalence is little known in Africa for several reasons: The rarity of the disease, the high cost of treating it, the insufficient number of haematology specialists and the lack of adequate laboratories for the biological diagnosis of this disease [3], for example, a recent study carried out in Madagascar showed only a total of 19 hemophilia [4]. However, it has been established that haemophilia is a ubiquitous condition that affects one in 10,000 to 12,000 births, without distinction of race or geographical region [5]. In Cameroon, the World Federation of Hemophilia reported that in 2016, 176 out of 23,439,189 people had hemophilia [6]. The first cases detected and treated in Cameroon date back to the 1970s, in the haematology department of the Yaoundé Hospital and University Centre (CHUY) [7]. The clinical manifestations associated with haemophilia correspond to uncontrolled internal haemorrhagic episodes [8]. The severity and frequency of these manifestations depend directly on the level of circulating plasma factor VIII or factor FIX [9]. The numerous bleeding episodes that individuals with severe hemophilia experience can lead to long-term disability. Recurrent joint bleedings can result in severe arthropathy, muscle atrophy, pseudo-tumors, and lead to chronic pain and impaired mobility that often requires surgery and arthroplasty to improve joint function. Haemophilia A and haemophilia B display similar clinical characteristics; however, several studies have reported possible differences [10]. The possible different clinical evolution of haemophilia B was initially suggested in 1959 by Quick [11]. He observed that haemophilia B, even in its most severe form, can be less incapacitating and disabling than haemophilia A. In some studies, severe haemophilia B has been defined with a factor IX < 2% that could contribute to a less severe bleeding tendency compared to haemophilia A, usually defined with a factor VIII < 1% [12] [13] [14]. It has also been reported that haemophilia B patients have a less severe arthropathy [15] and less hospital admission rate [12] than haemophilia A patients. Hemophilia is diagnosed in the laboratory by coagulation tests and screening and testing the

inhibitor development, now the most serious complication in hemophilia, is vital for any comprehensive hemophilia treatment program to be able to provide medical treatment and eradication of inhibitors [16]; however, most centres around the world do not have inhibitor testing capacities. Genetic assessment of hemophilia is important to define disease biology, establish diagnosis in difficult cases, predict risk of inhibitor development, and provide a prenatal diagnosis if desired. Wherever possible, genotype analysis should be offered to all patients with hemophilia [17]. The mainstay of treatment for hemophilia involves replacing the missing blood coagulation factor VIII in people with hemophilia and factor IX when bleeding episodes occur (on-demand treatment) or by scheduled infusions several times per week (prophylaxis treatment). Both plasma-derived (pd) and recombinant clotting factor concentrates are suitable for these different strategies of hemophilia management [18]. Nowadays, many studies are being carried out on polymorphisms within the genes coding for factors VIII and IX [19] [20] [21]. Both factor VIII and IX genes contain two types of polymorphisms. Polymorphisms have a scientific interest of their own and are useful in fields as varied as forensics [22] [23] and the study of human evolution [24]. They have clinical relevance in the context of hereditary disorders in that they can be used to detect a defective (or normal) patient through an affected family. Linkage studies have been used to study carrier status and prenatal diagnosis in hemophilia A and B [25]. The factor VIII gene contains several SNPs, many of which fall into the subcategory of restriction fragment length polymorphisms (RFLPs), such as the alleles of BclI and HindIII RFLPs, which have very high frequencies in Black Americans compared to other ethnic groups [26]. Depending on the nature of the mutation that causes the disease, the affected clotting factor may be completely absent from the patient's body, or present but in a dysfunctional form [27]. The human factor IX gene contains several SNPs, most of which fall incidentally into the RFLP subcategory, such as the MseI SNP located at −698, so involvement has been described in cases of severe haemophilia [27]. These differences result in varying degrees of severity of the disease. Advances in molecular genetic technologies are becoming routinely integrated into many genetic diagnostic laboratories. Full F8 or F9 gene screening is performed by polymerase chain reaction (PCR) and Sanger sequencing, or next-generation sequencing [28] [29]. Use of these techniques is evolving and increasing internationally. The approach and use of a specific technique depend on the available technical expertise and resources. That's why we used the RFLP-PCR method that was available to us It was noted that very few studies on polymorphisms of factor VIII and IX genes have been carried out in Africa, particularly in Cameroon. Hence our interest in determining HindIII and MseI in the Biomolecular Profile of Hemophilia in Cameroon: A Primer Study.

