Original Article

Association of CT Dinucleotide Repeat Polymorphism in the 5′-Flanking Region of the Guanylyl Cyclase (GC)-A Gene with Essential Hypertension in the Japanese

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Guanylyl cyclase (GC)-A (natriuretic peptide receptor [NPR]-1), the receptor for atrial and brain natriuretic peptide, is important in the regulation of blood pressure in animal models and, possibly, in humans. In this study, we examined the association between dinucleotide repeat polymorphism within the 5′-flanking region of the GC-A gene and essential hypertension in a group of Japanese subjects. By genotyping 177 hypertensive and 170 normotensive subjects, we identified 5 allele types with 6, 9, 10, 11 and 12 CT dinucleotide repeats, respectively, around position −293, upstream of the ATG codon in the human GC-A gene. The frequency of the (CT)*n*=6 allele was significantly higher among hypertensive than normotensive subjects, while the frequencies of the other allele types did not differ between the two groups. We also examined the linkage between G/A polymorphism at position −77 (rs13306004), downstream of the (CT)*n* polymorphism, and found that the (CT)*n*=6 allele was tightly linked to an A at position −77, while all other (CT)*n* alleles were linked to G. Promoter-reporter analyses carried out in cultured human aortic smooth muscle cells using a luciferase gene fused to the 5′-flanking region of the GC-A gene revealed that the promoter containing (CT)*n*=6 drove less transcriptional activity than that containing (CT)*n*=10. Finally, site-directed mutation showed that the (CT)*n* and G/A polymorphisms act synergistically to affect GC-A promoter activity. Our results thus define the (CT)*n* polymorphism in the 5′-flanking region of the GC-A gene as a potent and novel susceptibility marker for hypertension. (*Hypertens Res* 2008; 31: 89–96)

***Key Words*: guanylyl cyclase (GC)-A, polymorphism, hypertension, transcriptional activity**

Introduction

Atrial and brain natriuretic peptide (ANP and BNP, respectively) bind to and activate guanylyl cyclase (GC)-A (also termed natriuretic peptide receptor [NPR]-1) (*1–6*), thereby inducing relaxation of the vasculature and inhibition of vascular smooth muscle cell proliferation through the action of the second messenger cGMP (*7*). The important physiological role played by the natriuretic peptide/GC-A system in the regulation of arterial blood pressure (BP) and blood volume is now well documented in studies carried out in a variety of

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This work was supported by research grants from the Japanese Ministry of Education, Science and Culture and the Japanese Ministry of Health and Welfare.

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Received March 29, 2007; Accepted in revised form August 13, 2007.

Table 1. Characteristics of the Normotensive and Hypertensive Groups

	Normotensive	Hypertensive	*p*
Subject (*n*)	170	177	
Gender (male/female)	113/57	91/86	<0.05
Age (years)	54.9±14.1	57.3±14.1	n.s.
SBP (mmHg)	125.2±10.9	161.1±18.8	<0.001
DBP (mmHg)	74.4±9.5	92.9±13.7	<0.001
HR (beat/min)	70.6±12.3	72.9±13.1	n.s.
BMI (kg/m^2)	23.1±2.6	24.1±3.5	0.01
T-cho (mg/dL)	198±36.6	202.2±34.9	n.s.
HDL-cho (mg/dL)	47±17	49±17	n.s.
Cre (mg/dL)	0.96±0.3	0.96±0.99	n.s.
UA (mg/dL)	5.3±1.4	6.0±1.3	n.s.
FBS (mg/dL)	123.9±42.0	114±31.3	n.s.
PRA (ng/dL)	1.02±0.98	0.99±1.44	n.s.
PAC (ng/dL)	61.1±39.4	67.0±42.5	n.s.
ANP (pg/mL)	28.8±22.4	38.3±32.9	0.006
BNP (pg/mL)	20.9±35.0	39.4±32.9	0.002
UCG			
IVST (mm)	9.86±1.37	10.7±2.25	0.001
PWT (mm)	9.88±1.38	10.7±2.27	0.001

SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; BMI, body mass index; T-cho, serum total cholesterol; HDL-cho, serum high-density lipoprotein cholesterol; Cre, serum creatinine; UA, uric acid; FBS, fasting blood glucose; PRA, plasma renin activity; PAC, plasma aldosterone concentration; ANP, atrial natriuretic peptide; BNP, brain natriuretic peptide; UCG, ultrasonic echocardiography; IVST, interventricular septal thickness; PWT, posterior wall thickness; n.s., not significant. Data are means±SD.

genetically engineered mouse models. For instance, targeted deletion of GC-A leads to chronic hypertension (*8–10*), whereas its overexpression leads to a "dose-dependent" fall in BP (*11*). Although hypertension is a multifactorial disease controlled by multiple genes and environmental factors, these results demonstrating GC-A's critical role in the regulation of BP suggest that variations in GC-A gene expression could contribute significantly to the pathogenesis of essential hypertension in humans.

The GC-A gene is located on chromosome 1q21-22 in humans and is comprised of 22 exons spanning 16 kb (*12*). Recently, several polymorphisms were identified within the 5′-flanking region of the GC-A gene. Nakayama *et al*. identified an insertion/deletion polymorphism (8 bp deletion) at position −60 and a (CT)*n* dinucleotide repeat polymorphism at position −293 of the gene in a Japanese population (*13*, *14*). In addition, Knowles *et al*. also identified the (CT)*n* dinucleotide repeat site as well as nine other polymorphic sites in the noncoding region of the gene (*15*). These polymorphisms in the 5′-flanking region and other noncoding regions could affect the transcriptional activity of the GC-A gene and are thus potentially involved in the pathogenesis of essential hypertension. Indeed, Nakayama *et al*. showed that there is an association between the insertion/deletion polymorphism at position −60 and essential hypertension (*13*). With respect to variations in the (CT)*n* dinucleotide repeat at position −293, Nakayama *et al*. reported 3 allele types containing 10, 11 and 12 CT repeats, respectively, and found no association between these variations and essential hypertension (*14*). On the other hand, Knowles *et al*. reported alleles with 6, 10 or 11 CT repeats at the same position, but did not test for an association between this variation and any diseases. Thus, the association between the (CT)*n*=6 allele at position −293 of the GC-A gene and essential hypertension has not yet been evaluated.

In the present study, therefore, we examined the association between (CT)*n* repeat polymorphism at position −293 of the GC-A gene and essential hypertension in our Japanese subjects. We identified 5 allele types having 6, 9, 10, 11 and 12 CT repeats, respectively, and found that the (CT)*n*=6 genotype, which results in a reduction in GC-A promoter activity in human smooth muscle cells, was significantly associated with essential hypertension. We propose that the (CT)*n* polymorphism at position −293 of the GC-A gene is a potent and novel susceptibility marker for essential hypertension.

Methods

Patient Population

A total of 177 patients with essential hypertension were selected from the outpatient clinics at Kyoto University Hospital and its affiliated hospitals in Kyoto according to the following criteria: 1) patient age >20 years; 2) onset of

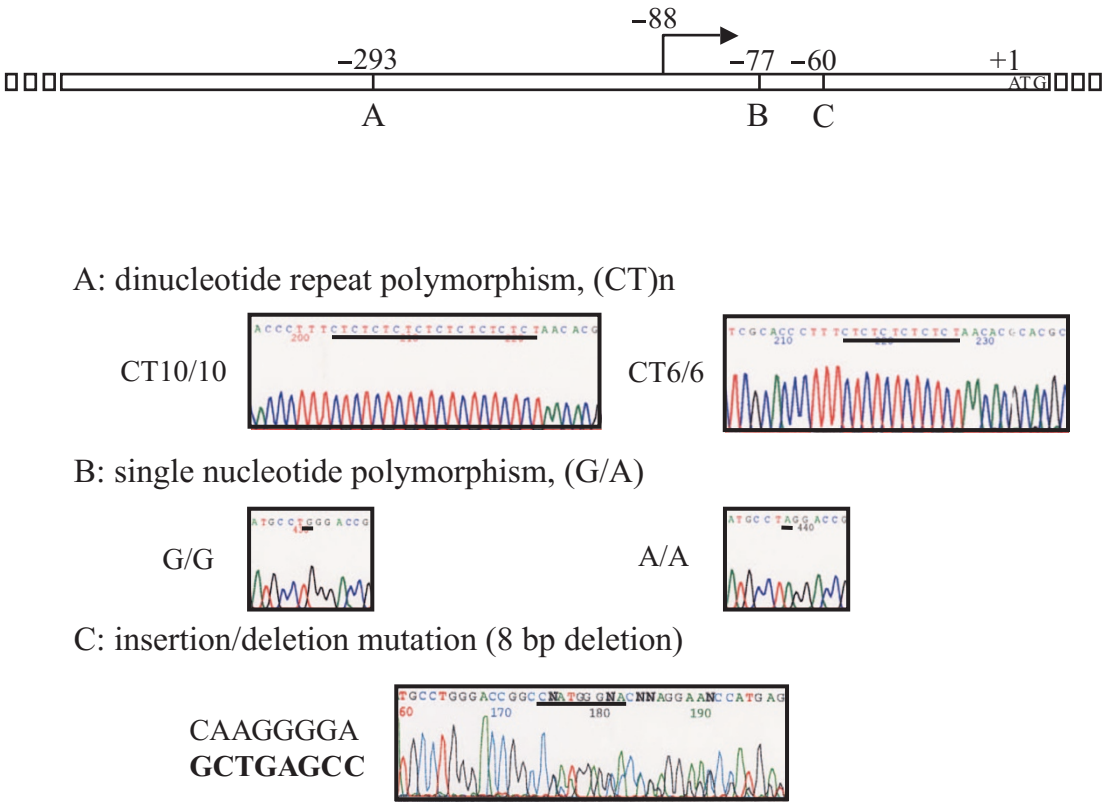

Fig. 1. *Schematic representation of the 5′-flanking region of the GC-A gene. The first methionine codon (ATG) is assigned position +1. The arrow at position −88 indicates the transcription start site. The nucleotide sequences show (CT)n dinucleotide repeat polymorphisms (homozygous CT10/10 and CT6/6) (A), G/A polymorphisms (homozygous G/G, A/A) (B) and the 8-bp deletion (heterozygous) (C).*

hypertension occurred at <60 years of age; 3) established hypertension defined either by long-term treatment of the disease or, in those previously untreated, by systolic/diastolic BP >140/90 mmHg on two consecutive office visits; and 4) absence of a secondary form of hypertension, as determined through extensive workup. BP was measured using a sphygmomanometer while the patient was in a supine position. A group of 170 normotensive control subjects were selected from the same clinics according to the following criteria: 1) subject age >30 years; 2) systolic/diastolic BP <140/90; and 3) absence of antihypertensive treatment (*16*, *17*). At the time of recruitment, informed consent was obtained from each person according to the protocol approved by the Human Study Committee of Kyoto University. The investigation conforms to the principles outlined in the Declaration of Helsinki.

Clinical Parameters

Serum total cholesterol, serum high-density lipoprotein (HDL) cholesterol, serum creatinine, uric acid, fasting blood glucose, plasma renin activity and plasma aldosterone concentrations were determined using standard methodology in an accredited laboratory. ANP and BNP were determined by specific radioimmunoassay as we reported previously (*18*, *19*). Interventricular septal thickness and left ventricular posterior wall thickness were determined by echocardiography (SONOS 5500; Hewlett Packard, Andover, USA).

Genotype Analysis

Genomic DNA was extracted from whole blood using DNA Quick II (Genomic DNA Separation Kit, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan) according to the manufacturer's protocol, after which the GC-A promoter region containing the (CT)*n* polymorphism was amplified by polymerase chain reaction (PCR) using appropriate sense (CCTGGATTGGCTCTTCTGTCAC; −554 to −533) and antisense (TACCACGGCTACCGTCAGGTTG; +99 to +120) primers. The thermocycling protocol entailed an initial denaturation at 95°C for 10 min followed by 30 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 1 min, with a final extension at 72°C for 5 min. After amplification, the double stranded PCR product (674 bp) was sequenced using ABI PRISM 310 and ABI PRISM 3100 automated sequencers with BigDye terminator cycle sequencing chemistry (Applied Biosystems, Foster City, USA) and template-specific forward

and reverse primers.

Plasmid Construction

To construct luciferase reporter plasmids containing GC-A promoters with the (CT)*n*=10 or (CT)*n*=6 allele at position −293 bp, the nucleotide sequence spanning the GC-A promoter (−1430 to −44 bp) was amplified by PCR from the genomic DNA using the appropriate sense (GCTTAATGC ATGTTGCATTACGG) and antisense (GCTCAAGCTTGG CCTCCTCGGTC) primers. After digestion with *SacI* and *HindIII*, the PCR products were subcloned into the *SacI* and *HindIII* sites of the pGV-B2 luciferase reporter vector (Toyo Inc., Tokyo, Japan), and the sequences of the plasmid constructs were verified. Mutant plasmids (CT6/−77G or CT10/−77A) were generated using a Quick-Change Site-Directed Mutagenesis Kit (Stratagene, La Jolla, USA), and their sequences were also confirmed by DNA sequencing.

Cell Culture

Human aortic smooth muscle cells (hAoSMCs) were grown in HuMedia-SG2 (KURABO Industries, Ltd., Osaka, Japan) supplemented with SmGM-2 SingleQuots (Cambrex Bio Science Walkersville, Inc., Walkersville, USA) at 37°C under a 5% CO_2-enriched atmosphere. For experimentation, hAoSMCs were used between passages 6 and 9.

Transient Transfection and Luciferase Reporter Assays

hAoSMCs (0.5–1.0 × 10^6 cells) were transfected by electroporation using Nucleofector (Amaxa Biosystems, Gaithersburg, USA) according to the manufacturer's protocol. The promoter/luciferase reporter gene (3 μg) was cotransfected with 150 ng pRL-TK harboring the renilla luciferase gene situated downstream of the thymidine kinase promoter. After 24 h, the cells were harvested and lysed using passive lysis buffer (Promega, Madison, USA). Firefly and renilla luciferase activities were then measured in 20-μL samples of the cell extract using a luminometer (LUMINOUS CT-9000D; Diatron, Tokyo, Japan) and a dual luciferase reporter assay system (Promega, Madison, USA). The firefly luciferase activity was normalized by the renilla luminescence activity.

Statistical Analysis

All values for clinical parameters are expressed as the means±SD. Allele frequencies were calculated from the genotypes of all subjects, and the Hardy-Weinberg equilibrium was assessed by χ^2 analysis. Individual differences in allele frequencies were tested using a 2-sided Fisher's exact test for each allele by combining the other alleles into one category (*20*). A multivariate logistic regression analysis was performed with hypertension as the dependent variable, and the following independent variables: subjects with the (CT)*n*=6 allele and subjects without the (CT)*n*=6 allele; gender; and BMI. For the clinical data, the gender distribution between the hypertensive and normotensive groups was assessed by χ^2 analysis. Other group data were compared using ANOVA followed by Fisher's Protected Least Significant Difference (PLSD) test. Luciferase activities are expressed as the means±SEM. Multiple groups were compared using one-factor ANOVA, followed by Fisher's PLSD test to assess specific group differences. For all tests, values of $p<0.05$ were considered statistically significant.

Table 2. Genotype and (CT)*n* Allele Frequency in the Normotensive and Hypertensive Groups

	NT (n=170)	HT (n=177)
Genotype frequency, *n*		
6/6	0	1
6/9	0	1
6/10	1	6
6/11	1	1
9/11	1	0
10/10	80	86
10/11	68	64
10/12	2	0
11/11	16	16
11/12	1	2
Allele frequency, *n* (%)		
6	2 (0.6)	10 (2.8)*
9	1 (0.3)	1 (0.3)
10	231 (68)	242 (68)
11	103 (30)	99 (28)
12	3 (0.9)	2 (0.6)

NT, normotension; HT, hypertension.*The (CT)*n*=6 allele was more frequent among the hypertensive group (2.8%) than in the normotensive group (0.6%) (Fisher's exact test: p=0.037). Hence, gender and body mass indexes were significantly higher in the hypertensive group than in the normotensive group, a multivariate logistic regression analysis was also performed, which revealed that, even after adjustment for these potential confounding factors, the association between (CT)*n*=6 allele and hypertension was statistically significant (odds ratio: 5.31, 95% confidence interval: 1.10 to 25.6; p=0.037).

Results

Clinical Characteristics of the Study Subjects

The clinical characteristics of the 177 individuals in the hypertensive group and the 170 in the normotensive group are summarized in Table 1. The systolic and diastolic BPs and body mass indexes (BMIs) were significantly higher in the

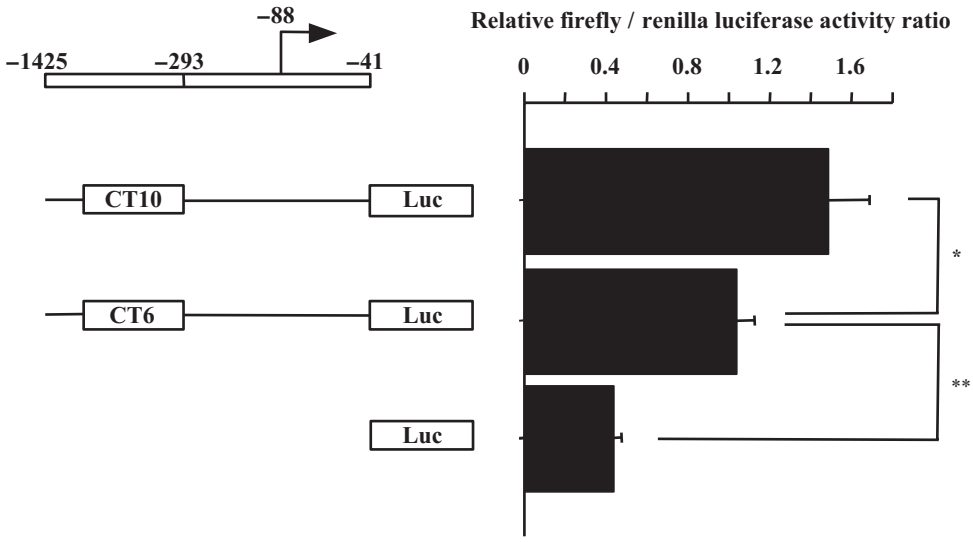

Fig. 2. *Transcriptional activities of the indicated (CT)n reporter constructs in human AoSMCs. For each construct, the mean firefly/renilla luciferase activity ratios from three or four independent experiments (n = 12 individual wells) in hAoSMCs are shown. Results are expressed as the means ± SEM. *p = 0.007 ((CT)n = 6 vs. (CT)n = 10), **p < 0.001 ((CT)n = 6 vs. promoterless pGVB2).*

hypertensive group than in the normotensive group. On the other hand, there were no significant differences between the groups with respect to age, heart rate, total serum cholesterol, serum HDL cholesterol, serum creatinine, uric acid, fasting blood glucose, plasma renin activity or plasma aldosterone concentration. Plasma ANP and BNP levels in the hypertension group were significantly higher than in the normotensive group. In addition, echocardiography revealed the interventricular septal thickness and posterior ventricular wall thickness to be significantly thicker in the hypertensive group than in the normotensive group.

Correlation of (CT)*n*=6 at Position −293 of the GC-A Gene with Essential Hypertension

We initially confirmed the presence of a (CT)*n* dinucleotide repeat polymorphism at position −293 (Fig. 1A), a G/A polymorphism at position −77 (rs13306004; Fig. 1B) and an insertion/deletion polymorphism at position −60 (8 bp deletion; Fig. 1C) within the 5′-flanking region of the GC-A gene in a Japanese population. No other polymorphisms were detected in this region. When we analyzed the (CT)*n* repeat polymorphism in our study subjects, we identified 5 allele types containing 6, 9, 10, 11 and 12 CT repeats, respectively. Table 2 shows the observed frequencies of these genotypes among the study subjects. The observed and expected frequencies of the genotypes in the normotensive group were in good agreement with the predicted Hardy-Weinberg equilibrium values. On the other hand, the frequency of the (CT)*n*=6 allele was significantly higher among subjects in the hypertensive group than those in the normotensive group (Fisher's exact test, $p=0.037$). Moreover, a multivariate logistic regression analysis reveled that the association between hypertension and the (CT)*n*=6 allele was significant even after adjustment for gender and BMI (odds ratio: 5.31, 95% confidence interval: 1.10 to 25.6; $p=0.037$). The (CT)*n*=6 allele was identified in 9 hypertensive and 2 normotensive subjects (1 homozygous and 8 heterozygous in the hypertensive group and 2 heterozygous in the normotensive group). Among subjects with the (CT)*n*=6 allele, plasma levels of ANP and BNP did not differ statistically between the hypertensive group and the normotensive group. In addition, plasma ANP and BNP levels did not differ between subjects with and those without the (CT)*n*=6 allele.

It was suggested in an earlier report that there may be a linkage between the (CT)*n* and G/A polymorphisms in some healthy subjects (*15*). Bearing that in mind, we next examined the linkage between the (CT)*n* and G/A polymorphisms in our study subjects. As previously reported, we found that only the (CT)*n*=6 allele was linked to the presence of an A at position −77, while the other (CT)*n* alleles were linked to the presence of a G at that position, indicating that (CT)*n* polymorphism at position −293 is tightly linked to the G/A polymorphism at position −77 of the GC-A gene.

We also examined the previously reported (*13*) association between the insertion/deletion variants (8 bp deletion) in the GC-A promoter region and essential hypertension. However, we observed no significant association between the insertion/deletion variants and essential hypertension in our study subjects (2 deletion variants in the hypertensive group and 2 in the normotensive group).

