jraas

Journal of the Renin-Angiotensin-Aldosterone System
12(3) 129–132

DOI: 10.1177/1470320310381911
jra.sagepub.com
SAGE

Improvement in *ACE* I/D polymorphism detection

Paola Bolli, Elena Sticchi, Betti Giusti, Claudia Saracini, Rosanna Abbate and Cinzia Fatini

Abstract

The *ACE* I/D polymorphism has been reported to influence predisposition to cardiovascular disease. Conflicting results in its detection may be due to mistyping of I/D genotypes as D/D genotypes occurring in the traditional genotyping method. In order to resolve mistyping troubles and to permit a rapid and accurate analysis, we performed a stepdown PCR reaction followed by detection using Nanogen technology, and we compared these results with those obtained from traditional genotyping methods, such as conventional and confirmatory PCR. The Nanogen stepdown method showed a 100% sensitivity and 99.6% specificity, when compared with the confirmatory PCR. Our experiments provide evidence that, by using the Nanogen stepdown method, the DD mistyping was markedly decreased, thus representing a useful tool suitable for performing large-scale screening or research.

Keywords

Angiotensin-converting enzyme ID polymorphism, Nanogen technology, polymorphism detection, stepdown PCR

Introduction

The ID polymorphism, in the gene encoding for angiotensin-converting enzyme (*ACE*), consists of the insertion (I) or deletion (D) of an Alu-type sequence in intron 16 of the gene. It has been reported that the D allele modulates serum levels of circulating enzyme with a dose-dependent effect,[1] and represents a predisposing factor to cardiovascular disease.[2] Data from genetic association studies have reported conflicting results about the strength of this association, probably due to a different genetic background of populations or different number of samples analyzed. However, it has also been hypothesized that the conflicting results may be attributed to methodological and technical variations in detecting the polymorphisms.[3]

In particular, conventional PCR is known to have a tendency to preferentially amplify the D allele in comparison to the I allele in a competitive amplification reaction, when both alleles are present (ID genotype).[4] This leads to mistyping of ID genotypes as DD in approximately 4–5% of the samples. To avoid any mistyping of ID as DD, a confirmatory PCR amplification using insertion-specific primers is performed for all homozygotes for the D allele;[5] this analysis, performed by agarose gel electrophoresis, highlights the presence of amplified products only in the presence of the I allele. Nevertheless, unamplified samples may also be associated with problems in the PCR reaction, rather than from the absence of the insertion allele, thus generating doubts in genotyping results.

In order to resolve mistyping problems, Chiang and colleagues[6] tested a stepdown PCR method in 60 patients with cardiovascular disease. This new method requires an initial PCR annealing temperature higher than the melting point of the primers, followed by annealing temperatures reduced stepwise to the melting point, thus resulting in higher amplification specificity. The development of a rapid and accurate genotyping technique, performed to analyze the *ACE* I/D polymorphism, may be relevant in both clinical and epidemiological studies.

Aims of our study were: (1) to perform the *ACE* I/D polymorphism detection through the stepdown PCR method[6] applied to a high-throughput technology such as electronic microchip technology (Nanogen technology) and; (2) to compare genotyping results from this method with those from conventional and confirmatory PCR.

Department of Medical and Surgical Critical Care and Center of Research, Transfer and High Education, 'DENOTHE', University of Florence, Italy

Corresponding author:
Paola Bolli, Department of Medical and Surgical Critical Care and Center of Research, Transfer and High Education, 'DENOTHE', University of Florence, Viale Morgagni 85, 50134 Florence, Italy.
Email: paobolli@hotmail.com

*CTGGAGAGCCACTCCCATCCTTTCT*CCCATTTCTCTAGACCTGCTGCCTATACAG
TCACTTTT[**TTTTTTTTTTTTGAGACGGAGTCTCGCTCTGTCGCCCAGG**
CTGGAGTGCAGTGGCGGGATCTCGGCTCACTGCAAGCTCCGCCTC
CCGGGTTCACGCCATTCTCCTGCCTCAGCCTCCCAAGTAGCTGGGA
CCACAGGCGCCCGCCACTACGCCCGGCTAATTTTTTGTATTTTTAG
TAGAGACGGGGTTTCACCGTTTTAGCCGGGATGGTCTCGATCTCCT
GACCTCGTGATCCGCCCGCCTCGGCCTCCCAAAGTGCTGGGATTA
CAGGCGTGATACAGTCACTTTT]ATGTGGTTTCGCCAATTTTATTCCAGCT
CTGAAATTCTCTGAGCTCCCCTTACAAGCAGAGGTGAGCTAAGGGCTGGAGC
TCAAGSCATTCAAMCCCCTACCAG*ATCTGACGAATGTGATGGCCACRTC*

Figure 1. Corrected *ACE* gene sequence (in bold insertion sequence (Alu), in italics and underlined primer sequence).

Material and methods

In order to perform the analysis, we collected 426 blood samples from patients with abdominal aortic aneurysm. All subjects gave informed consent and the study complies with the Declaration of Helsinki and was approved by the local ethics committee.

