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Development of the Genotyping Protocol for the Study of Genetic Polymorphisms of CYP450 2C9 (CYP2C9 * 2, * 3) and VKORC1 1639G> A

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Abstract

Two genes are mainly involved in the variability of the response to Acenocoumarol: one encoding the vitamin K epoxide reductase (VKORC1) pharmacological target of AVKs, and the other encoding the cytochrome P450 2C9 (CYP2C9), responsible for the metabolism of coumarin derivatives to inactive metabolites.The objective of our work is to develop a genotyping technique in order to study this genetic polymorphism within the Algerian population.Genotyping is performed by RFLP (restriction fragment length polymorphism) PCR over 4 steps: DNA extraction, amplification, enzymatic digestion and electrophoresis. The FDA has approved that the use of genetic testing may help healthcare professionals better predict steady-state doses of vitamin k antagonists in order to optimize the management of patients treated with this drug.

Keywords: acenocoumarol; genetic polymorphism; VKORC1; CYP2C9; PCR RFLP.

1. Introduction

Genetic variations have been identified as major determinants of the response to acenocoumarol. The polymorphisms of VKORC1 1639G> A and CYP2C9 * 2, * 3 are very common in the general population and vary according to ethnicity [1, 2, 3]. Patients with these polymorphisms are hypersensitive to acenocoumarol, which results in a low steady-state dose, an early response to initiation of treatment, and an increased risk of overdose compared to patients with the wild allele.

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The potential value of genotyping (VKORC1 and CYP2C9) at the individual level lies in the identification of hypersensitive patients, thus contributing to the development of safer personalized medicine [4, 5, 6, 7]. The objective of this work is to develop a genotyping technique for the study of genetic polymorphisms of CYP450 2C9 (CYP2C9 * 2, * 3) and VKORC1 1639G> A in patients treated with acenocoumarol at the EHU from Oran.

2. Materials and methods

This is a study starting in January 2017, carried out at the Pharmaceutical Development Research Laboratory LRDP in collaboration with the molecular biology and cytogenetics laboratory of Mohamed Boudiaf University in Oran. The genetic study is carried out using the RFLP (restriction fragment length polymorphism) PCR technique, the extracted DNA sample is then amplified and cut by restriction enzymes. The size of the fragments obtained is analyzed using the electrophoresis technique in figure 1.

