Detection of Single Nucleotide Polymorphisms Associated with Venous Thrombosis Using Multiplex Minisequencing Reaction

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Abstract: Venous thrombosis (VT) is a multifactorial disease with an interplay of acquired and genetic thrombotic risk factors. In this paper we describe technique referred to as a multiplex minisequencing reaction, which consist of analyses of four single nucleotide polymorphism (SNP) that predispose to thrombosis. Hereditary risk factors detected by the multiplex minisequencing reaction include G1691A mutation in factor V gene called Factor V Leiden (FVL), prothrombin gene G20210A mutation and MTHFR (methylentetrahydrofolate reductase) gene C677T and A1298C mutation.

Key-Words: Minisequencing, SNP, Thrombophilia, Risk factors, Genotyping, Venous thrombosis

1 Introduction

Venous thrombosis (VT) is a multifactorial disease with an interplay of acquired and genetic thrombotic risk factors. In this paper we describe technique referred to as a multiplex minisequencing reaction, which consist of genotyping of four single nucleotide polymorphism (SNP) that predispose to thrombosis.

The G1691A mutation in the factor V gene called factor V Leiden (FVL) is the most common genetic risk factor for deep vein thrombosis. The consequence of this mutation is disorder in hemostasis - activated protein C resistance (APCR). Activated protein C (APC) is a natural anticoagulation molecule [1,2]. Heterozygotes for FVL mutation have 3-7-fold increased risk of venous thrombosis, homozygotes are characterized by 50-100-fold increased risk. [3,4]. Female carriers of FVL are at higher risk of obstetric complications, such as pre-eclampsia, eclampsia, recurring pregnancy loss, abruption of the placenta, and intrauterine retardation of the fetal growth [3]. The frequency of FVL in the studied healthy populations varies from 3% to 12% in Europe. Prevalence of FVL among patients with venous thromboembolic disease is about 21% [3].

The G20210A mutation in prothrombin gene is due to a G to A transition in a nucleotide position 20210 in the prothrombin gene. One of the consequences of such a mutation is an increased concentration of prothrombin in plasma (>1.15 j/ml) [5]. The F2 G20210A polymorphism is common in Europe with a prevalence of 1.7-3% amongst healthy individuals. In the presence of the positive family history prevalence is increased 6.2-18% [6]. Heterozygotes for G20210A mutation is 3.5-fold increased risk of venous thrombosis [4].

Two common non-synonymous variants, the C677T (Ala222Val) and A1298C (Glu429Ala) MTHFR (methylentetrahydrofolate reductase) gene are associated with a decreased enzymatic activity. The C677T (Ala→Val) mutation in MTHFR gene is responsible for a high level of homocysteine. Hyperhomocysteinemia is a risk marker for venous thrombosis but with significantly lower than FVL and G20210A prothrombin mutation [7]. Furthermore, the C677T mutation in MTHFR gene increases the risk associated with FVL [8].

Genetic analysis of these variants has become a standard in thromboembolic diagnostics. The most common methods used to determine genotypes are Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP) and allele specific oligonucleotide hybridization (ASO) [9]. Minisequencing method is based on primer extending by one fluorescently labeled dideoxynucleotide (ddNTP) complementary to amplicon sequence. The multiplex minisequencing reaction is excellent solution for fast detection of any SNP in single assay. SNaPshot reaction also require less DNA and PCR amplicons and is also simple and quickly in increasing use method [10].
2 Material and methods

The venous blood samples was collected in two EDTA tubes from patients with thromboembolic incident. DNA was isolated using QIAamp® DNA Mini Kit (Qiagen) Kit according to the manufacturer’s recommendations. The next stage was multiplex polymerase chain reaction (PCR) using Multiplex PCR Kit (Qiagen). PCR was carried out according to manufacturer’s instructions, but the final reaction volume was reduced to 10µl. The sequences of used PCR primers are present in Table 1. All used primers were tested in singleplex reactions. The thermal cycling consisted of a first denaturation step at 95°C for 15 min, followed by 32 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 90 s, and extension at 72°C for 90 s and with a final extension at 72°C for 10 min. The products were purified by ExoSAP - exonuclease I (Exo) to remove leftover primers and phosphatase (SAP) to remove the dNTPs. 1,5µl ExoSAP was added to 4,5µl PCR product and incubated at 37ºC for 30 min than at 80°C for 15 min. The product of multiplex PCR were visualized by gel agarose electrophoresis in preliminary study to check amplicon size and correctness of designed primers. The multiplex minisequencing reactions were carried out using ABI PRIM® SNaPshot® Multiplex Kit. 25 cycles were applied including denaturation step (96°C, 10s.), primers attach step (57°C, 5s.) and primers extension step (60°C, 30s.) The sequences of used minisequencing reaction primers are present in Table 3. Reagents volume for minisequencing reaction was added according to manufacture’s instruction. Afterwards, the samples were purified by digestion with alkaline phosphatase to get rid of the ddNTPs. 5µl of product were purified by adding 0,5µl SAP.

Capillary electrophoresis and data analysis. 1µl of the purified products was mixed with 10 µl Hi-Di™ formamide (Applied Biosystems) and 0,35 µl of GeneScan™ 120 LIZ internal size standard (Applied Biosystems). After digestion at 95°C for 5 min the mixture was further analyzed by capillary electrophoresis on an 3130 Genetic Analyzer (Applied Biosystems) with performance-optimized polymer 4 (POP-4) (Applied Biosystems). The data were analyzed by the use of GeneMapper ID v.3.2 (Aplied Biosystems).