2. Methodes

2.1. Study Site

The study was conducted at the Centre Hospitalier et Universitaire de Yaoundé

(CHUY) located in the central region of Cameroon. The CHUY has a unit for the care of haemophilia patients in Cameroon, and also has a data bank on haemophilia patients. The aforementioned unit was set up thanks to the twinning programme established between the Haematology and Blood Transfusion Department of CHUY and the Haematology Unit of the University Hospital of Geneva in Switzerland since 2009.

2.2. Enrolment Procedure, Sample Collection and DNA Extraction

Oral, written and informed consent was obtained prior to any sampling from the interviewees or their legal guarantor. Indeed, before obtaining this consent, the interviewees were informed of the objectives of the study, explaining that the information collected would be strictly anonymous and confidential. They were also informed of the guarantees taken with regard to the security of the information collected. Before obtaining their consent (signature), patients or parents of under-age patients were truly informed about the nature of the study, the risks and benefits, as well as the possibility of interrupting their participation or that of their child in the study at any time. In order to guarantee the confidentiality of the information collected, all patients selected for the study were registered with an identification code. Moreover, this study received approvals of the National Ethics Committee of Cameroon (CE N˚ 0565/CRERSHC/2018) and that of the Ethics Committee of the Faculty of Medicine and Biomedical Sciences of Yaounde (FMSB) as well as authorisations by the directors to conduct a study in the different centres (CHUY and the Biotechnology Centre of the University of Yaounde I (BTC-UYI)).

A total of 55 patients were enrolled in the study. These were all collected after completing a questionnaire on anthropometric and medical record information. Finger-prick blood was collected and spotted on filter paper then left to dry at room temperature for 24 hours, then introduced into the envelopes containing silica gel and transported to the molecular biology laboratory. In the laboratory blood spots on the filter paper were excised with a sterile pair of surgical scissors. DNA was extracted from the dried blood spots by bioling in Chelex-100 in buffered Tris-EDTA as previously described. A quantitative spectrophotometric assay of DNA was performed using a Cary 60 UV-visible spectrophotometer. Absorbance was measured at wavelengths of 260 and 280 (A 260 and A 280, respectively) nm. The absorbance quotient (OD 260/OD 280) provides an estimate of DNA purity. An absorbance quotient value of 1.8 ratio (R) 2.0 was considered to be good, purified DNA. A ratio of 1.8 is indicative of protein contamination, where as a ratio of 2.0 indicates RNA contamination. The integrity of genomic DNA was tested by resolving DNA extracts on a 0.8% agarose gel by electrophoresis, followed by visualization with ethidium bromide staining. Each DNA sample was graded, according to the electrophoretic migration of sample DNA compared with a known molecular weight marker (Fermentas, Thermo Scientific). The DNA was stored in a Tris-EDTA buffer at −20˚C until analysis and allelic discrimination analysis.

2.3. Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP)

Two polymorphisms, HindIII of factor VIII and MseI of factor IX, were investigated using PCR-RFLP modified protocol and primer design of Graham *et al.* [30] [31]. The primer sequences used to amplify the factor VIII and Factor IX genes are found in Table 1. The amplification was done in a T3 thermal cycler (Biometra, UK). Each PCR cycle was performed in a total volume of 25 µL containing: nuclease-free water, 10× ThermoPol buffer, 10 mM dNTPs (200 µM of each deoxyribonucleotide), 20 pmol of primer, 5 U/µL of Taq polymerase and 100 ng of DNA. For factor VIII gene, after initial denaturation at 95˚C for 5 min, 35 cycles of amplification were carried out with denaturation at 95˚C for 1min, annealing at 62˚C for 55 s and extension at 72˚C for 1 min, followed by a final extension at 72˚C for 10 min. For factor IX gene after initial denaturation at 95˚C for 7 min, 30 cycles of amplification were carried out with denaturation at 91˚C for 1 min, annealing at 60˚C for 1 min and extension at 70˚C for 3 min, followed by a final extension at 70˚C for 7 min. The RFLP reaction conditions for digestion with HindIII (for HindIII polymorphism) and MseI (for MseI polymorphism) (New England Biolabs, USA) were set at 37˚C, both for 16 h each. The products of the digestion reaction were separated on a 2% agarose gel stained with ethidium bromide.

2.4. Statistical Analysis

The variables of interest were the presence of HindIII and MseI SNPs on the factor VIII (F8) and IX (F9) genes respectively. The main variables studied were the severity and presence of osteoarticular complications. Family history, age in years and place of residence were also studied. The data collected was qualitative in nature. A descriptive analysis was also carried out. In order to look for possible associations between the severity on the one hand and the complications on the other hand with the variables of interest, statistical tests (Chi^2, Pearson and Fischer exact) were used. The statistical analysis was carried out using STATA 15 software. The degree of significance was set at 5%. A p-value of 0.05 or less was considered statistically significant.

Table 1. Primer sequences for the amplification of the F8 and F9 genes.

Genes	Primer Code	Nucleotide Sequences (Primers)	Size after PCR	Restriction Enzymes (RE)	Results from RFLP
Factor VIII **(F8)**	F8-S	**5'GTGAGTAGCAGTGTGGGCAGA 3'**	608 bp	***HindIII***	(−): 427 and 181 bp (Wild-type) (+): 427, 100 and 81 bp (Mutant)
	F8-A	**5'CTGAAATGAAACGGGTGGAAC 3'**			
Factor IX **(F9)**	F9-S	**5'GATAGAGAAACTGGAAGTAGACCC 3'**	369 bp	***MseI***	(−): 83, 73 bp (Wild-type) (+): 57, 73 bp (Mutant)
	F9-A	**5'TTAGGTCTTTCACAGAGTAGAATTT 3'**			

bp: base pair.

3. Results

3.1. Study Population Characteristics

We were able to recruit 55 (37.16%) patients out of the 148 haemophilia patients registered at the CHUY until December 2018. The youngest patient was one year old and the oldest was 41 years old. The average age was 15 years. Patients between 6 and 15 years old were the most numerous (45.45%). The Western region and the Central region were the most represented with 24 patients (43.64%) and 13 patients (23.64%) respectively. The vast majority of patients 83.64% (46 patients) resided in Yaoundé. Of the 55 haemophilia patients, 42 (76.36%) had type A haemophilia and 13 (23.64%) had type B haemophilia, 41 patients (74.55%) had a severe form, 10 (18.18%) had a moderate form and 4 (7.27%) a mild form. Of the 55 patients recruited, 38 (69.09%) already had an osteoarticular complication and the remaining 17 (30.91%) had no complication. In addition, 34 patients (61.82%) had a family history of haemophilia, while the remaining 21 (38.18%) had no family history of haemophilia (Table 2).

3.2. Frequencies of HindIII and MseI Polymorphism of F8 and F9 Respectively among Study Participants

After RFLP-PCR we have obtained For HindIII Polymorphism, The amplified segment was 608 bp in size. Genotypically positive (+) showed 427- and 100-bp fragments. Genotypically negative (−) showed 427-bp and 181-bp fragments. And for MseI Polymorphism the amplified segment was 557 bp in size. Genotypically positive (+) showed 176-bp fragments. Genotypically negative (−) 176-, and 81-bp fragments. Alleles were assigned for presence (+) and absence (−) (Figure 1).

In this study, 96.36% of hemophilia patients had HindIII polymorphism, and 27.27% of hemophilia patients had MseI polymorphism (Figure 2).

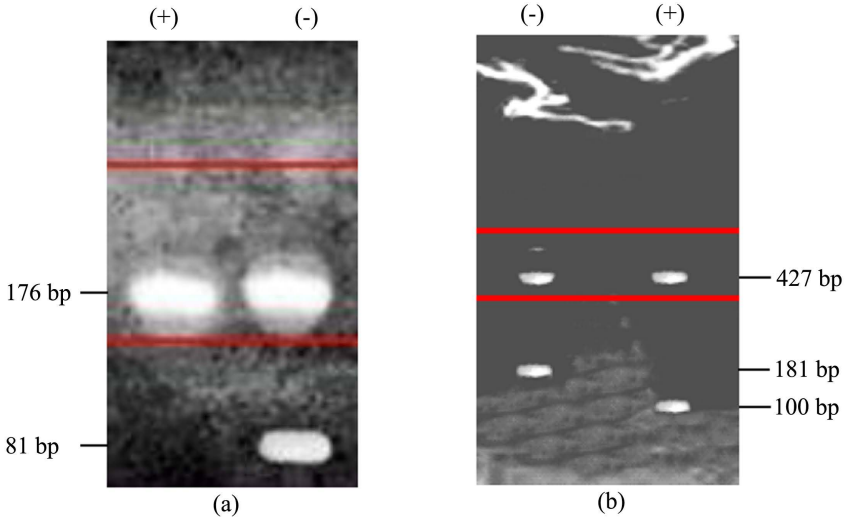

Figure 1. RFLP-PCR profile with HindIII (F8) and MseI (F9). Legend: (a) RFLP-PCR profile with MseI (F9) and (b) RFLP-PCR profile with HindIII (F8).

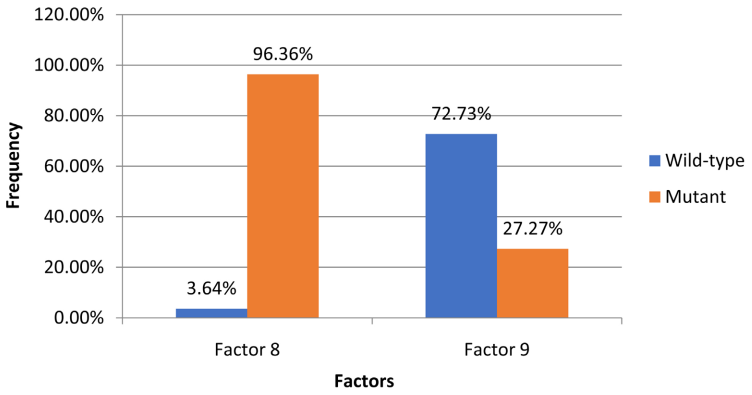

Figure 2. Distribution of HindIII and MseI polymorphisms on factors 8 and 9 respectively, in haemophiliac patients (N = 55).

Table 2. Baseline characteristics of the study population.

Patient characteristics	Number of cases	Frequency (%)
Age in categories		
Less than 6 years old	8	14.55
6 - 15 years old	25	45.45
More than 15 years old	22	40
Total	55	100
Place of residence		
Others	9	16.36
Yaoundé	46	83.64
Total	55	100
With family history		
No	21	38.18
Yes	34	61.82
Total	55	100
Type of haemophilia		
A	42	76.36
B	13	23.64
Total	55	100
With osteo-articular complications		
No	17	30.91
Yes	38	69.09
With severity		
Light	4	7.27
Moderate	10	18.18
Severe	41	74.55
Total	55	100

3.3. Association between HindIII and MseI Polymorphism and Severity of Hemophilia

The study between the HindIII polymorphism of the F8 gene and the severity of haemophilia showed a high significance (P = 0.001) and we observed that all patients with the severity had the HindIII polymorphism of the F8 gene. Moreover, our study revealed that there is no significant association between the HindIII polymorphism of the F8 gene and the complications of haemophilia. On the other hand, our study portrayed that there is no significant association between the MseI polymorphism of the F9 gene and the severity as well as complications of haemophilia (Table 3).