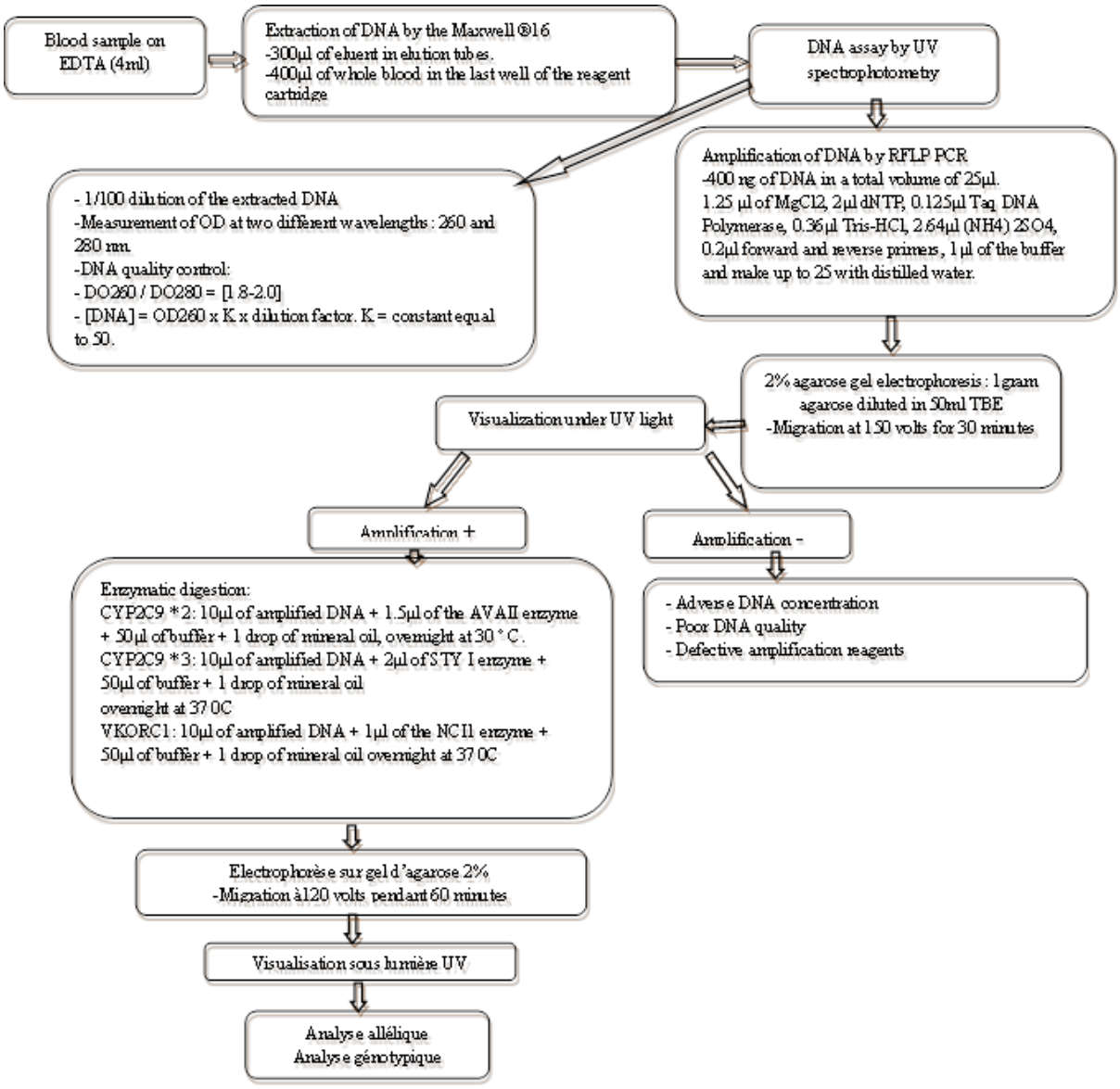

Figure 1: Experimental protocol for the genotyping of the CYP2C9 * 2, CYP2C9 * 3 and VKORC1 genes.

3. Results

3.1. Sampling for genotyping

The genetic study was carried out on a sample of 4ml whole blood taken from an EDTA tube. The number of white blood cells in the blood should be between 4.2 x 106 and 1.2 x 107 / ml.

3.2. DNA extraction

First, we extracted DNA from patient samples using an extraction device, the Maxwell ®16, in the following steps:

- The frozen samples were brought to room temperature then they were homogenized by rotary movements.
- Decontamination of the device by the UV lamp.
- The list of samples is entered into the machine.
- Using a micropipette, 300µl of the elution solution was put into the elution tubes.
- The extraction reagent cartridges have been placed in the special reagent rack.
- 400µl of whole blood was taken and placed in the last well of each cartridge.
- The purification plungers were placed in the first well of each cartridge.
- Reagent cartridges and elution tubes are placed in the device which can analyze 16 samples at a time.
- Start of DNA extraction.
- At the end of the extraction, the DNA is recovered in the elution solution. The extracted DNA can be stored in sterile ependorfs labeled and sealed with parafilm at -200 ° C.

3.3. Assay of extracted DNA

The concentration of the purified DNA should be measured in order to verify the quality of the extraction.

