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PCR-RFLP of the Vitamin D Receptor Gene in Human Immunodeficiency Virus Patients Deficient in Vitamin D3 in Cote d'Ivoire

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Authors' contributions

This work was carried out in collaboration among all authors. Author LB wrote the protocol of the study, the first draft of the manuscript and supervised the technical aspects of the study. Authors AJAA and LOMB supervised blood samples collection and managed the biological analyses of the study. Authors KLS and GAB supervised the analyses and performed the statistical analyses of the study. Authors YGY and MFA managed a part of the literature searches and corrected the first draft of the manuscript. Author JAD designed the study, managed a part of the literature searches investigator and the final correction of the manuscript. All authors read and approved the final version of the manuscript.

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ABSTRACT

Aims: This study was to identify mutations in patients' vitamin D receptor (VDR) gene in Côte d'Ivoire, precisely in Human immunodeficiency virus (HIV) patients deficient in vitamin D_3.
Methodology: Fifty (50) DNA extractions from peripheral blood mononuclear cells collected from HIV positive and vitamin D_3 deficient patients were analyzed after verifying their integrity by quantification of genomic DNA and migration from agarose gel. The use of the restriction enzymes Dpn I, Bg III and Pst I made it possible to carry out the PCR-RLFP of the fragments Fok-1 in exon 2, Bsm-1 and Apa-1 in intron 8 and Taq-1 in exon 9.
Results: The analysis of the DNA fragments Fok-1 in exon 2 and Bsm-1 in intron 8 of the VDR gene from HIV positive patients deficient in vitamin D_3 showed a significantly high prevalence of mutant genotype (100% and 98%) respectively p = 0.0001. Furthermore, in this study, a prevalence of 6% of mutant genotype was observed in Taq-1 of exon 9 of the VDR gene.
Conclusion: The high prevalence of mutant genotypes observed in the DNA fragments of Fok-1 in exon 2 and Bsm-1 in intron 8 of the VDR gene studied confirms the presence of mutations in the VDR gene of these patients. It would, therefore, be necessary to sequence the DNA fragments with mutations in order to identify the mutations that affect the VDR gene and that are responsible for the vitamin D_3 deficiency observed in these patients.

Keywords: Vitamin D receptor (VDR); HIV; PCR-RFLP; Côte d'Ivoire.

1. INTRODUCTION

HIV infection is a global public health problem. Africa is the most affected continent with 25.6 million people living with HIV in 2016 [1]. In Côte d'Ivoire, the prevalence of the disease had decreased from 3.4% in 2013 to 2.8% in 2017 [2,3].

The active form of vitamin D_3 (1,25-dihydroxyvitamin D_3) ensures its biological activity with the aid of its nuclear receptor (VDR) [4]. It plays a major role in the regulation of phosphocalcic homeostasis and the metabolism of minerals. It is involved in other biological functions such as cell growth, differentiation, proliferation, apoptosis, adapted and inborn immune responses [5]. There is a link between vitamin D_3 deficiency and susceptibility to acute infections as well as the more unfavorable course of certain chronic infections such as HIV infection [6].

VDR is involved in the gene regulation of a large number of cell types including cells involved in innate and adaptive immunity [7]. It is widely expressed in various tissues [8] and, therefore, represents an important therapeutic target in the treatment of various disorders [9]. As ligand binding is a key step in VDR signaling, the vitamin D_3 deficiency observed in HIV positive patients could also be linked to mutations in the vitamin D nuclear receptor (VDR) [10,11]. Indeed, the analysis of the gene coding VDR on chromosome 12 showed the diversity of possible alterations of this gene are that often mutations concerning exon 7 and 9 which codes the part of the receptor that binds the hormone, the exons 2 or 3 which encode the region by "Zinc-fingers" allowing the receptor to bind to nuclear DNA. These mutations are responsible for the vitamin D_3 deficiency observed in HIV positive patients [12]. Therefore, the existence of genetic polymorphism of VDR also constitutes an important factor of individual susceptibility to the biological effects of vitamin D_3 [13,14].

