Original Article

A Single Nucleotide Polymorphism in the Carboxylesterase Gene Is Associated with the Responsiveness to Imidapril Medication and the Promoter Activity

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Imidapril is an angiotensin-converting enzyme inhibitor that is widely used in treating hypertension, although the responses vary among individuals. We investigated whether a single nucleotide polymorphism at position −816 of the carboxylesterase 1 (CES1) gene, which activates imidapril in the liver, is involved in the responsiveness to imidapril medication. A total of 105 Japanese hypertensives with systolic/diastolic blood pressures (SBP/DBP) of 140/90 mmHg or higher were prescribed 5–10 mg/day of imidapril. At baseline, blood pressure levels were not different between patients with and those without the −816*C* allele (*AA vs. AC+CC* groups). After 8 weeks of treatment, we classified the responders and non-responders based on the decline in their blood pressures, and found that the responder rate was significantly higher in the *AC+CC* group than in the *AA* group ($p=0.0331$). Also, the reduction in SBP was significantly greater in the *AC+CC* group than in the *AA* group (24.7±11.8 *vs.* 17.6±16.8 mmHg, $p=0.0184$). Furthermore, an *in vitro* reporter assay revealed that the −816*C* construct had significantly higher promoter activity ($p<0.0001$). These findings suggest that the A(−816)C polymorphism affects the transcriptional activity, and that this may account for the responsiveness to imidapril. (*Hypertens Res* 2005; 28: 719–725)

***Key Words*: angiotensin converting enzyme inhibitor, imidapril, hypertension, carboxylesterase, single nucleotide polymorphism**

Introduction

Hypertension is considered to be a multifactorial disease to which genetic, environmental, and demographic factors contribute interactively (*1*). Hypertension is one of the established risk factors for cardiovascular diseases (*2*). Angiotensin-converting enzyme (ACE) inhibitors reduce blood pressure by antagonizing the production of angiotensin II, which causes vasoconstriction, aldosterone secretion, and sympathetic activation in the renin-angiotensin system (*3*). ACE inhibitors also reduce the rate of breakdown of bradykinin, a potent vasodilator in the kallikrein-kinin system. Both of these actions of the ACE inhibitors contribute to the blood pressure-lowering effect (*4*).

The responsiveness to ACE inhibitors varies among patients, as with any medication, and this variation may be explained by individual differences in the genetic back-

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Table 1. Clinical Characteristics at Baseline in the CES1 Genotype Groups

	CES1 A(−816)C genotype group		
	AA (64)	*AC*+*CC* (41)	*p* value
Age (years)	60.4±11.2	58.3±13.7	0.3760
BMI (kg/m^2)	24.1±3.2	25.2±4.0	0.1425
Creatinine (mg/dl)	0.7±0.2	0.8±0.2	0.5482
TC (mg/dl)	199.3±37.4	213.2±31.1	0.0437
TG (mg/dl)	156.5±130.3	144.6±83.2	0.6055
HDL (mg/dl)	56.1±16.3	55.8±13.3	0.9360
SBP (mmHg)	166.3±14.1	162.9±14.9	0.2430
DBP (mmHg)	92.8±11.3	90.6±12.5	0.3643
Adverse effects (*N* [%])	9.0 (14.1)	4.0 (9.8)	0.5133

CES1, carboxylesterase 1 gene; BMI, body mass index; TC, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein; SBP, systolic blood pressure; DBP, diastolic blood pressure. The data are shown as mean±SD, unless otherwise indicated. Statistical significance was calculated with Fisher's exact test for gender and adverse effect frequencies, and with Student's *t*-test for other factors. Coughing was counted as a typical adverse effect of angiotensin converting enzyme inhibitors.

grounds (*5*). In order to clarify these genetic backgrounds, many investigations have been undertaken, with special interest paid to genes of the renin-angiotensin system. Among them, the insertion/deletion (I/D) polymorphism of the ACE gene has been of particular interest, mostly because it affects the serum ACE level (*6*). Although this polymorphism is not significantly associated with the blood pressure level (*7*), associations with the effectiveness of captopril (*5*), benazepril (*8*), and fosinopril (*9*) have been reported. Also, angiotensinogen gene M235T polymorphism and type-1 angiotensin-II receptor gene A1166C polymorphism have been shown to be associated with the responsiveness to ACE inhibitors (*10*). However, there are some controversial results for those studies (*11*, *12*).

Imidapril is as effective as captopril (*13*) or the calcium channel blocker nifedipine (*14*) for the treatment of hypertension. One study reported that the responder rates to imidapril were around 50% (*13*), and another that 29% of patients achieved a systolic/diastolic blood pressure (SBP/DBP) reduction to below 140/90 mmHg within an average of 26 days of treatment (*15*). It has been reported that the ACE I/D polymorphism may influence the ability of imidapril to prevent restenosis after percutaneous transluminal coronary angioplasty (*16*). However, the responsiveness to imidapril showed no association with the ACE I/D genotype (*17*), and the genes and the genotypes that are associated with the response to this drug remain largely unknown.

The carboxylesterases (CES, esterase type B, EC 3.1.1.1) catalyze the hydrolysis of a variety of drugs or prodrugs containing ester- and amide-bonds to the respective free acids and alcohol. Since ester derivatives of therapeutic agents have been in use as prodrugs, carboxylesterases are major determinants of the pharmacokinetic behavior of most prodrugs (*18*). Imidapril is one of the newer ACE inhibitors characterized by carboxyl functional groups and requiring hepatic activation to form the active metabolite, imidaprilat (*19*). Among two CES gene products purified from human liver, CES1 (hCE-1), but not CES2 (hCE-2), can convert imidapril into active metabolites (*20*).

There are two CES1 genes, CES1A1 (AB119997) and CES1A2 (AB119998), on chromosome 16. These genes—including their promoter regions—show very high sequence homology. We searched for single nucleotide polymorphisms (SNPs) on the promoter regions of CES1A1 and CES1A2 and found a SNP A(−816)C that was located only on the CES1A2 promoter region (Yoshimura *et al*., under submission). Here, we report on a short prospective study investigating whether this SNP is associated with responsiveness to imidapril.

Methods

Study Population and Inclusion Criteria

A total of 119 Japanese hypertensive patients who visited the Showa University Hospital, Tokyo, Japan, during the study period from February 2002 through October 2003 were eligible for this study. The inclusion criteria were as follows: 1) SBP ≥140 mmHg or DBP ≥90 mmHg, 2) no antihypertensive treatment for at least 8 weeks before baseline, 3) no clinical or biological signs of secondary hypertension, and 4) no diseases requiring treatment, such as coronary heart disease, hyperlipidemia, or diabetes mellitus. After excluding 13 patients due to loss of follow-up and 1 due to genotyping error, 105 patients (mean age: 59.6±12.2 years; 59 men and 46 women) were assessed. The study was approved by the Ethical Review Committee of Showa University Hospital, and written informed consent was obtained from all participants.

Treatment Procedure and Evaluation Criteria

All patients were prescribed 5–10 mg/day of imidapril at

Week 0. At Week 8, patients who fulfilled one of the following conditions were defined as "responders" while the others were defined as "non-responders": 1) SBP <140 mmHg and DBP <90 mmHg, or 2) SBP decreased by 20 mmHg or more and DBP decreased by 10 mmHg or more, or 3) mean blood pressure (defined as the mean of [SBP + 2 × DBP]/3) decreased by 13 mmHg or more.

Blood Pressure and Other Measurements

Blood pressure was measured by trained medical staff using a mercury sphygmomanometer with a stethoscope in the sitting position after a rest of 15 min or more. The body mass index (BMI) was calculated as weight (kg)/height $(m)^2$.