- First, we must proceed to a dilution of 1/100 th of the concentrated DNA, for this, we put 10µl of DNA in a sterile ependorf and we supplemented with sterile distilled water until 1ml, then we vortexed the mixture and then let the ependorfs homogenize in a drum for 48 hours.
- Subsequently, we measured the absorbance of the daughter solutions using a spectrophotometer at two different wavelengths: 260 and 280 nm.
- The device determined the value of OD260, OD280, of each sample, the OD260 / OD280 ratio, the latter must be between 1.8 and 2 and the DNA dosage is calculated according to the following formula:

[DNA] = OD 260 X K x dilution factor. K = constant equal to 50.

3.4. Amplification

We then proceeded to amplify the gene segments using the Biometra® T GRADIENT thermocycler. During this phase, we experimented with the modified protocol of Anupriya Kaur [8]. From a sample of 400 ng of DNA in a

total volume of 25µl. The analysis was carried out at the molecular biology and cytogenetic laboratory of Mohamed Boudiaf University in Oran.

Table 1: Quantitative composition of the reaction mixture

Reagent	Concentration	Volume to be sampled
- MgCl2	2.5mM	1.25 µl of MgCl2 at 50 mM
-dATP, dCTP, dGTP, dTTP	200µM of each base	2 µl of the dNTP mixture at 10mM
- Taq DNA Polymerase	0.025units / µl	0.125 µl of Taq DNA Polymerase at 5 units / µl
-Tris-HCl (pH 8.8 à 25°C)	75mM	0.36 µl of the 58.8 mM Tris-HCl solution
-$(NH4)_2$ SO4	20mM	2.64 µl of 189 mM (NH4) 2SO4 solution
-Forward primer*	0.16µM	0.2 µl of the 20 µM daughter solution
-Reverse primer*	0.16µM	0.2 µl of the 20 µM daughter solution
-DNA buffer		1µl
-DNA	400 ng	According to the concentration of DNA
--Sterile distilled water		QSP 25 µl

Forward et reverse primers utilisés pour chaque polymorphisme :

CYP2C9*2 : Forward primer : 5'TACAAATACAATGAAAATATCATG-3'

Reverse primer : 5'CTAACAACCAGGACTCATAATG3'

CYP2C9*3 : Forward primer : 5'AGGAAGAGATTGAACGTGTGA-3'

Reverse primer : 5'GGCAGGCTGGTGGGGAGAAGGTCAA-3'

VKORC1 : Forward primer : 5'ATCCCTCTGGGAAGTC AGC-3'

Reverse primer : 5'CACCTTCAACCTCTCCATCC-3'

Remarque:

- Each amplification cycle theoretically results in the doubling of the amount of fragments located between the two primers so that the amount increases exponentially. 2^n copies are obtained for n cycles and in practice a yield of 70%.
- In order to overcome any possible contamination, a negative control containing the reaction mixture without DNA is added.

In vitro amplification (PCR) program: takes place in three phases

- First phase: (one cycle) a denaturation step at 94°C for 5 minutes.
- Second phase: repeated in 34 cycles, each cycle includes the following steps:
- A denaturation step at 94°C for 45 seconds;
- An hybridization step at 54.3°C for 1 minute;
- An elongation step at 72°C for 1 minute 30 seconds.
- Third phase: Final extension at 72°C for 8 minutes followed by storage of DNA at 15^0C for unlimited time.

3.5. Handling

The temperature cycle was introduced in the Biometra® T GRADIENT thermocycler, we prepared our master mix, then we aliquoted it in the PCR tubes, and we added the DNA of the samples, the last PCR tube only contains the master mix, it served as a negative control. We placed the PCR tubes in the thermocycler, and started amplification. At the end of the polymerization chain, the amplified DNA is recovered.

3.6. Analysis of DNA fragments amplified by agarose gel electrophoresis

The amplimeters of the CYP2C9 and VKORC1 genes are tested by electrophoresis on agarose gel, the migration is carried out at 150 volts for 30 minutes, the visualization of the migration bands is made under UV by fluorescence with Ethidium Bromide, the size of the expected bands is 690 bp, 130 bp and 636 bp for CYP2C9*2, CYP2C9*3 and VKORC1 respectively. The presence of bands under UV light in the sample wells signifies the presence of amplified DNA.