In addition to its role in mineral metabolism, 1,25-dihydroxivitamin D3 exerts an immuno-modulatory activity through the vitamin D receptor (VDR) by activating monocytes, stimulating cell-mediated immunity and suppressing lymphocyte proliferation, antibody production, and cytokine synthesis. VDR mediates genomic actions of 1,25 (OH)2D3, by acting as a transcription factor that modulates the expression of several1,25(OH)2D3 response genes. Variations in the VDR gene have been associated with susceptibility and progression to several immune diseases mainly HIV infection. they may alter gene function and compromise the role of 1,25 [OH] 2D, leading to decreased activation of the immune system [15].

Genetic variations in VDR might decrease VDR expression, influencing the binding of 1,25 [OH]2D to VDR and inducing apoptosis, consequently affecting CD4+ T-cells recovery [16,17].

In Côte d'Ivoire, no molecular study has been done to this effect to better understand vitamin D_3 deficiency. This study aimed to identify mutations in the VDR gene in HIV positive patients.

2. SAMPLES AND METHODS

This is an experimental study that took place between November 2015 and December 2016 in the Department of Biochemistry of the Institut Pasteur in Côte d'Ivoire. A cohort of 50 blood samples was taken from an EDTA (ethylene diamine tetra-acetic) tube in 50 adult HIV-positive patients deficient in 25-hydroxyvitamin D_3 after obtaining written informed consent from each patient.

2.1 Assay for 25 (OH) D_3

The assay of 25-hydroxyvitamin D3 was performed using UV detection in high performance liquid chromatography (HPLC) with a Waters® device, after extracting soluble vitamins in hexane protected away from light according to the method of Zaman [18]. The serum reference values of 25 (OH) D_3 are: Deficient (< 20 ng / mL); Insufficient (20-29 ng / mL); Sufficient (30-100 ng / mL); Toxic (> 100 ng / mL) [19].

2.2 Extraction of Genomic DNA in Peripheral Blood Mononuclear Cells

Mononuclear cells (approximately 10^7 cells) were previously isolated from peripheral blood using the separation method of Ficoll flotation [20].

The DNA, extracted from mononuclear cells from the blood by the method of Maurya [21] was collected and washed three times with 70% ethanol, then dried at room temperature under the laminar flow hood for 30 min. It was then dissolved in 100 µL of TE buffer (10 mM Tris HCl [pH 7.5], 1 mM sterile EDTA) and incubated for at least four hours at 50°C with gentle shaking during the incubation. The purified DNA extracts obtained were aliquoted in 1.5 mL Eppendorf tubes and stored at -20°C.

2.3 Amplification of the DNA Fragments Fok-1, Bsm-1, Apa-1 and Taq-1 of the VDR Genes

Fok-1, Bsm-1, Apa-1 and Taq-1 are among the six major polymorphisms of the VDR gene studied and associated with susceptibility of viral infection. They may alter gene function and compromise the role of 1,25 [OH] 2D, leading to decreased activation of the immune system [15].

The most studied fragments of the VDR gene are Fok-1 (rs2228570) in exon 2, Bsm-1 (rs1544410) and Apa-1 (rs7975232) in intron 8 and Taq-1 (rs731236) in Exon 9 [22]. The Fok-1 polymorphism occurs near the 5'-UTR region of the gene in the DNA binding domain and plays an essential role in message stability and in post-transcriptional processes [23]. Suneetha's study reported an association between the Bsm-1, Apa-1 and Taq-1 polymorphisms, and the stability of messenger RNA from VDR (mRNA) [24].

The following mixture was prepared in a 25 µL final volume: water milliQ , specific primers (10 µM), 5×HOT FIREpol® Blend Master ($MgCl_2$ 2,5 mM, dNTPs 200 µM, taq DNA polymérase), DNA matrix (2 µL) [25].

The primer pairs used were: Fok-1F 5'-AGCTGGCCCTGGCACTGACTCTGCTCT-3' and Fok-1R 5'-ATGGAAACACCTTGCTTCTTCTCCCTC-3';

Bsm-1F 5'-AACCAAGACTACAAGTACCGCGTCAGTGA-3' and Bsm-1R 5'-AACCAGCGGGAAGAGGTCAAGGG-3';

Apa-1F 5'-GTGGGATTGAGCAGTGAG-3' and Apa-1R 5'-ATCATCTTGGCATAGAG-3'; Taq-1F 5'-CAGAGCATGGACAGGGAGCAA-3' and Taq-1R 5' GCAACTCCTCATGGCTGAGGTCTC-3'.