Genotyping of the A(−816)C SNP

A TaqMan assay was performed according to the manufacturer's protocol (Applied Biosystems, Foster City, USA). Briefly, in a total volume of 5 μl, 5.0 ng of genomic DNA, 200 nmol/l of TaqMan MGB probes (FAM, 5′-CATCAC CCCTACTGC-3′; VIC, 5′-CATCACACCTACTGCT-3′), and 900 nmol/1 of each primer (F, 5′-CCTTAATTTGGT GATTTCACATTGC-3′; R, 5′-CAAGACATGGTTCAGCT TCTCAAG-3′), designed based on a sequence in the UCSC database [http://genome.ucsc.edu/cgi-bin/hgGateway]), were heated at 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min, with a final soak at 50°C. Although this primer set would amplify both the CES1A1 and A2 promoter regions, the probe set can discriminate only the CES1A2 allele where the SNP exists (Yoshimura *et al*., under submission). The fluorescent signal was read and the genotype was determined as described elsewhere (*21*). The genotyping was done after collecting the clinical data so that the clinicians were not aware of the patients' genotypes.

Promoter Assay

Genomic DNA was extracted from 16 Japanese healthy volunteers' whole peripheral blood using a Genomix DNA extraction kit (SRL, Tokyo, Japan). Selective amplification of the CES1A2 promoter region was performed using the nested polymerase chain reaction (PCR) method as described elsewhere (Yoshimura *et al*., under submission). We amplified the 922-bp fragment including the −816 site using the primers 5′-CC*ACGCGT*GCCCAGAGCACTCTGTATC-3′ and 5′-GG*GCTAGC*CCCAAGCCGCGGAAGCAGC-3′, which include *MluI* and *NheI* sites, respectively. The fragments possessing A or C at the position −816 and identical for the rest of the sequences were selected. Each of the fragments was digested with MluI and NheI to be subcloned into the respective sites of pGL3-Basic luciferase reporter vector (Promega, Madison, USA). The derived plasmids were designated CES1 −816*A* and CES1 −816*C*, respectively.

HepG2 cells (a human hepatoma cell line) were cultured in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, USA) supplemented with 10% fetal bovine serum (Sanko Junyaku, Tokyo, Japan). In 24-well dishes (TPP, Trasadingen, Switzerland), 1.3×10^5 cells per well were plated and transfected with 0.45 μg of CES1 −816*A* or CES1 −816*C* luciferase reporter vector and 4.5 ng of *Renilla* luciferase expression vector pRL-TK (Promega), using Lipofectamine 2000 (Invitrogen, Carlsbad, USA). The cells were harvested after 48 h, and dual luciferase assays (Promega) were performed according to manufacturer's instructions. The firefly luciferase activity was normalized to the *Renilla* luciferase activity. Data were collected in two independent experiments, using 4 wells for each SNP. The mean of two measurements was taken as the activity of a well.

Statistical Analysis

The allele frequencies were calculated using a gene counting method, and Hardy-Weinberg equilibrium was confirmed using Fisher's exact test. The SBP decline levels at Week 8 were adjusted to the baseline SBP levels and were compared between the three A(−816)C genotype groups: *AA*, *AC* and *CC*. We found no significant difference between the *AC* and *CC* groups ($p=0.9072$). To elucidate the effect of the *C* allele (the dominant model for the *C* allele), we combined the *AC* and *CC* genotypes (*AC*+*CC* group) for all subsequent analyses.

The baseline characteristics were compared between the *AA* and *AC*+*CC* genotype groups, or between the responders and non-responders using Student's *t*-test for differences of means and Fisher's exact test for the categorical variables. The odds ratio and the 95% confidential interval (95% CI) were calculated using a logistic regression model. The decline levels of the blood pressure between the genotype groups were compared using general liniar model with adjustments for age, sex, and BMI. The DBP level was adjusted to age, and the differences were indicated as least square means (LSM)±SEM. Differences between the −816*A* and −816*C* allele in the responder rate and in the promoter activity were compared by the Student's *t*-test. A two-sided *p* value less than 0.05 was considered to be statistically significant. All analyses were performed using SAS software version 8.2 (SAS Institute Inc., Cary, USA).

Results

Genotyping of CES1A2 A(−816)C and Frequencies in a Japanese Hypertensive Population

We sequenced the promoter regions of two CES1 genes of 16 Japanese and found a SNP that was located on the promoter region of CES1A2 (AB195642) but not that of CES1A1 (AB195643) (Yoshimura *et al*., under submission). CES1A2 is a typical TATA-less promoter and seemed to have several transcription initiation sites. We therefore chose one of the

Table 2. Characteristics at Baseline in the Responders and the Non-Responders

	Responder (N=58)	Non-responder (N=47)	p value
Age (years)	59.8±12.5	59.3±11.8	0.8384
BMI (kg/m^2)	24.6±3.9	24.4±3.0	0.8036
Creatinine (mg/dl)	0.71±0.21	0.77±0.17	0.1480
TC (mg/dl)	205.9±33.3	203.4±38.4	0.7204
TG (mg/dl)	149.0±107.3	155.4±122.7	0.7769
HDL	57.0±13.7	54.7±16.7	0.4565
SBP (mmHg)	166.3±16.1	163.3±12.0	0.2721
DBP (mmHg)	94.7±10.7	88.6±12.3	0.0073

BMI, body mass index; TC, total cholesterol; TG, triglyceride; HDL, high-density lipoprotein; SBP, systolic blood pressure; DBP, diastolic blood pressure. The data are shown as mean±SD, unless otherwise indicated. Statistical significance was calculated with Fisher's exact test for gender and adverse effect frequencies, and with Student's t-test for other factors.

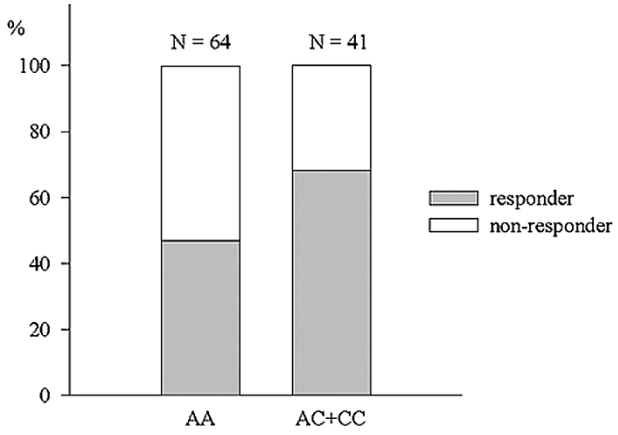

Fig. 1. *The responder ratio according to the A(−816)C genotype group. 46.9% of* AA *group patients and 68.3% of* AC+CC *group patients became responders after 8-week imidapril treatment (*p=*0.0440). The total numbers of patients in the* AA *and* AC+CC *groups are indicated above the bars.*

reported initiation sites (44 bp upstream from the ATG codon) (*16*) and set it as +1. In this way, this SNP site was designated as A(−816)C.

The A(−816)C SNP genotype distributions were 61.0% for the *AA* genotype (n=64), 28.6% for the *AC* genotype (n=30) and 10.5% for the *CC* genotype (n=11). The allelic frequencies of the *A* and *C* alleles were 75.2% and 24.8%, respectively. They were in Hardy-Weinberg equilibrium (p=0.8718). Because the incidence of the −816*C* allele in the responder group (69.2%) was significantly higher than that of the −816*A* allele (50.6%) (p=0.0242), we combined the *AC* and *CC* genotypes (*AC+CC* group) in the subsequent analyses to elucidate the effect of the *C* allele (the dominant model for the *C* allele).

Clinical Characteristics at Baseline in the A(−816)C Genotype Groups

The clinical characteristics at baseline were not significantly different between the *AA* and *AC+CC* genotype groups (Table 1). Although the total cholesterol level was slightly different between the genotype groups, when we analyzed the data without excluding the 13 patients for whom the follow-up at Week 8 was lost, the significance disappeared (mean±SD: 202.8±38.2 mg/dl and 212.6±30.4 mg/dl for the *AA* and *AC+CC* genotype groups, respectively; p=0.1508).

Characteristics in the Responders and the Non-Responders at Baseline

After 8 weeks of treatment, the patients were categorized either as responders or non-responders according to the criteria described in the Methods. The genotype distribution and the baseline clinical parameters in responders and non-responders are summarized in Table 2. The mean DBP level was higher in the responders than in the non-responders (p=0.0073). The other clinical factors were not significantly different.

We next compared the responder ratios between the A(−816)C genotype groups after the 8-week imidapril treatment (Fig. 1). While 68.3% of the *AC+CC* group patients responded efficiently to the treatment (n=28), more than half of the *AA* group patients remained non-responders (53.1%; n=34) (p=0.0440). A logistic analysis with an *AA* group as a reference (odds ratio=1.0) yielded an odds ratio of 2.4 (95% CI: 1.1 to 5.5) for the likelihood of *AC+CC* genotype patients becoming responders.

Blood Pressure Reduction According to Genotype

The SBP and DBP levels were significantly decreased in both genotype groups (Fig. 2). Although the baseline level was not

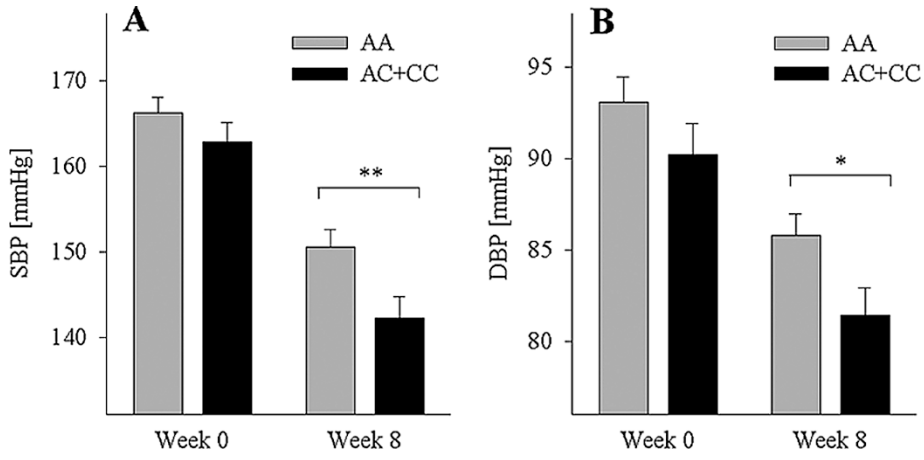

Fig. 2. *The differences in the blood pressure levels at the baseline and at Week 8 between the A(−816)C genotype groups. The SBP levels (A) are shown as the mean±SD.* **p = *0.0045. The DBP levels (B) were adjusted to age and are shown as LSM±SEM.* *p = *0.0230.*

different between the two A(−816)C genotype groups (Table 1), the SBP at Week 8 was significantly lower in the *AC+CC* group than in the *AA* group (mean±SD: 150.6±17.8 mmHg *vs.* 142.3±11.6 mmHg, $p=0.0045$; Fig. 2A). The DBP level was also lower in the *AC+CC* group, although the difference did not reach the level of statistical significance (85.5±9.9 mmHg *vs.* 81.9±10.4 mmHg; $p=0.0711$). Because a linear regression analysis yielded a significant correlation between the DBP level and age, both at Week 0 and 8 (the elder the subjects were, the lower the DBP was; $p<0.0005$), the adjustment for age revealed a significant difference between the two genotype groups in the DBP level at Week 8 (LSM±SEM: 81.4±1.5 mmHg [*AC+CC*] *vs.* 85.8±1.2 mmHg [*AA*]; $p=0.0230$) (Fig. 2B).

The subjects with higher baseline blood pressure values showed greater blood-pressure reductions. Even after an adjustment to the baseline SBP level, the decline in SBP was significantly greater in the *AC+CC* group (LSM±SEM: −22.1±2.4 mmHg) than in the *AA* group (−14.7±1.9 mmHg; $p=0.0174$). The reduction in DBP, after adjusting the baseline DBP level and age, was also greater in the *AC+CC* group, although the difference did not reach the level of statistical significance (−9.9±1.3 mmHg [*AC+CC*] *vs.* −6.6±1.1 mmHg [*AA*]; $p=0.0581$).

Promoter Activity and the A(−816)C SNP

The significantly higher incidence of the −816*C* allele in the responder group (69.2%) compared to that of −816*A* allele (50.6%) ($p=0.0242$) led us to hypothesize that this SNP has some physiological function(s). Because this SNP is situated in the promoter region, we conducted an *in vitro* reporter assay using two constructs that were identical except for the SNP site. The promoter activity of the CES −816*C* was significantly higher than that of the CES −816*A* (means±SEM: 3.09±0.32 *vs.* 2.00±0.26; $p<0.0001$) (Fig. 3).

Discussion

We here conducted an 8-week-imidapril medication program for 105 Japanese hypertensives to investigate individual differences in the treatment response. The overall responder rate at Week 8 was 54.3%. Within 4 weeks, 24.5% of patients (25/102) achieved an SBP/DBP reduction to below 140/90 mmHg. This is similar to the responder rate of a large-scale study conducted in Europe, in which 29% of patients achieved the target blood pressure reduction within an average of 26 days of imidapril medication (*15*).

Imidapril is a long-acting, non-sulfhydryl ACE inhibitor prodrug, which, after oral administration, rapidly converts to its active metabolite, imidaprilat. Among the human CES genes registered in the GenBank database to date as CES1 or its analogues could be classified as CES1A1 (*22*–*25*) or as CES1A2 (*16*, *26*). Because the gene products of CES1A1 (HU1a) and CES1A2 (HU1b) are almost identical, with the exception of a few amino acids encoding a signal peptide, the protein structure and the enzymatic activity should also be indistinguishable. The CES1 gene product, but not that of CES2, purified from human liver can activate imidapril (*20*). Whether CES3 (*27*) and CES4/PCE-3 (*16*) can activate imidapril remains to be elucidated.

The structure (*26*) and the promoter activity (*28*) of the CES1A2 gene have been previously determined. Because of the large resemblance between the CES1A1 and CES1A2 promoter sequences, the SNPs on this region (*29*) are hardly distinguishable. We reported that several SNPs exist exclusively on the promoter regions of CES1A1 or CES1A2, respectively (Yoshimura *et al*., under submission). One of them, A(−816)C, is located only on the CES1A2 promoter and not on that of CES1A1.

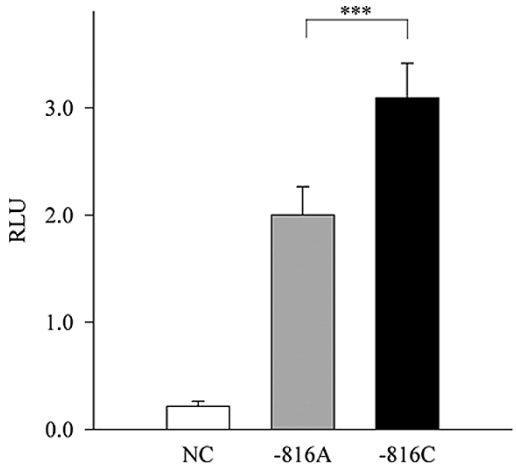

Fig. 3. *The allele frequencies of −816*A *and −816*C *in the responders and the corresponding levels of promoter activity. The promoter activities of the CES1 −816*A *and −816*C *constructs are shown as the mean±SEM. Data were collected in two independent experiments, using 4 wells for each SNP. The mean of two measurements was taken as the activity of a well.* ***p*<*0.0001. NC, negative control (pGL3-Basic vector); RLU, relative luciferase unit.*