3 Results

We develop a multiplex minisequencing reaction for identification four hereditary risk factors: FV Leiden, G20210A mutation in prothrombin gene, C677T and A1298C mutation in MTHFR gene. The synthetic standard, which consist of wild homozygotes, a heterozygotes and mutant homozygotes for these mutation , produced the same results, which confirmed the validity of the method. Fig.1 and Fig.2 presents the results of synthetic control INTROL ™ Thrombosis Genotype Panel (Maine Molecular Quality Controls, INC.) for four heterozygous. Fig.3 and Fig.4 presents the results of patient with three wild homozygous and heterozygotes Table 2. presents the size of amplicons minisequencing reaction.

Table. 1 PCR primer sequence.

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>Forward</th>
<th>Reverse</th>
<th>Amplicon size</th>
</tr>
</thead>
<tbody>
<tr>
<td>FVLeiden</td>
<td>ACAGTGACGTTGACATCATGAGAG</td>
<td>GGAGACCTAACATGTTCTAGCCAG</td>
<td>166 bp</td>
</tr>
<tr>
<td>G20210A prothrombin</td>
<td>TATTCTGGGCTCTGGGAACCAATC</td>
<td>CAGAGAGCTGCCCATGAATAGCAC</td>
<td>123 bp</td>
</tr>
<tr>
<td>MTHFR C677T</td>
<td>CTGAAGCACTTGAAGGAGAAGGTG</td>
<td>GCCTTCACAAGCCGGAAGATGTG</td>
<td>95 bp</td>
</tr>
<tr>
<td>MTHFR A1298C</td>
<td>CCTCTTCATCCAAGAGCAAGCTC</td>
<td>CTCCAGCATCACTCATTGTGAC</td>
<td>154 bp</td>
</tr>
</tbody>
</table>

Table.2 Amplicon size minisequencing reaction [bp].

<table>
<thead>
<tr>
<th>Polymorphism</th>
<th>FII F</th>
<th>FII R</th>
<th>FVL F</th>
<th>FVL R</th>
<th>MTHFR C677T F</th>
<th>MTHFR C677T R</th>
<th>MTHFR A1298C F</th>
<th>MTHFR A1298C R</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild alleles [bp]</td>
<td>G 29</td>
<td>C 36</td>
<td>G 25</td>
<td>C26,5</td>
<td>C 31</td>
<td>G 40</td>
<td>A 49</td>
<td>G 49</td>
</tr>
</tbody>
</table>
Table 3. Minisequencing reaction primer sequence.

<table>
<thead>
<tr>
<th>polymorphism</th>
<th>Minisequencing reaction primer sequence (5’→3’)</th>
<th>Forward</th>
<th>Reverse</th>
</tr>
</thead>
<tbody>
<tr>
<td>FVLeiden</td>
<td>GAGCAGATCCCTGGACAGGC</td>
<td>CAAGGACAAATACCTGTATTCCT</td>
<td></td>
</tr>
<tr>
<td>G20210A</td>
<td>ATGGTTCCCAATAAAAATGGACTCTCAGT</td>
<td>TGCCCATGAATAGCACTGGGAGCATTGAGGCT</td>
<td></td>
</tr>
<tr>
<td>prothrombin</td>
<td>G20210A</td>
<td>ATGGTTCCCAATAAAAATGGACTCTCAGC</td>
<td></td>
</tr>
<tr>
<td>gene</td>
<td>MTHFR C677T</td>
<td>GACTAGCACCTTGAGAAGGAGTCTCTGAGGGAG</td>
<td>(GACT)(_n)TCAAAAGAAAAGCTGATGATGAAATCG</td>
</tr>
<tr>
<td></td>
<td>MTHFR A1298C</td>
<td>(GACT)(_n)GAAGATGTGGGGGGAGGAGCTAGAGACACTT</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1 Electroferogram of synthetic control of heterozygous for FVL, prothrombin G20210A mutation and MTHFR gene C677T and A1298C mutation, forward

Fig. 2 Electroferogram of synthetic control of heterozygous for FVL, prothrombin G20210A mutation and MTHFR gene C677T and A1298C mutation, reverse

Fig. 3 Electroferogram of wild homozygous for FVL, prothrombin G20210A mutation, MTHFR gene C677T and heterozygotes for A1298C mutation, forward

Fig. 4 Electroferogram of wild homozygous for FVL, prothrombin G20210A mutation, MTHFR gene C677T and heterozygotes for A1298C mutation, reverse
Conclusion

Method enable to detect several SNPs in one analysis is very useful to rapid identification risk factors for venous thrombosis. Patients with first episode of deep vein thrombosis are usually treat with oral anticoagulation for six weeks to six months. The frequency of recurrence can by 12 to 18 % after 2 years. 5% of these patients dies from pulmonary embolism [11]. Genetic polymorphisms can considerably increase chance of another episode of venous thrombosis. These cases should be taken intro consideration, do if the patients with genetic risk factors should be treat with oral anticoagulation to the rest of they life. All methods creating multiple genetic markers in a single assay reduce costs of molecular tests. Unquestionable advantages of this method is possibility to multiplex. Simultaneous analysis of two strands of DNA confirm validity of obtained results.

SNaPshot technique is successful applied in routine work in our laboratory for detection of four thrombosis risk factors.

References:


