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Characterization of the VKORC1 -1639G>A (rs9923231) polymorphism-associated genotypes

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Mirsada Causevic, Honours BSc, PhD[1], Amina Sahbaz, MD[1], Edin Begic, MD, MA, PhD[1]

[1]Pharmacology Department, Sarajevo Medical School, University Sarajevo School of Science and Technology, Sarajevo, Bosnia and Herzegovina

Mirsada Causevic
University Sarajevo School of Science and Technology

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We use this protocol and it is working.

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Abstract

The procoagulant factors (FII, FVII, FIX, FX) and anticoagulant factors (proteins C and S), which play an important role in blood coagulation, are vitamin K-dependent factors. That means that their activation depends on the optimum level of the reduced form of vitamin K in the human body. The formation of the reduced form of vitamin K is controlled by the enzyme called vitamin K epoxide reductase (VKOR), which transforms the oxidized form of vitamin K, the vitamin K epoxide, into the reduced form, the vitamin K hydroquinone. Then, the vitamin K hydroquinone functions as a cofactor in the activation of blood factors which are essential for blood clotting.

The VKOR is the enzyme which represents the molecular target of warfarin and other coumarin-type anticoagulants, also called vitamin K antagonists (VKAs). They inhibit the VKOR enzyme thereby indirectly reducing the levels of the functional clotting factors and, thus, achieving appropriate anticoagulation.

The VKOR enzyme is encoded by the vitamin K epoxide reductase complex subunit 1 (*VKORC1*) gene, which is a polymorphic gene. A non-coding, single nucleotide polymorphism (SNP), in the promoter region of the *VKORC1* gene, regulates the transcription of the *VKORC1* and, hence, the translation and the expression level of the VKOR protein/enzyme. This, in turn, affects the level of the functional clotting factors, in an individual's body, which has consequences for an individual's endogenous, physiological thrombogenicity or antithrombosis, as well as his/her sensitivity to anticoagulant drugs.

Many studies have demonstrated an association between a gene sequence in the promoter region of the *VKORC1* gene (genotype) and the resulting levels of the VKOR enzyme and functional clotting factors (phenotype) -

1. **GG** or ***VKORC1 -1639GG*** **genotype**: the presence of the wild-type sequence or GG genotype, in an individual, results in high or standard level of the VKOR enzyme, high or standard level of the functional clotting factors, high or standard endogenous thrombogenicity, which requires high or standard dosing of an anticoagulant drug, e.g. warfarin;
2. **GA** or ***VKORC1 -1639GA*** **genotype**: the presence of the heterozygous, variant sequence or GA genotype results in an intermediate level of the VKOR enzyme, intermediate level of the functional clotting factors, intermediate endogenous thrombogenicity, which may require reduced dose of an anticoagulant drug; and
3. **AA** or ***VKORC1 -1639AA*** **genotype**: the presence of the homozygous, variant sequence or AA genotype results in low level of the VKOR enzyme, low level of the functional clotting factors, low level of endogenous thrombogenicity, which requires low dose of an anticoagulant drug.

This protocol provides a detailed, step-by-step guide to characterization of the *VKORC1* -1639G>A (rs9923231) polymorphism-associated genotypes by using a previously published protocol by Elizabeth A. Sconce and colleagues (DOI: 10.1182/blood-2005-03-1108), with modifications. It utilizes polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method, followed by direct, Sanger sequencing.

Attachments

Figure1_VKORC1.jpg 233KB

Figure2_Sanger-seque... 124KB

Image Attribution

Amina Sahbaz, MD, Pharmacology Department, Sarajevo Medical School, University Sarajevo School of Science and Technology, Sarajevo, Bosnia and Herzegovina