Figure 2: Analysis of DNA fragments amplified

The absence of UV light bands in the sample wells means the DNA was not amplified. The presence of bands under UV light in the control well means that there is contamination with foreign DNA.

3.7. Enzymatic digestion

Enzymatic digestion is carried out using the following enzymes:

- AVAII: for CYP2C9*2

In the wild-type allele, this enzyme cuts the gene into two amplicons, while the mutated allele abolishes the restriction site of the enzyme. We put 1µg of DNA (10µl of amplified DNA) with 15 units of this enzyme (1.5µl of the enzyme AVAII 10 U / µl) in 50µl of buffer, we added a drop of mineral oil to avoid evaporation of the mixture then we incubated it overnight in the oven at 30^0C.

- STY I: for CYP2C9 * 3

In the wild-type allele, this enzyme lacks a restriction site, while in the mutated allele, the enzyme finds its restriction site resulting in two amplicons. We put 1µg of DNA (10µl of amplified DNA) with 20 units of this enzyme (2µl of STY I 10 U / µl enzyme) in 50µl of buffer, we added a drop of mineral oil to avoid evaporation of the mixture then we incubated it overnight in the oven at 370C.

- NCI 1: for VKORC1

In the wild-type allele, this enzyme cuts at two restriction sites, in the mutated allele, the enzyme abolishes one restriction site, and therefore two amplicons are obtained.

We incubated 1µg of DNA (10µl of amplified DNA) with 10 units of this enzyme (1µl of NCI1 10 U / µl enzyme) in 50µl of buffer, we added a drop of mineral oil to avoid evaporation of the mixture then we incubated it overnight in the oven at 370C.

Table 2: Sizes of the products of enzymatic digestion

	Amplicon size	**Wild allele**	**Minor allele**
CYP2C9*2	690	521	690
		169	
CYP2C9*3	130	130	104
			26
VKORC1	636	472	522
		114	114
		50	

3.8. DNA electrophoresis

Electrophoresis of the PCR products is carried out on a 2% agarose gel at 120 volts from the negative pole to the positive pole, for 60 minutes labeled with 0.5 µg / ml of ethidium bromide in Tris borate EDTA (TBE) buffer (pH = 8.3) according to the following steps: The agarose gel is prepared by diluting one gram (1g) of agarose powder in 50ml of TBE, then was put in the microwave for 60 to 90 seconds to dissolve.

The rubber gaskets are placed around the edges of the tank, then, the tank is introduced into the electrophoresis tank and the comb is put in its place. 5µl of Ethidium Bromide was added to the gel and poured into the vessel, then allowed to gel for half an hour. After gelation, the comb is removed. The electrophoresis tank is immersed in TBE. In the first well, we put 5µl of the size marker; in the following wells, we put 10 µl of DNA amplified with 5 µl of loading buffer. The power generator is set to 120 volts, and then electrophoresis is started

Figure 3: DNA electrophoresis

4. Recommendations

The study of the genetic polymorphisms of CYP450 2C9 (CYP2C9 * 2, * 3) and VKORC1 1639G> A in Algeria, appears currently essential for the optimization of treatment with acenocoumarol. Antitamin k dose prediction algorithms; established in different countries of the world; and which incorporate several factors such as age, size and genetic determinants have shown their effectiveness especially at the start of treatment with acenocoumarol, prospective studies are still necessary in order to approve their interest in the individualization of doses of acenocoumarol to long-term treatments [9,10].

5. Conclusion

This research work is part of a process to improve the quality of the prescription and the safety of use of acenocoumarol. Through this work we have prepared the ground to study these polymorphisms in Algerian patients as well as to see their influence on the dosage of acenocoumarol at equilibrium in order to establish a dose prediction algorithm.

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