Interestingly, the responsiveness was significantly different depending on the CES1A2 A(−816)C genotypes; the *AC*+*CC* group patients who possessed the −816*C* allele had a higher responder rate (odds ratio: 2.4 [95% CI, 1.1 to 5.5]). Also, the reductions in SBP and DBP were greater in the *AC*+*CC* group than in the *AA* group ($p=0.0174$ and $p=0.0581$, respectively). Accordingly, an *in vitro* reporter assay revealed that the promoter with −816*C* had significantly higher transcription activity than that with −816*A* ($p<0.0001$), and this might explain the significantly higher incidence of the −816*C* allele in the responders (69.2%; $p=0.0242$). The higher DBP at baseline in the responders might indicate that diastolic hypertensives are more likely to become responders under this protocol. This raises a concern that the responder rate may be affected by the difference in the baseline blood pressure. However, this possibility is unlikely, since there was no significant difference in the blood pressures between the *AC*+*CC* and *AA* groups, and only higher DBP but not SBP was found in the responder group compared to the non-responder group.

The difference in responsiveness to imidapril treatment between the genotype groups was clear among the patients who had a baseline SBP/DBP of 160/100 mmHg or higher ($n=81$). Among these severe hypertensives, the responder rates were 46.2% in the *AA* group, while high as 79.3% in the *AC*+*CC* group ($p=0.0027$). It is possible that the differences in the therapeutic effect of imidapril between the A(−816)C genotype groups are seen more clearly in severe hypertensives. If so, one plausible reason is that the higher transcriptional activity of the CES1 gene would contribute to a higher enzymatic activity, which would be necessary for severe hypertensives to convert imidapril for normalizing blood pressure.

Indeed, the promoter activity was higher in patients with the −816*C* allele than in those with the −816*A* allele, suggesting that A(−816)C was directly responsible for the difference in transcription activity. It should be determined in a future study whether the difference in promoter activity is associated with the plasma level of the CES1 enzyme or the activity of this enzyme in the liver. Although there were no putative transcription factor binding sites that exactly matched, the sequence around −816 is resembled a putative SREBP1-binding site (5′-ATC-ACCCCAC-3′) (*30*) or sterol regulatory element (SRE, 5′-GAC-ACCCCTA-3′) (*31*), especially when it has *C* on −816. We do not know whether this sequence would act as an SRE; the answer to this question awaits further investigation.

In the present study, we studied the CES1A2 gene and examined a SNP A(−816)C in the promoter region that was found to be associated with the responsiveness to imidapril. The relation between CES1A2 polymorphism and imidapril responsiveness warrants further investigation. In particular, measurement of imidapril and imidaprilat, which was not conducted due to limitations of the current study, may provide more direct information. It is also worth investigating whether there are other polymorphisms in the CES1A2 gene or in the other CES genes including CES1A1 that may affect the responsiveness to imidapril. Moreover, considering that many ACE inhibitors, including enalapril, trandolapril, and perindopril, are also activated by carboxylesterase, it would be of special interest to study the combination or specificity of the interactions of these prodrugs and the CES genes and their polymorphisms.

Conclusion

In conclusion, we have shown that the reduction in blood pressure by imidapril is influenced by the A(−816)C genotype, which is associated with the promoter activity of the gene. It is necessary to further investigate whether our results can be reproduced in different subjects and populations. If our results can be consistently replicated, then SNP genotyping might be routinely used to make a more effective choice of antihypertensive medication in individual cases.

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