4. Discussion

The vast majority of haemophilia patients registered at CHUY until December 2018 were not able to participate in the study. Indeed, several authors have been confronted with this problem. This was the case of Driscoll *et al.* who worked on 29 men including 13 black and 16 white men in 1988 in the United States [25]. Similarly, Lalloz *et al.* worked on 25 men [32]. In Africa, Diop *et al.* worked on 54 men with haemophilia. Finally, in Cameroon, different authors have worked

Table 3. Association between HindIII and MseI polymorphism and severity of hemophilia.

	Complication				Severity				
	No n %	Yes n %	Total n %	P	Mild n %	Moderate n %	Severe n %	Total n %	P
SNP HindIII of F8 gene									
Wild-type	1	1	2		2	0	0	2	
	5.88	2.63	3.64		33.33	0	0	3.64	
Mutant	16	37	53		4	9	40	53	
	94.12	97.37	96.36		66.67	100	100	96.36	
Total	17	38	55		6	9	40	55	
	100	100	100	0.56	100	100	100	100	**0.001**
SNP MseI of F9 gene									
Wild-type	13	27	40		4	7	29	40	
	76.47	71.05	72.73		66.67	77.78	72.5	72.73	
Mutant	4	11	15		2	2	11	15	
	23.53	28.95	27.27		33.33	22.22	27.5	27.27	
Total	17	38	55		6	9	40	55	
	100	100	100	0.68	100	100	100	100	0.89

P: P value; **n %**: proportions in percentage.

on samples generally ranging from 9 to 40 patients. According to Doncarli *et al.*, this difficulty is due to the irregular follow-up of minor forms of haemophilia in treatment centres [33]. In our context, the reason for this small sample could be due on the one hand to the refusal of some patients to participate in the study, on the other hand to the fact that many of them did not reside in the city of Yaoundé where we conducted the study, and also that some of the patients registered had already died at the time of the study. Three-quarters of our sample had haemophilia type A. This corroborates the global statistics. For example, Doncarli *et al.* in one of the largest cohort studies in 2005 in France found a trend almost identical to our sample [33]. Furthermore, the vast majority of patients were suffering from severe forms as opposed to the much less common mild forms. On this subject, the literature consulted is not consensual. Some authors such as Antonarakis *et al.* [34] in an international study in 1995 found that mild haemophilia is the most widespread. Diop *et al.* in 2003 found that the moderate form was the most widespread [2]. Doncarli *et al.* 2005 in France [33] and Tagny *et al.* in 2014 in Cameroon [7] found a trend rather similar to ours, with the severe form more widespread, followed by the moderate form and finally the mild form. Almost all of our patients had HindIII SNP, which corroborates the results of Husain *et al.* in 2009 and Moharrami *et al.* 2015 [35] [36]. Indeed, these two authors found a very high frequency of HindIII SNP (>75%) in black Americans, low in whites and Chinese and absent in Japanese. This study does not show any significant link between the HindIII SNP of the F8 gene and the complications of haemophilia. However, the relationship between the HindIII SNP of the F8 gene and the severity of haemophilia showed a highly significant association (P = 0.000). In addition, patients with severe haemophilia all had the F8 gene HindIII SNP. This SNP would therefore be strongly linked to the severity of haemophilia. In this respect, very few studies in the world have mentioned this aspect. In our study, the MseI SNP was only slightly found. This aligns with the results of Winship *et al.* in 1993 and Bowen 2002 among Caucasians and Thais [21] [27]. Our study reveals no significant link between the MseI SNP of the F9 gene and the severity or complications of haemophilia.

5. Conclusion

Our study revealed that the frequency of HindIII SNP of the F8 gene was higher in the participants and so there is a possible association of HindIII SNP of the F8 gene with the severity and complications of haemophilia, however, we found no significant association of MseI SNP of the F9 gene with the severity and complications of haemophilia.

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Authors' Contributions

WFM, CT, JPKC, CNE, contributed to the design of the study. CNE, SEE, JPKC, coordinated the study. CNE, AC, VM supervised the enrolment of patients and sample collection. JPKC, CNE, CTF, performed the molecular analysis. SEE, Analyzed data. CNE, SEE, JPKC, writing up the manuscript. All authors contributed in the revision of the manuscript and approved the final version of the manuscript prior to submission.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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