Genotyping of the Angiotensin I-Converting Enzyme Gene Insertion/Deletion Polymorphism by the TaqMan Method, *Werner Koch,* Wolfgang Latz, Marianne Eichinger, Claudia Ganser, Albert Schömig, and Adnan Kastrati* (Deutsches Herzzentrum München and 1. Medizinische Klinik, Klinikum rechts der Isar, Technische Universität München, Munich, Germany; * address correspondence to this author at: Deutsches Herzzentrum München, Lazarettstrasse 36, D-80636 Munich, Germany; fax 49-89-1218-3053, e-mail wkoch@dhm.mhn.de)

Intron 16 of the angiotensin I-converting enzyme gene (*ACE*) contains an insertion/deletion (I/D) polymorphism that is characterized by the presence (I allele) or absence (D allele) of a 289-bp incomplete *alu* type repeat sequence *(1, 2)*. The D allele has been related to higher concentrations of *ACE* mRNA in cells and increased ACE concentration and activity in plasma and serum *(1, 3–6)*. There is great continuing interest in the link between the *ACE* I/D polymorphism and interindividual variations in physiologic properties and disease susceptibility. Reported associations include physical activity and endurance, drug response, and neuropathologic, cardiac, and cardiovascular diseases *(7–20)*. However, a considerable number of findings are in disagreement with the existence of such relationships *(21–31)*, and more work is required before the role of the *ACE* I/D polymorphism in health and disease can be firmly established.

Conventional genotyping of the *ACE* I/D polymorphism involves PCR, using primers that flank the insertion sequence, and exploits the different migration velocities of I- and D-allele–specific PCR products during electrophoresis in gel matrices *(32)*. Here we describe an assay for genotyping of the *ACE* I/D polymorphism that is based on the TaqMan technique *(33)*. The TaqMan method involves 2 allele-specific fluorogenic oligonucleotide probes and combines DNA amplification and genotype detection in a single assay. Usually, TaqMan genotyping assays are designed and used for the analysis of single-nucleotide polymorphisms or small insertions/deletions *(34–36)*. The new TaqMan assay for the *ACE* I/D polymorphism extends the scope of the TaqMan genotyping technique to the analysis of a polymorphism that is distinguished by the presence or absence of a relatively large sequence block. Special to this assay is the requirement of 3 different PCR primers to obtain the 2 types of allele-specific PCR products, instead of the 2 primers used in conventional TaqMan assays. The binding sites of primers and probes on the I- and D-allele–specific DNA templates are shown in Fig. 1A. Primer pair AC111/ACE112 gave rise to a D-allele–specific PCR product of 78 bp, and primer pair AC112/ACE113 gave rise to an I-allele–specific PCR product of 71 bp. The 367-bp–long I-allele–specific amplicon produced by primer pair AC111/ACE112 was not detectably multiplied in TaqMan reactions that included DNA of genotype II or ID, as indicated by sample analysis using electrophoresis in a polyacrylamide gel and subsequent treatment of the gel with ethidium bromide solution. Allele-specific signaling