These primers are those previously published by Chakraborty [26] and Rashedi [27]. They have been synthesized by Sigma Aldrich (France). Stock solutions of 100 µM of each primer received were reconstituted according to Sigma Aldrich's instructions.

For carrying out the PCRs, each stock solution (100 µM) was diluted 1:10 to give a concentration of 10 µM.

The Appled Biosystems thermal cycler (2720 Thermal Cyber, Singapour) was programmed to perform 35 cycles of 94°C for five minutes (first cycle), followed by 94°C for 30 seconds, or one minute (29 following cycles), 51°C for Fok-1and Bsm-1, 53°C, 69°C for Apa-1 and Taq-1 respectively × 30 seconds, and

Table 1. PCR-RFLP fragments obtained

Gene	Enzymes	Digestion site 5'-3'	Fragments obtained	
			Wild type (n: bp)	Mutant (n: bp)
Exon 2: Fok-1 267 bp	*Dpn I* *Diplococcus pneumoniae*	GA^TC	**1:** 267 bp	**1**: 267 bp
Intron 8: Bsm-1 822 bp	*Bg III* *Bacillus globigii*	A^GATCT	**2:** 435 bp & 387 bp	**1**: 822 bp
Intron 8: Apa-1 195 bp	*Pst I* *Providencia stuartii*	CTGCA^G	**2:** 130 bp & 65 bp	**1**: 195 bp
Exon 9: Taq-1 745 bp	*Pst I* *Providencia stuartii*	CTGCA^G	**2:** 421 bp & 324 bp	**1**: 745 bp

n= number; bp= base pair

72°C × one minute followed by 72°C × seven minutes at the end of 35 cycles.

The PCR products, obtained after amplification were migrated to a 1.5% agarose gel containing SBYR Green I and visualized by UV translumination (Gel Doc TMEC imager). Size of the fragment: Presence or absence of band made it possible to judge the effectiveness of PCR.

2.4 PCR-RFLP of Amplified VDR Gene Sequences

Fok-1 PCR products were digested by *Dpn I* [(50°C, 16,3 µL milliQ water, 2 µL Buffer 10X, 0,2 µL Acetyl BSA (10 µg/µL), 1 µL PCR products, 0,5 µL *DpnI* (10U/µL)]. Bsm-1 PCR products were digested by *Bg III* [(50°C, 16,3 µL milliQ water, 2 µL Buffer 10X, 0,2 µL Acetyl BSA (10 µg/µL), 1 µL PCR products, 0,5 µL *Bg III* (10 U/µL)], while Apa-1 and Taq-1 PCR products were digested by *Pst I* [(50°C, 16,3 µL milliQ water, 2 µL Buffer 10X, 0,2 µL Acetyl BSA (10 µg/µL), 1 µL PCR products, 0,5 µL *Pst I* (10 U/µL)] [28] (Table 1).

The wild-type was defined when these different enzymes cleave the DNA fragments. The presence of two DNA fragments indicates that the enzyme has recognized its cleavage site.

Then ten microliter (10 µL) of the reaction mixture were used for migration on 2% agarose gel containing SYBR® green I and visualized using a UV transilluminator. The size of the fragments was determined using a 100 bp scale.

2.5 Statistical Methods

The R software was used for the statistical analysis of proportions by the Chi-square comparison test. Thus, a value of $P \leq 0.05$ is considered statistically significant.

3. RESULTS

- The lengths of the fragment product PCR obtained after the amplification were 267 bp for exon 2 (Fok-1), 822 bp for intron 8 (Bsm-1), 195 bp for intron 8 (Apa-1) and 745 bp for exon 9 (Taq-1), respectively.
- After PCR-RFLP using *Dpn* I, no restriction site was found, so only one fragment of 267 bp is obtained. For the others [*Bg* III (intron 8), *Pst* I (intron 8), *Pst* I (exon 9)], two DNA fragments were found in each case (435 bp /387 bp; 130 bp / 65 bp; 421 bp / 324 bp respectively).
- The analysis of DNA fragments in exon 2 Fok-1 of the PCR-RFLP of the VDR gene from HIV positive patients deficient in vitamin D_3 showed a high prevalence of 100% (50/50) of mutant genotype. The same is true for the DNA fragments in intron8 Bsm-1 of the VDR gene with a prevalence of 98% (49/50), $P < 0.0001$. In addition, in the study of the polymorphism in intron 8 Apa-1 and in exon 9 Taq-1 of the VDR gene, a high prevalence of wild genotype was observed: 100% and 94% respectively, with a mutant genotype Taq-1 prevalence of 6% (Table 2).