Materials

Agarose - Sigma A9539-100G;
Boric acid - Sigma B6768-500G;
DNA dye - Ethidium bromide - Sigma E1510-10ML;
DNA ladder (1) - BenchTop 100bp DNA Ladder - Promega G8291;
DNA ladder (2) - MiniSizer 50bp DNA Ladder - Norgen 11200;
dNTPs - dATP, dCTP, dGTP and dTTP - at a stock concentration of 100mM each - Promega U1330;
EDTA (ethylenediaminetetraacetic acid) - Sigma EDS-500G;
Ethanol - Sigma 32205-1L;
HCl - Sigma 30721-2.5L;
Isopropanol - 2-propanol - Sigma 59304-500ml;
NaCl - Honeywell-Fluka 31434-1KG;
MspI restriction endonuclease enzyme - New England BioLabs R0106S;
Proteinase K - Sigma P2308;
SDS (sodium dodecyl sulfate) - Sigma L3771-100G;
GoTaq G2 DNA Polymerase - Promega M7845;
Trizma base - Sigma RDD008-1KG.

Troubleshooting

Safety warnings

This protocol uses human whole blood, as a starting material for extracting human, genomic DNA. Human whole blood could be a source of hepatitis B (HepB) virus infection (if taken from persons with HepB virus infection) to the individuals who work with human blood and, as such, represents health and safety, or biological hazard.

Ethidium bromide, which is used during the preparation of agarose gels, is a DNA intercalating dye and it can, therefore, act as a mutagen. It should be handled with care and only when wearing gloves and safety glasses.

Human, genomic DNA extraction

1 Approximately 2-3 ml of human whole blood was collected in ethylenediaminetetraacetic acid (EDTA)-containing tubes and stored at -20°C until use. Genomic DNA extraction from the human whole blood, that is, leukocytes, was carried out according to the protocol described by **Subbarayan PR and colleagues** (DOI: 10.2144/02336bm10), with modifications.

> Note
>
> All solutions should be prepared with ultra-pure H_2O and sterilized and all pipette tips used should be sterile, filter tips.

1.1 Day 1
Upon thawing of the blood, for every blood sample, five (5) sterile, 1.5 ml eppendorf tubes were labeled and 300 µl of the whole blood was added. This was the amount of the starting material that yielded ample amount of human, genomic DNA at the end of the DNA extraction protocol.

1. To every 300 µl of the whole blood, add 1.2 ml of 20 mM Tris-HCl, pH 7.5, mix by inverting the tube, and incubate at room temperature (RT) for 10 minutes.
2. Centrifuge the tubes at 14,000xg, at RT for 30 seconds.
3. Remove the supernatant away from the pellet and throw it away.
4. Place 1.5 ml of 20 mM Tris-HCl, pH 7.5, to every pellet, mix by inverting the tube, with gentle flicking of the tube with fingers, to wash the pellet.
5. Incubate the pellets with the fresh buffer at RT for 10 minutes.
6. Centrifuge the tubes at 14,000xg, at RT for 30 seconds.
7. Repeat the washing of the pellets 4-5 times, until they are clearly visible in a tube, with off-white color.
8. After the final wash, remove the supernatants, as previously.
9. Merge all five (5) pellets into 1.8 ml of lysis buffer (0.1M Tris-HCl, pH 8.0, 0.2M NaCl, 5mM EDTA, pH 8.3, 0.4% SDS). Disperse the pellets in the lysis buffer by using a glass homogenizer, with gentle movements, until the pellets are uniformly dispersed, without major cell clumps in the lysis buffer.
10. Divide the lysate into three (3) sterile, 1.5 ml eppendorf tubes, with 600 µl of lysate in each tube.
11. Add proteinase K to each tube, at a final concentration of 0.1 mg/ml.
12. Incubate the lysates at 55°C overnight, with gentle shaking, if possible.