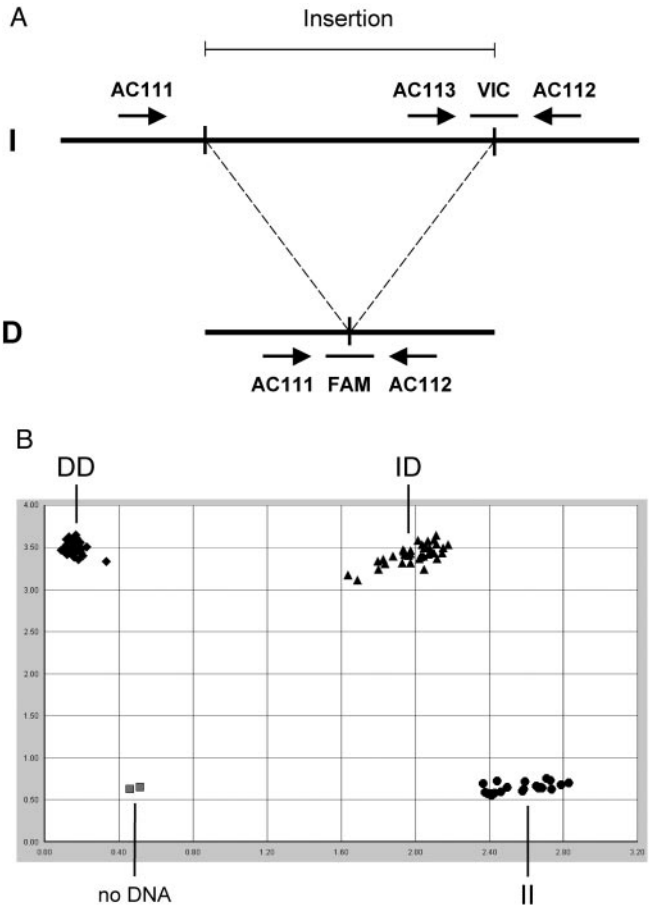

Fig. 1. Genotyping of the *ACE* I/D polymorphism with the TaqMan method.

(*A*), schematic presentation of the binding sites of primers ACE111, ACE112, and ACE113 and probes VIC-AC100 (*VIC*) and FAM-AC100 (*FAM*) in the I allele (*I*) and D allele (*D*) of *ACE*. The insertions were found to be 288 bp long (GenBank and EMBL accession nos. A28005 and X62855) or 289 bp long (AF118569 and our own sequence analysis). The D-allele–related sequence has been deposited under the accession number AY436326. (*B*), screen shot showing a typical genotyping result, as obtained on the ABI Prism 7000 Sequence Detection System after completion of the cycling reactions. The graphic represents a read-out of a 96-well microtiter plate, which included, at the outset of the experiment, 90 reactions with DNA samples of unknown genotype and, as controls, reactions containing no DNA (2 wells), DNA of the II genotype (2 wells), and DNA of the DD genotype (2 wells).

of the probes was accomplished by the reporter dyes VIC (proprietary dye of Applied Biosystems) and 6-carboxyfluorescein (FAM), which were attached to the 5′ ends of the I- and D-allele–specific oligonucleotides, respectively. Minor groove binder (MGB) groups were conjugated with the 3′ ends of the oligonucleotides to facilitate formation of stable duplexes between the probes and their single-stranded DNA targets *(37)*. Primers and probes were synthesized by Applied Biosystems and had the following structures:

Primer ACE111: 5′-CCCATCCTTTCTCCCATTTCTC-3′
Primer ACE112: 5′-AGCTGGAATAAAATTGGCGAA-AC-3′
Primer ACE113: 5′-CCTCCCAAAGTGCTGGGATTA-3′

I-Allele–specific probe (VIC-ACE100): VIC-5′-AGGCG-TGATACAGTCA-3′-MGB
D-Allele–specific probe (FAM-ACE100): FAM-5′-TGC-TGCCTATACAGTCA-3′-MGB

Genomic DNA was extracted from peripheral blood leukocytes by use of the NucleoSpin Blood Quick Pure reagents (Macherey-Nagel). Reactions were carried out on 96-well microtiter plates. The assay volume was 22 μL, which contained 11 μL of the Absolute QPCR ROX Mix (ABgene); 150 nM each of the primers ACE111, ACE112, and ACE113; 150 nM I-allele–specific probe VIC-ACE100, 75 nM D-allele–specific probe FAM-ACE100, and 10–50 ng of DNA. The 2-step thermocycling procedure consisted of 35 cycles of denaturation at 92 °C for 15 s and primer annealing and extension at 57 °C for 1 min. After cycling on a GeneAmp PCR System 9600 or 9700 (Applied Biosystems), genotype calling was carried out on the ABI Prism 7000 Sequence Detection System (Applied Biosystems). A typical result is shown in Fig. 1B, which demonstrates the capability of the new TaqMan assay to differentiate between the *ACE* I/D genotypes. We verified the ability of the TaqMan system to provide correct genotype data by separate analysis of a limited number of samples (n = 150), using DNA sequencing or an established PCR genotyping system for the *ACE* I/D polymorphism, as described previously *(30, 38)*.

Using the new method, we determined the *ACE* I/D genotype in 1500 individuals of Caucasian origin who were included in a study designed to examine a possible association of the *ACE* I/D polymorphism with adverse events commonly occurring after interventions in coronary arteries. Written informed consent was obtained from all participating patients. The study protocol was approved by the institutional ethics committee, and the investigations were in accordance with the principles of the current version of the Declaration of Helsinki *(39)*. In this series of patients, 299 (19.9%) were homozygous II, 737 (49.1%) were heterozygous ID, and 464 (30.9%) were homozygous DD. The observed *ACE* I/D genotype distribution was in Hardy–Weinberg equilibrium ($P = 0.84$) and in accordance with results obtained in other Caucasian populations *(4, 5, 9, 15, 19, 20, 24, 26, 28, 31)*.

The widely used conventional technique for *ACE* I/D genotyping, a combination of PCR and gel electrophoresis, is prone to misclassification of ID heterozygotes as DD homozygotes because of preferential amplification of the smaller D-allele–related sequence *(28, 40, 41)*. To take into consideration the possibility of mistyping, it has been recommended samples typed as DD be subjected to a second, independent PCR including a primer pair that permits amplification only in the presence of the I allele but not the D allele *(28, 40, 41)*. With the conventional method, accurate genotyping requires several time-consuming reaction steps, repeated transfer of material, and manual data acquisition.

The new TaqMan genotyping system for the *ACE* I/D polymorphism is relatively simple to use and requires little hands-on time because it is a single-tube assay and allows for automated reaction setup, genotype determination, and data processing. Because post-PCR sample handling is not involved, the chance of sample mix-up or the possibility of carryover contamination is greatly reduced. The microtiter plate format and the option to process multiple plates in parallel make this method particularly suitable for approaches demanding high-throughput genotyping, such as clinical association studies involving large numbers of individuals. In addition, the TaqMan system for the *ACE* I/D polymorphism may serve as a prototype for genotyping assays to be designed for other polymorphisms that are characterized by large insertions/deletions.

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The factor V Leiden and prothrombin G20210A polymorphisms are established risk factors for thrombosis *(1, 2)*. General screening for these polymorphisms in persons with additional risk factors has been discussed *(3)*, but a significant proportion of familial cases with deep vein thrombosis/venous thromboembolism is not explained by carriage of either of these mutations *(4)*. There is accumulating evidence that multiple coexisting defects are present in persons with the most marked tendency to thrombosis *(5)*. The current lack of a clear consensus regarding the clinical roles for several of the additional polymorphisms studied *(1, 2)* could reflect that most studies have addressed these independently.

We developed a pyrosequencing-based genotyping protocol for parallel analysis of the β-fibrinogen (−455G/A and −854 G/A), prothrombin (G20210A), coagulation factor V Leiden (G1691; Arg506Gln), coagulation factor VII (−401G/T and −402 G/A), coagulation factor XIII (G163T; Val34Leu), plasminogen activator inhibitor-1 (*PAI-1*; −675 4G/5G), methylenetetrahydrofolate reductase (*MTHFR*; C677T; Ala222Val), glycoprotein IIIa (*GPIIIa*; C1565T; Leu33Pro; also known as PlA1/PlA2), and endothelial nitric oxide synthase (*eNOS*; G894T; Glu298Asp) polymorphisms, together with the cytochrome P450 2C9 [*CYP2C9*1* (wild type)], *CYP2C9*2* (C430T; Cys144Arg), *CYP2C9*3* (A1075C; Ile359Leu), and *CYP2C9*4* (T1076C; Ile359Thr) isoforms, which modulate the effect of warfarin in antithrombotic therapy.

To start with subnanogram amounts of genomic DNA, we developed an outer nested PCR for simultaneous amplification of 11 gene fragments covering these single-nucleotide polymorphisms (SNPs). Genomic DNA samples were arrayed in 96-well plates together with negative controls. PCR primers were designed based on available GenBank entries and searched against publicly available nucleotide databases to ensure specificity for the selected primer annealing regions. Individual primer pairs (outer