First, we carried out a stepdown PCR by using a PCR protocol modified from that reported by Chiang[6] in order to reduce differences between D and I allele amplification intensity as far as possible. Moreover, as we observed some differences in the *ACE* gene sequence in close proximity to the I/D polymorphism (rs4646994), we designed two novel primers, different from those reported by Rigat[4] (Figure 1) and also used in the stepdown protocol from Chiang[6] (Table 1). Additionally, the insertion sequence (Alu) was 289 bp long, following Villar,[7] and not 287 bp, as Rigat described.[4]

The stepdown PCR reaction was performed in a final volume of 25 µL containing 100 ng of genomic DNA, 0.7 µl of 10 µM of each primer, 0.2 mM of each dNTP, 1.5 µl $MgCl_2$ (25 mM), 1.25 µl DMSO (5%) and 0.5 units of Taq DNA polymerase (GoTaq, Promega) in a 1× PCR reaction buffer. The amplification was performed by a PCR reaction in an MJ thermocycler (MJ Research) by denaturing first at 95°C for 5 minutes and then repeating the following cycle: denaturing at 95°C for 1 minute, annealing at 70°C for 1 minute and extension at 72°C for 1 minute. This cycle was repeated 5 times before reducing the annealing temperature to 65°C and then to 60°C (each cycle repeated 5 times) and then to 60°C for 1 minute 5 seconds for 25 cycles, with a final extension at 72° for 10 minutes.

Before proceeding further with the microarray analysis (NanoChip® Molecular Biology Workstation; 10 × 10 NanoChip cartridge; Nanogen), we sequenced the PCR product, in order to verify its good quality (ABI310 automated sequencer).

After the amplification, the PCR products were desalted with Nucleo Fast System (96-well plates) from Macherey-Nagel. A 30 µl volume from purified samples was mixed with 30 µl of 100 mM histidine and placed in a 96-well Nunc plate (Nalge Nunc International). A 0.3 M NaOH solution, required for the denaturation of the PCR product, a 50 mM histidine solution, required for background subtraction, and three heterozygous control samples, for the normalization, were also added to the plate. The samples, prepared as described previously[8], according to NanoChip guidelines, were electronically addressed to the pads on the cartridge. After addressing the amplicons on the array, the chip was rinsed with 150 µl of high salt buffer (50 mM sodium phosphate and 500 mM sodium chloride, pH 7.4) for 3 minutes and next with 100 µl hybridization mix containing the D-allele probe (5'-Cy3-TGACTGTATATAGGCA-3') and I-allele probe (5'-Cy5-GTGACTGTATCACG-3') was added before loading the cartridge into the instrument for the fluorescence scanning at the specific discrimination temperature. The mutation analysis was performed with software that calculates the fluorescence values from each sample. The fluorescence signal ratio, deriving from both wild-type and mutant labelled probes, after fluorescence signal normalization by a heterozygous control sample, allows the genotype discrimination.[9]

Results

The genotyping results generated by the Nanogen technology were compared with those obtained by the conventional and confirmatory PCR.

By the conventional PCR method, 180 patients were genotyped as DD, 186 as ID and 54 as II. With the confirmatory PCR, 11 (6.1%) mistyped DD samples have been demonstrated (Table 2). The stepdown method, applied to the Nanogen technology, showed one conflicting sample (0.2%) in comparison with the confirmatory PCR (K = 0.995, agreement test for the stepdown-Nanogen method

Table 1. Traditional and new primers.

Traditional Primers	Sequence	Primer size	CG content	Amplicon size
Forward	5'-CTGGAGACCACTCCCATCCTTTCT-3'	24	54%	482
Reverse	5'-GATGTGGCCATCACATTCGTCAGAT-3'	25	48%	
New Primers	**Sequence**	**Primer size**	**CG content**	**Amplicon size**
Forward	5'-CTGGAGA**G**CCACTCCCATCCTTTCT-3'	25	56%	483
Reverse	5'-GA**Y**GTGGCCATCACATTCGTCAGAT-3'	25	48%	

Table 2. Genotype distribution of the *ACE* gene in 420 patients using three PCR methods.

PCR Method	DD	ID	II	*p*-value*
Traditional PCR	180	186	54	0.492
Confirmatory PCR	169	197	54	0.99
Stepdown PCR-Nanogen technology	170	196	54	0.99

*Hardy–Weinberg equilibrium

and confirmatory PCR). In order to evaluate if this conflicting result (ID in the confirmatory PCR vs DD in the stepdown-Nanogen method) was due to mistakes in the PCR protocol or to difficulties in the normalization process of the microarray analysis, we proceeded to the 'mistyped sample' stepdown PCR amplification and gene sequencing (ABI310 automated sequencer). The sequencing analysis revealed an ID genotype, so demonstrating that the 'mistyped result' might be due to a failure in the fluorescence normalization and not to the stepdown PCR protocol.