4. DISCUSSION

In this study, the analysis of DNA fragments Fok-1 in exon 2 and Bsm-1 in intron 8 of the VDR gene in HIV-positive patients deficient in vitamin D_3 showed a significantly high prevalence of mutant genotype (100% and 98% respectively).

Indeed, this strong presence of the mutant genotype of DNA fragments Fok-1 could be due to the polymorphism at exon 2 of the VDR gene [29].

Table 2. Distribution of DNA samples according to the genetic profile

ADN Fragments of VDR genes	PCR-RFLP (n = 200)		
	Genotypes		P
	Wild n (%)	Mutant n (%)	
Exon 2: Fok-1 n= 50	**0 (0)**	**50 (100)**	**< 0.0001**
Intron 8: Bsm-1 n= 50	**1 (2)**	**49 (98)**	**< 0.0001**
Intron 8: Apa-1 n= 50	**50 (100)**	**0 (0)**	**< 0.0001**
Exon 9: Taq-1 n= 50	**47 (94)**	**3 (6)**	**< 0.0001**

It is a substitution of a nucleotide T (Thymine) for C (Cytosine) at the two potential translation initiation sites [30]. This mutation, which was a substitution of thymine by cytosine (T/C substitution), affects the polymorphism Fok-1 which alters the VDR gene function and disrupts the immunoregulatory activity of 1,25-dihydroxivitamin D3. This disruption leads to a vitamin D deficiency in the patient and therefore a decreased activity of his immune system [15].

The polymorphism of Fok-1 occurs near the 5'-UTR region of the gene in the DNA binding domain and plays a critical role in message stability and in post-transcriptional processes [23]. In addition, this polymorphism which occurs at the 3 'region of the VDR gene, the region in which exon 2 of the VDR gene is located, is associated with vitamin D_3 deficiency and the progression of HIV infection [31]. As for the polymorphism of Bsm-1, it does not modify the sequence of the VDR but could influence the expression of the VDR by altering the stability of the mRNA [32].

On the other hand, a high prevalence of wild genotype was observed in the DNA fragments of Apa-1 in intron 8 and Taq-1 at exon 9 of the VDR gene of 100% and 94%, respectively. In addition, a prevalence of 6% of mutant genotype was observed in Taq-1. This Taq-1 polymorphism is localized in exon 9 at codon 352. Indeed, polymorphisms have been identified in this region of the VDR gene. They could influence the expression of VDR by altering the stability of the mRNA [33].

5. CONCLUSION

The analysis of the DNA fragments of the VDR gene in HIV-positive patients deficient in vitamin D_3 revealed the presence of mutations in exon 2, intron 8 and exon 9. The high prevalence of genotype mutants observed in the DNA fragments Fok-1 at exon 2 and Bsm-1 at intron 8 of the VDR gene studied confirms the presence of mutations in the VDR gene of these patients. It would, therefore, be necessary to sequence the DNA fragments with mutations in order to identify and better assess the mutations that affect the VDR gene and those responsible for the vitamin D_3 deficiency observed in these patients.

Therefore, studies have shown that polymorphisms affecting the VDR gene are associated with HIV replication [34] and represent an important factor in genetic susceptibility to a decrease in vitamin D_3 [35].

In perspective, the sequencing of these three VDR genes will make it possible to better identify the mutations in order to verify their implication in vitamin D_3 deficiency.

CONSENT

Before sample-collection, informed consent was obtained from patients for the use of their blood for research purpose after completing the standard analyzes prescribed by their doctor.

ETHICAL APPROVAL

The study was conducted in accordance with the Helsinki Declaration 2000 on HIV and AIDS research conducted in poor countries and in accordance with the local legislation regarding the national program on treatment management for people living with HIV/AIDS (Decree No. 411 of December 23, 2001). All results were given to doctors for patients' treatment.

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COMPETING INTERESTS

Authors have declared that no competing interests exist.

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