Original Article

An Alternative Fast and Convenient Genotyping Method for the Screening of Angiotensin Converting Enzyme Gene Polymorphisms

Chihiro TANAKA, Kei KAMIDE*, Shin TAKIUCHI*, Yoshikazu MIWA*, Masayoshi YOSHII*, Yuhei KAWANO*, and Toshiyuki MIYATA

Insertion/deletion (I/D) polymorphisms in intron 16 of the angiotensin converting enzyme gene (ACE) are associated with the plasma angiotensin converting enzyme (ACE) levels, and individuals with the DD allele have been reported to be more susceptible to cardiovascular disease than those without. The conventional genotyping method for the screening of I/D polymorphisms, which involves polymerase chain reaction (PCR)-gel electrophoresis, is laborious and time-consuming. In this study, we assessed the use of TaqMan-PCR genotyping for the screening of I/D polymorphisms as a replacement for the conventional method. We genotyped seven single nucleotide polymorphisms (SNPs) in linkage disequilibrium (LD) with the I/D polymorphisms, and calculated the LD coefficients of the I/D polymorphisms. We found that three polymorphisms, rs4331, rs4334 and rs4341, exhibited the highest LD coefficients ($D' = 1.000$; $r^2 = 0.967$) and that the genotyping of rs4341 by the TaqMan-PCR method yielded the best discrimination among the different genotypes. Genotyping of 511 samples took only 2 h and the amount of DNA required for each test was only 6 ng by the TaqMan-PCR method using rs4341. In the course of this study, we identified a novel additional polymorphism (a deletion of six amino acids) in exon 13, near rs4316. The deletion allele encoded the testicular ACE, but not the plasma ACE. We concluded that genotyping of the rs4341 ACE polymorphism by the TaqMan-PCR method is a fast and convenient alternative method for direct I/D genotyping. We also concluded that testicular ACE may manifest a deletion of six amino acids that may result in deleterious function of this enzyme. (Hypertens Res 2003; 26: 301–306)

Key Words: angiotensin converting enzyme, insertion/deletion polymorphism, genotyping, testicular angiotensin converting enzyme

Introduction

The angiotensin converting enzyme gene (ACE) carries a 287-base pair (bp) Alu-repeat sequence insertion/deletion (I/D) polymorphism in intron 16. This polymorphism is associated with the plasma angiotensin converting enzyme (ACE) levels (1). Individuals with the DD genotype have been reported to be more susceptible to cardiovascular disease (2–4), including myocardial infarction (5), arterial hypertension (6, 7) and left ventricular hypertrophy (8), than those with the ID or II genotype. The I/D polymorphism is generally considered to be a genetic marker for cardiovascular disease development, though controversy exists regarding this issue (9, 10). The physiological function of the I/D polymorphism has not been clarified. Intronic localization of the I/D

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polymorphism suggests that other functional polymorphisms also exert an influence on the control of ACE transcription and/or its enzymatic activity. Consequently, many studies have attempted to identify a functional variant of ACE (11, 12).

The conventional genotyping method for the screening of I/D ACE polymorphisms consists of two steps, polymerase chain reaction (PCR) and agarose gel electrophoresis, both of which are laborious and time-consuming. In addition, DD genotyping requires confirmation by insertion allele-specific amplification (13). An alternative fast, convenient and reliable genotyping method is required to replace this method for studies using large numbers of samples.

The recent sequencing of 24 kilobase (kb) of the ACE in DNA samples from six European-Americans and five African-Americans revealed 37 polymorphisms (14). Of these polymorphisms, 17 single nucleotide polymorphisms (SNPs) exhibited complete linkage disequilibrium (LD) with the I/D polymorphism, suggesting that genotyping for one of the SNPs could replace I/D genotyping. In this study, we genotyped seven ACE SNPs and identified one SNP for use in an alternative genotyping method. We also identified a novel six amino acid deletion polymorphism that may result in an alternative genotyping method. We also identified a genotyped seven SNPs could replace I/D genotyping. In this study, we genotyped seven SNPs and identified one SNP for use in an alternative genotyping method. In this study, we genotyped seven SNPs and identified one SNP for use in an alternative genotyping method.

The SNPs genotyping in this study, as designated by the rs number, can be found on the public genotype database (http://www.ncbi.nlm.nih.gov/SNP). The PCR mixture used for the SNPs genotyping, we designed TaqMan probes and primers using Primer Express software (version 1.5; Applied Biosystems, Foster City, USA). The sequences of the allele-specific probes and primers used in the TaqMan assay are shown in Table 1, and information regarding the SNPs genotyped in this study, as designated by the rs number, can be found on the public genotype database. The PCR mixture used for the genotyping requires confirmation by insertion allele-specific amplification (13). An alternative fast, convenient and reliable genotyping method is required to replace this method for studies using large numbers of samples.

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Methods

Genotyping of SNPs by TaqMan-PCR Assay

Peripheral blood samples were collected with written informed consent from 511 Japanese patients with hypertension and 32 normotensive volunteers. This study protocol was approved by the ethical committee of the National Cardiovascular Center. Genomic DNA was extracted using a NA-3000 nucleic acid isolation system (Kurabo, Osaka, Japan). To perform the SNPs genotyping, we designed TaqMan probes and primers using Primer Express software (version 1.5; Applied Biosystems, Foster City, USA). The sequences of the allele-specific probes and primers used in the TaqMan assay are shown in Table 1, and information regarding the SNPs genotyped in this study, as designated by the rs number, can be found on the public genotype database (http://www.ncbi.nlm.nih.gov/SNP). The PCR mixture used (7.5 µl) contained 6 ng of genomic DNA, 800 nmol/l of primers, the appropriate concentration of probes (see Table 1), and 3.75 µl of TaqMan Universal PCR Master Mix, No UNG (Applied Biosystems). Amplification was done according to the manufacturer’s protocol, with a minor modification. The fluorescence intensity of the PCR products was measured using an ABI PRISM 7900HT Sequence Detection System (Applied Biosystems).

Table 1. Primers and Probes Used in the TaqMan-PCR Assay

<table>
<thead>
<tr>
<th>dbSNP ID</th>
<th>Allele 1/allele 2</th>
<th>Primer</th>
<th>Final conc. (nmol/l)</th>
<th>Probe</th>
<th>Final conc. (nmol/l)</th>
<th>Anneal/extend temp. (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs4291</td>
<td>T/A</td>
<td>gtttccggcacaactgcc</td>
<td>800</td>
<td>FAM-cctcttcacaaagaga-MGB</td>
<td>200</td>
<td>58</td>
</tr>
<tr>
<td>rs4316</td>
<td>C/T</td>
<td>cctcttctgtcgtctgc</td>
<td>800</td>
<td>FAM-tgctcccgccacg-MGB</td>
<td>107</td>
<td>58</td>
</tr>
<tr>
<td>rs4330</td>
<td>A/C</td>
<td>aaggttaaaccagcttgc</td>
<td>800</td>
<td>FAM-cctttaagacaccttg-MGB</td>
<td>200</td>
<td>58</td>
</tr>
<tr>
<td>rs4331</td>
<td>A/G</td>
<td>ggccttcatataaaggtcga</td>
<td>800</td>
<td>FAM-aacgggcaagc-acgtctgc-TAMRA</td>
<td>200</td>
<td>60</td>
</tr>
<tr>
<td>rs4334</td>
<td>A/C</td>
<td>ggaacgtgctcctagctc</td>
<td>800</td>
<td>FAM-ctcttctcatcacc-MGB</td>
<td>200</td>
<td>58</td>
</tr>
<tr>
<td>rs4341</td>
<td>G/C</td>
<td>aacagagggagctgaaggtctg</td>
<td>800</td>
<td>FAM-ctcaagggcatc-MGB</td>
<td>200</td>
<td>58</td>
</tr>
<tr>
<td>rs4351</td>
<td>G/A</td>
<td>acgctccccctgtcgtctc</td>
<td>800</td>
<td>FAM-tggtcttggccac-MGB</td>
<td>200</td>
<td>58</td>
</tr>
</tbody>
</table>

Polymorphic sites in the probe are shown in bold. dbSNP ID, SNP identification number on the public genotype database, db SNP.
ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).

**Determination of ACE I/D Genotype**

Twenty nanograms of genomic DNA was amplified by PCR as described by Rigat et al. (16) with a minor modification. The PCR mixture was treated at 95 °C for 15 min for activation of HotStar Taq polymerase (QIAGEN, Hilden, Germany), followed by 30 cycles of denaturation at 95 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 1 min with use of a HotStar Taq Master Mix Kit (QIAGEN). The PCR products were subjected to a 1.5% agarose gel electrophoresis, stained with SYBR Green I (Molecular Probes, Eugene, USA), and detected using LAS-1000plus (Fuji Film, Tokyo, Japan). The DD genotype was confirmed by insertion allele-specific amplification (13).

