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Modified method for analysis of InDel *ACE1* gene polymorphism using the temperature melting curves of PCR products

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Aim. The *ACE1* gene is a key to the renin-angiotensin system, as it encode the ACE1 enzyme, which regulates blood pressure and water-electrolyte balance. The InDel (I/D) polymorphism of the *ACE1* gene causes a different level of enzyme synthesis and is associated with various pathological conditions, including the course of COVID-19. The aim of our study was to develop an efficient method for the analysis of I/D polymorphism of the *ACE1* gene by analyzing the melting temperature curves of specific PCR products. **Methods.** Genotyping was carried out by the PCR method using DNA isolated from peripheral blood leukocytes. The primers were designed using BLAST SEARCH and synthesized at METABION (Germany). The reaction was carried out using the "HOT FIREPol EvaGreen qPCR Mix Plus" mixture. Amplification was performed on iQ5TM Multicolor Real-Time PCR Detection System (BIO-RAD), melting curves were analyzed using BIO-RAD iQ5 Optical System Software V 2.0. **Results.** The proposed method, using one pair of primers, turned out to be effective for detecting genotypes based on the melting temperature curves of PCR products: the peak at 83–85 °C corresponded to deletion, at 86–89 °C to insertion, and in the presence of both peaks, the genotype was defined as heterozygous. **Conclusions.** The proposed method of genotyping the I/D polymorphism of the *ACE1* gene is simple and reliable, and can be used for the analysis of genetic markers of cardiovascular pathology and the prognosis of the course of COVID-19.

Keywords: ACE1, InDel, melting temperature curve analysis.

Introduction

The *ACE1* gene plays a pivotal role in the renin-angiotensin system (RAS), encoding the ACE enzyme, which converts angiotensin I to angiotensin II — a potent vasoconstrictor that elevates blood pressure and stimulates aldosterone secretion, thereby regulating water and electrolyte balance [1]. The most common I/D polymorphism of the *ACE1* gene is defined by the presence or absence of an Alu sequence in the 16th intron. This polymorphism influences the plasma ACE level, modulating pathophysiological processes via the RAS and the kinin-kallikrein system [2, 3]. The expression level of ACE1 varies depending on the genotype: DD — homozygote for Alu deletion (allel D) exhibit the highest ACE level, while II — homozygote for Alu insertion (allel I) have the lowest [3]. Moreover, this polymorphism serves as a marker of cardiovascular disease risk and treatment efficacy and is associated with the severity of COVID-19 [2, 4]. Our study aimed to develop an efficient and simple method for detecting the InDel polymorphism in *ACE1* gene using the analysis of PCR products melting temperature curves.

Methods

The DNA samples from peripheral blood leukocytes were isolated with the Nucleic Acids Extraction Kit "U-Prep-100" (RECLICON LLC, Poland).

Specific oligonucleotide primers were designed using the BLAST search tool and Vector NTI software (Invitrogen). The synthesis of specific oligonucleotides was carried out by METABION (Germany). PCR was conducted using the pre-formulated "HOT FIREPol EvaGreen qPCR Mix Plus" (Solis BioDyne, Estonia), which includes the intercalating dye EvaGreen and HOT FIREPol Taq DNA polymerase. To enhance amplification efficiency and reaction specificity, the betaine solution (Sigma-Aldrich) was added to the reaction mixture to lower the melting point of the PCR product. The amplification and subsequent analysis of the melting temperature curve were performed using the iQ5TM Multicolor Real-Time PCR Detection System (BIO-RAD, USA), and data were analyzed with the BIO-RAD iQ5 Optical System Software V 2.0 (2006).

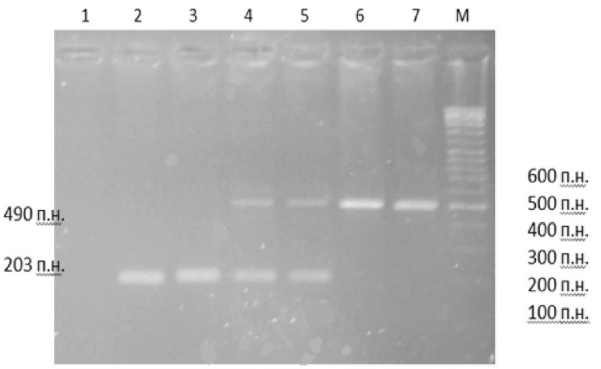

Fig. 1. Electrophoregram of the separation of PCR fragments of the *ACE1* gene product (I/D) in a 2% agarose gel: 1 — negative control; 2, 3 — homozygote for allele I (II); 4, 5 — heterozygote (I/D); 6, 7 — homozygote for allele D (DD); *M* — molecular weight marker (Ladder 100 b.p.)

Table 1. **The primers used in PCR reactions**

ACE1 Forward	5` CTG GAG AGC CAC TCC CAT CCT TTC T 3`
ACE1 Reverse	5` GAC GTG GCC ATC ACA TTC GTC AGA T 3`

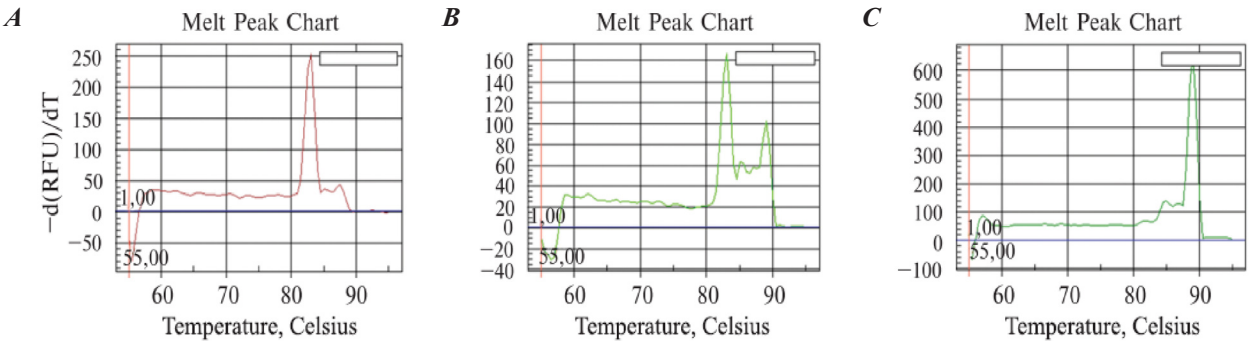

Fig. 2. Melting temperature curves of PCR products for different genotypes: (A) DD homozygote, (B) DI heterozygote, (C) II homozygote

Results

The primer design targets the I/D sequence by positioning it between the flanking sequences. The primer sequences are presented in Table 1.

To identify the DNA samples with different *ACE1* gene I/D genotypes, we have performed an endpoint analysis for detecting defferent PCR products. The PCR reaction was carried out in a final volume of 20 μL, consisting of 3 μL DNA (50ng), 10 pmol of each oligonucleotide primer, 4 μL "HOT FIREPol EvaGreen qPCR Mix Plus" 4 μL 5M betaine solution. The optimal temperature and time conditions were as follows: initial denaturation at 94 °C for 12 minutes, followed by 35 cycles of denaturation at 94 °C for 1 minute, annealing at 64 °C for 1 minute, extension at 72 °C for 1 minute, and a final elongation step at 72 °C for 2 minutes. Amplicons were differentiated based on their length and GC content.

Then, the aliquots (5 μL) containing each amplicon were subjected to the electrophoretic separation on an 2% agarose gel, stained with the intercalating dye ethidium bromide, and visualized using a transilluminator (Fig. 1). Electrophoresis was performed for 35 minutes at 260 V and 400 mA. The 2-log DNA Ladder from Thermo Fisher, with a range of 100 to 1000 bp in 100 bp increments, served as a molecular weight reference.

After amplification was completed, the melting curve analysis was performed by cooling the reaction to 55 °C and then heating slowly to 95 °C. The melting curve and fluorescent signals were analyzed with BIO-RAD iQ5 Optical System Software V 2.0 (2006).

Comparetive test results indicated that the curve peak between 83 °C and 85 °C corresponded to a deletion, while the peak between 86 °C and 89 °C indicated an insertion. The presence of both peaks signified a heterozygous genotype (Fig.2). The reference DNA samples produced consistent and reproducible genotyping results, confirming the method's efficacy.

In our study we have developed a validated version of the method for analyzing the InDel polymorphism of the *ACE1* gene using the melting temperature curves of PCR products. Similar methodologies have been employed by other researchers. For instance, Nissen *et al.*

applied a three-primer approach in RT-PCR for genotyping Danish patients, but the procedure looks like more complicated, than our Protocol [5]. It is necessary to know that the efficiency of melting curve analysis was shown not only for InDel variants but for SNP genotyping [6]. The similar method has been proposed recently for the investigation of impact of *ACEI* gene insertion/deletion polymorphisms on COVID-19 severity [7].

The technic proposed in our study was used for investigation the association of *ACE1* I/D polymorphism with the clinical course of COVID-19 in pediatric patients [8]. Therefore, we propose this version of method as a valuable tool for the analysis of *ACE1* I/D polymorphism as a genetic marker not only of cardiovascular disorders but also of susceptibility to COVID-19.

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Модифікований метод аналізу поліморфізму I/D гена ACE1 з використанням температурних кривих плавлення продуктів ПЛР

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Мета. Ген *ACE1* є ключовим у роботі ренін-ангіотензинової системі, оскільки він кодує фермент ACE1, який регулює артеріальний тиск та водно-електролітний баланс. Поліморфізм I/D гена *ACE1* зумовлює різний рівень синтезу фермента і є асоційованим із різними патологічними станами, включаючи перебіг COVID-19. Метою нашого дослідження було розробити ефективний метод для аналізу індел-поліморфізму гена *ACE1* за допомогою аналізу температурних кривих плавлення специфічних продуктів ПЛР. **Методи.** Генотипування здійснювали методом ПЛР з використанням ДНК, виділеної з лейкоцитів периферійної крові. Праймери розробили за допомогою

BLAST SEARCH та синтезували у компанії METABION (Німеччина). Реакцію проводили з використанням суміші "HOT FIREPol EvaGreen qPCR Mix Plus". Ампліфікацію виконували на iQ5TM Multicolor Real-Time PCR Detection System (BIO-RAD), аналізували криві плавлення за допомогою BIO-RAD iQ5 Optical System Software V 2.0. **Результати.** Запропонований метод, із використанням однієї пари праймерів, виявився ефективним для детекції генотипів на основі кривих температури плавлення продуктів ПЛР: пік при 83–85 °C відповідав делеції, при 86–89 °C — інсерції, а за наявності обох піків генотип визначали як гетерозиготний. **Висновки.** Запропонований метод генотипування I/D-поліморфізму гена *ACE1* є простим і надійним, може бути використаний для аналізу генетичних маркерів серцево-судинної патології та прогнозу перебігу COVID-19.

Ключові слова: ACE1, InDel, аналіз кривих температури плавлення.

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