1.2 Day 2

1. Prepare and label fresh, sterile 1.5 ml eppendorf tubes.
2. Remove the tubes containing cell/DNA lysates from a 55°C shaker, or a water bath, to which they were placed on Day 1, and let them cool down.
3. Centrifuge the tubes at 14,000xg, at RT for 5 minutes.
4. Carefully remove the supernatants, which now contain human, genomic DNA, and place them in fresh, sterile tubes.
5. Add equal volume of ispopropanol to the supernatants, mix by inverting the tubes and let the DNA precipitate at RT for 10 minutes. Precipitated DNA should become visible in a tube resembling a small piece of white thread.
6. Centrifuge the samples at 14,000xg, at RT for 5 minutes.
7. Carefully remove the ispopropanol away from the DNA pellet. Add 1 ml of 70% ethanol (which should be prepared with sterile, ultra-pure H_2O) to DNA pellets. Gently mix by inverting the tubes several times.
8. Centrifuge the samples at 14,000xg, at RT for 5 minutes. Remove the ethanol and air-dry DNA pellets for approximately 10 minutes. Ensure that no droplets of ethanol are left in the tubes by removing them carefully with a sterile pipette tip.
9. Add 50 µl of sterile 1xTE buffer (10mM Tris-HCl, pH 8.0, 1mM EDTA, pH 8.3), pre-warmed at 55°C, to every DNA pellet, in order to dissolve genomic DNA sample. Vortex to dislodge the pellet away from the wall of the tube.
10. Place the tubes at 55°C for 10 minutes, with gentle shaking, if possible.
11. Merge two of three tubes of every individual DNA samples into one.
12. Centrifuge the merged human, genomic DNA samples at 14,000xg, at RT for 1 minute, in order to pellet the undissolved DNA.
13. Place the supernatant containing the dissolved, human, genomic DNA into a fresh, sterile 1.5 ml eppendorf tube.
13. Determine the concentration of the DNA in tubes.
14. Freeze down the 3rd tube containing the individual DNA sample, without removing the pellet. Keep this as a backup DNA sample.

2 Concentrations of human, genomic DNA was determined by using Qubit 4 Fluorometer (Q33226, ThermoFisher Scientific).

For subsequent polymerase chain reactions (PCRs), 50 ng of genomic DNA was used in every PCR reaction.

Identification of different *VKORC1* genotypes

3 Identification of the *VKORC1 -1639G>A* (rs9923231) polymorphism-associated genotypes ("GG", "GA" and "AA") was performed by using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method, which was published by **Sconce EA and colleagues** (DOI: 10.1182/blood-2005-03-1108), with modifications.

3.1 For a 25 µl PCR reaction, the following was included:

1) sterile, ultra-pure H_2O;
2) 1xPCR buffer (for a GoTaq G2 DNA polymerase, M7845, Promega), from a stock of 5xPCR buffer;
3) 0.2 mM deoxyribonucleotide triphosphates (dNTPs) (U1330, Promega), from a stock of 2.5 mM;
4) 1 µM forward primer, from a stock of 25 µM;
5) 1 µM reverse primer, from a stock of 25 µM;
6) 50 ng of human, genomic DNA and
7) 2.5 U GoTaq DNA polymerase (M7845, Promega).

Sequences of the PCR primers were as follows:
1) Forward primer: 5'-GCCAGCAGGAGAGGGAAATA-3';
2) Reverse primer: 5'-AGTTTGGACTACAGGTGCCT-3'.

PCR cycles used for the amplification of the *VKORC1* gene promoter are based on the touchdown PCR protocol, which was published by **Darren J. Korbie and John S. Mattick** (DOI: 10.1038/nprot.2008.133).

They were the following ones:

1) 95°C for 3 min (1 cycle);
2) 95°C for 2 s, 60°C for 2 s, 72°C for 30 s (**2 cycles**);
3) 95°C for 2 s, 59°C for 2 s, 72°C for 30 s (**2 cycles**);
4) 95°C for 2 s, 58°C for 2 s, 72°C for 30 s (**2 cycles**);
5) 95°C for 2 s, 57°C for 2 s, 72°C for 30 s (**2 cycles**);
6) 95°C for 2 s, 56°C for 2 s, 72°C for 30 s (**2 cycles**);
7) 95°C for 2 s, 55°C for 2 s, 72°C for 30 s (**30 cycles**);
8) 72°C for 7 min (**1 cycle**);
9) 4°C for 2 h (**1 cycle**).

Expected result

The expected size of the PCR product is 290 base pairs (bp).

3.2 In order to characterize the *VKORC1 -1639G>A* (rs9923231) polymorphism-associated genotypes by using the PCR-RFLP method, the following reactions were set up with MspI restriction endonuclease enzyme:

1) 25 µl of the PCR product;
2) 3 µl of the 10xCutSmart Buffer (B7204S, New England Biolabs);

3) 1 µl of sterile, ultra-pure H_2O;

4) 1 µl of MspI enzyme (R0106S, New England Biolabs).

Total reaction volume: 30 µl.

Reactions were incubated in a 37°C water bath overnight.

DNA fragments were examined by using 2% agarose gel electrophoresis with 1xTBE buffer (89mM Trizma base, 89mM boric acid, 2mM EDTA, pH 8.3), with agarose gels containing 0.5 mg/ml ethidium bromide (E1510-10ML, Sigma). The results were viewed with a UV lamp (UVstar, Biometra, Analytik Jena). Gel images were captured by using BioDocAnalyze (BDA) camera (Biometra, Analytik Jena).

Expected result

The following pattern of DNA fragments can be expected after the MspI restriction endonuclease enzyme reaction - **Figure 1** (please see the attached file titled Figure1_VKORC1):

1) ***GG*** or ***VKORC1 -1639GG*** **genotype** is characterized by two (2) DNA fragments of 168 and 122 base pairs (bp);

2) ***GA*** or ***VKORC1 -1639GA*** **genotype** is characterized by three (3) DNA fragments of 290, 168 and 122 bp;

3) ***AA*** or ***VKORC1 -1639AA*** **genotype** is characterized by one (1), undigested DNA fragment of 290 bp.

Sanger sequencing of the *VKORC1* gene promoter

4 In order to confirm the results obtained by using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method, 12 samples, from different human volunteers, that is, 4 of each genotype (GG, GA, and AA), were directly sequenced using the Sanger method.

For Sanger sequencing, PCR reactions were performed as described above.

go to step #3.1

The amplified DNA was purified with a PCR Purification Kit (MinElute, 28004, QIAGEN). Next, DNA concentration was established by using Qubit 4 Fluorometer (Q33226,

ThermoFisher Scientific) and 50 µl of 1 ng/µl of each DNA sample was sent for sequencing (**Eurofins Genomics**).

The utilized forward and reverse primers' sequences were 5'-GCAGGAGAGGGAAATATC-3', and 5'-ACAGGGTTTCACCATGTTGG-3', respectively.

Expected result

Attached are electropherograms showing sequencing results from human volunteers with different genotypes:

1) ***GG*** genotype (please see the attached file titled Figure2_Sanger-sequencing, panel A);
2) ***GA*** genotype (please see the attached file titled Figure2_Sanger-sequencing, panel B);
3) ***AA*** genotype (please see the attached file titled Figure2_Sanger-sequencing, panel C).

In **Figure 2** (please see the attached file titled Figure2_Sanger-sequencing), the polymorphic sequence (CCGG), for the GG, GA and AA genotypes, starts at the 91st, 93rd and 94th nucleotide residue, respectively.

In the electropherogram which represents heterozygous, mutant or variant genotype (GA), there is an appearance of a letter "N" which symbolizes aNy nucleotide residue. This occurred due to the overlapping traces in the electropherogram at the 95th nucleotide residue: there is an overlap of traces for A = adenine (green) and G = guanine (black).

In addition, there is an appearance of a letter "N" at the 96th nucleotide residue, in the same electropherogram, probably due to the ambiguity related to the identity of the previous, 95th nucleotide residue. There is a clear trace for G = guanine nucleotide (black) at the 96th nucleotide residue.

Furthermore, it is unclear why the 92nd and 93rd nucleotides were called an "N", in the GA electropherogram, as a clear black trace (representing a G = guanine) is present at the 92nd nucleotide and a clear blue trace (representing a C = cytosine) is recorded at the 93rd nucleotide.

Protocol references

1. Subbarayan PR, Sarkar M, Ardalan B. Isolation of genomic DNA from human whole blood. BioTechniques. 2002 Dec;33(6):1231, 1234.

2. Sconce EA, Khan TI, Wynne HA, Avery P, Monkhouse L, King BP, et al. The impact of CYP2C9 and VKORC1 genetic polymorphism and patient characteristics upon warfarin dose requirements: proposal for a new dosing regimen. Blood. 2005 Oct 1;106(7):2329–33.

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