Discussion

Although the difference in genotype distribution between conventional PCR and stepdown PCR applied to Nanogen technology ($p = 0.76$) was not statistically significant, DD mistyping was markedly decreased in the Nanogen stepdown method in comparison to conventional PCR.

Moreover, when evaluating the Hardy–Weinberg equilibrium (HWE), required as a quality-control measure, in the conventional, confirmatory and stepdown PCR-Nanogen analysis groups, we did not detect deviation from HWE (Table 2).

The analytical accuracy of the developed Nanogen stepdown method has also been evaluated. The Nanogen stepdown method shows a 100% sensitivity and 99.6% specificity, when compared with the results from confirmatory PCR.

In addition, the stepdown PCR followed by Nanogen detection reduces the technician hands-on-time in comparison to the traditional genotyping method. Moreover, the liquid handling in all the reaction protocols was supported by the Biomek FX Workstation (Beckman Coulter, Fullerton, CA).

A limit of Nanogen technology is that it performs the normalization step by comparing the fluorescence values with those derived from heterozygous controls in order to assess the genotype discrimination, and thus it requires templates with a good intensity and good homogeneity between the alleles amplified in the heterozygous samples.

The stepdown method, modified from that described by Chiang[6] in order to limit as far as possible the heterogeneity in the intensity of the amplified alleles, has been shown to be more adaptable to electronic microchip technology than traditional PCR, since it improves the quality of templates. Indeed, traditional PCR produces templates with uneven intensity, because of the preferential amplification of the D allele.

Conclusions

In conclusion, our results demonstrate the reliability of *ACE* I/D detection through Nanogen technology, carried out after the amplification of the samples with a stepdown method, modified from that described by Chiang.[6] However, we are aware that small research labs may not be equipped with Nanogen technology. In such a case, the stepdown PCR that we suggest, followed by confirmatory PCR, should become the preferred method for the analysis of the *ACE* I/D polymorphism.

Stepdown PCR followed by Nanogen detection avoids the need for confirmatory PCR, so limiting the difficulties and uncertainties inherent in genotyping results; moreover, the application of Nanogen technology does not require the use of hazardous reagents, such as ethidium bromide, necessary for agarose gel preparation and permits high-throughput polymorphism detection.

Owing to the relevance of *ACE* I/D polymorphism detection in clinical and epidemiological studies, the development of a genotyping technique, more rapid and accurate than the traditional detection method, such as stepdown PCR applied to Nanogen technology, might provide a suitable tool for performing large-scale screening or research.

Funding

This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

Conflict of interest statement

None declared.

References

1. Cambien F, Poirier O, Lecerf L, Evans A, Cambou JP, Arveiler D et al. Deletion polymorphism in the gene for angiotensin-converting enzyme is a potent risk factor for myocardial infarction. *Nature* 1992; 359: 641–644.
2. Samani NJ, Thompson JR, O'Toole L, Channer K and Woods KL. A meta-analysis of the association of the deletion allele of the angiotensin-converting enzyme gene with myocardial infarction. *Circulation* 1996; 94: 708–712.
3. Ueda S, Heeley RP, Lees KR, Elliott HL and Connell JM. Mistyping of the human angiotensin-converting enzyme gene polymorphism: frequency, causes and possible methods to avoid errors in typing. *J Mol Endocrinol* 1996; 17: 27–30.
4. Rigat B, Hubert C, Corvol P and Soubrier F. PCR detection of the insertion/deletion polymorphism of the human angiotensin converting enzyme gene (DCP1) (dipeptidyl carboxypeptidase 1). *Nucleic Acids Res* 1992; 20: 1433

5. Ribichini F, Steffenino G, Dellavalle A, Matullo G, Colajanni E, Camilla T et al. Plasma activity and insertion/deletion polymorphism of angiotensin I-converting enzyme: a major risk factor and a marker of risk for coronary stent restenosis. *Circulation* 1998; 97: 147–154.
6. Chiang FT, Hsu KL, Chen WM, Tseng CD and Tseng YZ. Determination of angiotensin-converting enzyme gene polymorphisms: stepdown PCR increases detection of heterozygotes. *Clin Chem* 1998; 44: 1353–1356.
7. Villar J, Flores C, Pérez-Méndez L, Maca-Meyer N, Espinosa E, Blanco J et al. Angiotensin-converting enzyme insertion/deletion polymorphism is not associated with susceptibility and outcome in sepsis and acute respiratory distress syndrome. *Intensive Care Med* 2008; 34: 488–495.
8. Frusconi S, Giusti B, Rossi L, Bernabini S, Poggi F, Giotti I et al. Improvement of low-density microelectronic array technology to characterize 14 mutations/single-nucleotide polymorphisms from several human genes on a large scale. *Clin Chem* 2004; 50:775-777.
9. Sticchi E, Lenti M, Giusti B, Sestini I, Abbate R and Fatini C. LPA +93C>T and +121G>A polymorphisms detection by electronic microchip technology. *Mol Genet Metab* 2007; 91: 79–84.