**Statistical Analysis**

For each pair of polymorphisms, we calculated the D* value and r^2*, the two most commonly used measures of LD, by using SNPAlyze version 2.0 software (Dynacom, Shigehara, Japan).

**Results**

**Establishment of Alternative Genotyping Assay for I/D Polymorphism**

We selected 6 SNPs (rs4316, rs4330, rs4331, rs4334, rs4341 and rs4351) out of 17 SNPs that have been reported in LD with the I/D polymorphism (14). In addition, we genotyped one additional SNP, rs4291, present in the promoter region of the ACE that has been reported to be a functional SNP (17). We performed the genotyping of these 7 SNPs by the TaqMan-PCR assay. The experimental conditions are listed in Table 1. As shown in Table 1, the TaqMan-PCR probe with TAMRA was used only for genotyping of rs4331, because the adjacent region of rs4331 was GC rich. Two representative typing results are shown in Fig. 1. The 511 Japanese subjects could be clearly distinguished by genotype: i.e., as dominant allele homozygous, recessive allele homozygous, or heterozygous. The genotype of the I/D polymorphism was also determined for individuals by the conventional method.

Seven SNPs were examined and the I/D polymorphisms were all in tight LD. Their allele frequencies and LD coefficients are shown in Table 2. Three polymorphisms, rs4331, rs4334 and rs4341, were in strongest LD with the I/D polymorphism. Among them, the typing of rs4341 by the TaqMan-PCR method gave the best resolution for discrimination of the three genotypes (data not shown). Therefore, we concluded that the rs4341 polymorphism, which is located outside of the 100-bp region downstream of the I/D polymorphism, is an alternative genotyping target for use in place of the I/D polymorphism. In fact, genotyping of 511 samples took only 2 h, and the amount of DNA required for each test was only 6 ng by the TaqMan-PCR method using rs4341. We also tested LD coefficients between rs4341 and I/D polymorphism using 32 normotensive control samples. The results of these genotypings were completely concordant (D* = 1.000; r^2* = 1.000).

![Genotype data for the rs4341 and rs4316 ACE polymorphisms. The vertical and horizontal axes represent the fluorescence intensity for FAM and VIC, respectively. Reaction products without DNA are represented by a square. A: Genotyping of rs4341. B: Genotyping of rs4316. Samples indicated by the circle were ambiguously genotyped because of an 18-bp deletion near the rs4316 polymorphism.](image-url)
A Novel Polymorphism of Six Amino Acids Deletion in the Testicular ACE

On the genotyping of rs4316, four out of 511 individuals were ambiguously genotyped (Fig. 1B). As shown in Fig. 1B, fluorescence signals from the three individuals indicated by a circle were located to the area between the CT and TT allele. The sequence analysis revealed that these signals were due to an 18-bp deletion (Fig. 2). The deletion was present in the PCR amplicon but not overlapped with the sequences of primers and probes. Interestingly, the deletion was located in exon 13, which is exclusively expressed in testis. The deletion corresponded to six amino acids from Ala45 to Gln50 in testicular ACE. On rs4331, we observed two individuals carrying a G-to-A substitution in exon 15, which caused an amino acid exchange of Arg to Gln at position 729.

Discussion

We have genotyped seven SNPs present in ACE to find a convenient, alternative genotyping method for use in place of the widely used I/D polymorphism of ACE. In our analysis, three of seven SNPs showed equal LD coefficients. Based on the fluorescence separation among these three SNPs, we concluded that rs4341 was the most suitable SNP for genotyping by the TaqMan method. There are a couple of advantages to using the rs4341 genotyping instead of the conventional I/D typing. The TaqMan-PCR assay allows

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Table 2. Allele Frequency and Pairwise Linkage Disequilibrium Coefficients between ACE Polymorphisms Obtained from 511 Japanese Subjects

<table>
<thead>
<tr>
<th>dbSNP ID</th>
<th>Region</th>
<th>Allele 1/ allele 2</th>
<th>Allele frequency</th>
<th>rs4291</th>
<th>rs4316</th>
<th>rs4330</th>
<th>rs4331</th>
<th>rs4334</th>
<th>rs4340</th>
<th>rs4341</th>
</tr>
</thead>
<tbody>
<tr>
<td>rs4291</td>
<td>promoter</td>
<td>T/A</td>
<td>0.615/0.385</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs4316</td>
<td>exon 13</td>
<td>C/T**</td>
<td>0.622/0.378</td>
<td>0.944</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs4330</td>
<td>intron 14</td>
<td>A/C</td>
<td>0.623/0.377</td>
<td>0.944</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs4331</td>
<td>exon 15</td>
<td>A/G**</td>
<td>0.623/0.377</td>
<td>0.939</td>
<td>1.000</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs4334</td>
<td>intron 16</td>
<td>A/C</td>
<td>0.620/0.380</td>
<td>0.939</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rs4340</td>
<td>intron 16</td>
<td>I/D</td>
<td>0.628/0.372</td>
<td>0.938</td>
<td>0.996</td>
<td>0.987</td>
<td>1.000</td>
<td>1.000</td>
<td></td>
<td></td>
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<tr>
<td>rs4341</td>
<td>intron 16</td>
<td>G/C</td>
<td>0.621/0.379</td>
<td>0.940</td>
<td>1.000</td>
<td>1.000</td>
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<td></td>
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<tr>
<td>rs4351</td>
<td>intron 20</td>
<td>G/A</td>
<td>0.588/0.412</td>
<td>0.932</td>
<td>1.000</td>
<td>1.000</td>
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</tr>
</tbody>
</table>

* Within the linkage disequilibrium coefficients, the first and second lines represent $D$ (absolute value) and $r^2$, respectively. ** C/T in exon 13 and A/G in exon 15 are synonymous SNPs encoded by Pro27 (testicular) and Ala731, respectively. rs4291, rs4316, rs4330, rs4331, rs4334, rs4341 and rs4351 correspond to Rieder’s numbers 02400, 10514, 11866, 12257, 13145, 14480 and 18222, respectively (14). rs4292 corresponds to Villard’s number A-240T (17). SNP, single nucleotide polymorphisms; dbSNP ID, SNP identification number on dbSNP.

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**Fig. 2.** Partial sequence of exon 13. This region encodes testicular ACE. The dashed line indicates the signal peptide of testicular ACE. The rs4316 polymorphism (C/T) at the third Pro codon is indicated by an arrowhead. The 18-bp deletion, which corresponds to the area from Ala45 to Gln50, is indicated by double-underlining.
easy, automated data handling, and requires less time and fewer samples. In fact, genotyping of 511 samples took only 2 h and the amount of DNA required for each test was only 6 ng by the TaqMan-PCR method using rs4341. This constitutes a clear improvement in terms of both time and quantity of DNA compared to the conventional ACE I/D typing. One disadvantage of this method, however, is that adjacent variants of the target SNP may interfere with the accuracy of genotyping. Therefore, adjacent DNA variants must be considered when performing this test. As we were unable to find a variant similar to rs4341 in the Japanese population, we believe that rs4341 is the most suitable SNP for use in genotyping by the TaqMan-PCR method in place of the I/D polymorphism.

A recent study sequenced 24 kb of the ACE. Three common haplotypes (H1, H6 and H7) were defined in European-American individuals (14), and it was found that haplotypes H1 and H7 contain an Alu deletion allele, indicating a major genetic subdivision in the deletion clade. It was hypothesized that these two haplotypes are the result of an ancestral recombination event at a breakpoint located between intron 5 and exon 8 (18, 19). Both the Alu deletion and the rs4316 polymorphism in intron 16 are downstream of this breakpoint, and thus the use of the rs4316 polymorphism in the TaqMan-PCR assay is feasible.

During the course of this study, we identified a novel in-frame 18-bp deletion in testicular ACE that corresponds to the six amino acids from Ala45 to Gln50. Two forms of human ACE have been characterized by molecular cloning of their respective cDNA and genome structure (20–22). The larger form is present in endothelial cells and in the brush border of epithelial cells, while the smaller form is present in the germinal cells of the testis, and is referred to as testicular ACE. Exon 13 encodes for the 67 amino acids of the NH2-terminal region of testicular ACE. The deletion identified in this study is contained in this NH2-terminal region of testicular ACE (Fig. 2). Therefore, the deletion of six amino acids may result in abnormal enzyme function. Further studies will be needed to analyze the function of the deletion form of testicular ACE.

In conclusion, we propose that genotyping of rs4341 is a convenient alternative approach for ACE I/D genotyping.

References