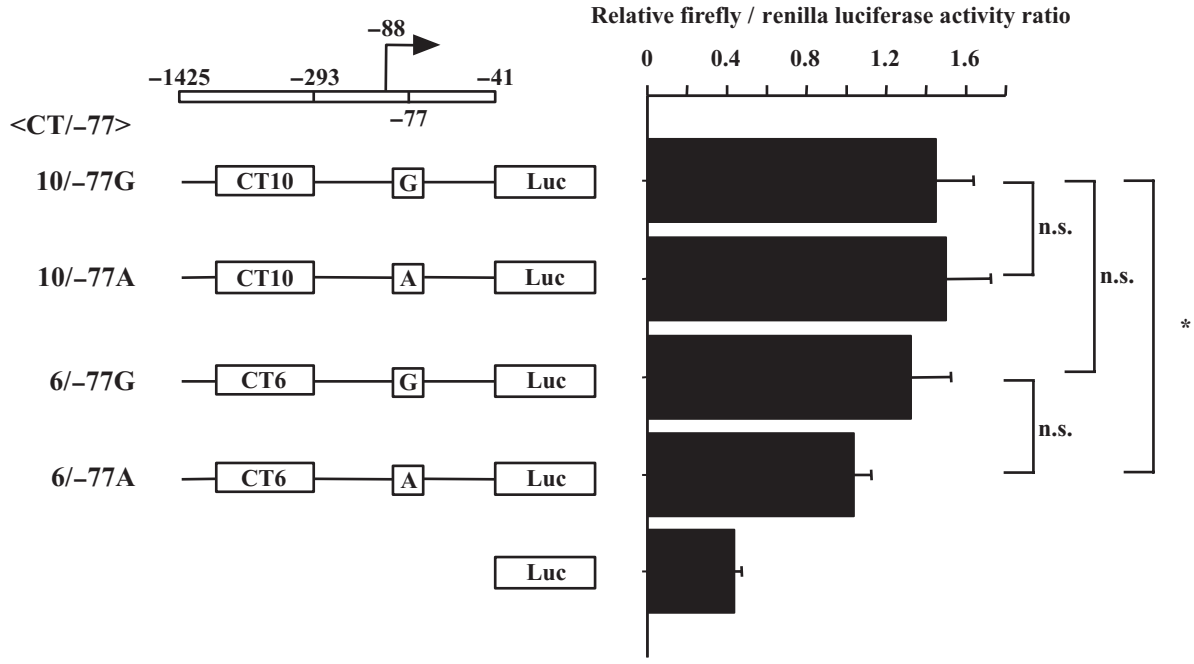

Fig. 3. *Transcriptional activities of reporter constructs mutated at position −77 within the GC-A promoter in human AoSMCs. 10/−77G and 6/−77A denote GC-A promoter constructs containing wild-type sequences; 10/−77A and 6/−77G respectively denote constructs containing G→A and A→G substitutions at position −77. *p = 0.046 (6/A vs. 10/G).*

The Transcriptional Activity Is Decreased in the GC-A Promoter Containing the (CT)*n*=6 Allele

Given that the (CT)*n*=6 allele appears to be significantly associated with essential hypertension, we hypothesized that having only 6 CT repeats at position −293 attenuates the transcriptional activity of the GC-A promoter. To assess the extent to which the (CT)*n* repeat polymorphism within the 5′-flanking region affects GC-A transcription in human cells, we transfected hAoSMCs with an expression plasmid harboring the GC-A promoter region upstream of the luciferase reporter gene. We found that the GC-A promoter containing 6 CT repeats showed significantly ($p<0.01$) less transcriptional activity than the promoter containing 10 CT repeats (Fig. 2).

In addition, since the (CT)*n* and G/A polymorphisms appear to be tightly linked, we next assessed their relative importance in the regulation of GC-A transcription. Examination of the effects on the GC-A promoter activity of substituting the −77G/A in the CT6/−77A and CT10/−77G alleles in hAoSMCs revealed that the transcriptional activity of the CT6/−77A-containing promoter was significantly diminished, as compared to the promoter containing CT10/−77G (Fig. 3). No significant difference was observed in the transcriptional activities of the CT10/−77G- and CT10/−77A-containing promoters. The activity of the CT6/−77G-containing promoter was somewhat weaker than that of the promoter containing CT10/−77G, and somewhat stronger than that of the promoter containing CT6/−77A, but neither difference was significant (Fig. 3). Taken together, these findings suggest that the 6 CT repeats at position −293 and the A at position −77 contribute synergistically to the reduction in GC-A promoter activity.

Discussion

In heterozygous and homozygous GC-A knockout mice, systolic BPs are elevated by about 10 and 30 mmHg, respectively, as compared to wild-type mice (*9*). Conversely, transgenic mice overexpressing GC-A show significantly lower BPs than wild-type mice (*11*). This suggests that the level of GC-A expression exerts an effect on the regulation of BP in mice and raises the possibility that gene variations that reduce GC-A expression contribute to the pathogenesis of hypertension in humans. Bearing that in mind, we examined 1) the polymorphisms within the 5′-flanking region of the human GC-A gene, 2) the association between the polymorphisms and essential hypertension in human subjects, and 3) the extent to which the polymorphisms affect transcriptional activity. We found that having only 6 CT repeats at position −293 of the GC-A gene is significantly associated with essential hypertension in the Japanese. In hAoSMCs, moreover, the GC-A promoter containing the (CT)*n*=6 allele shows significantly less transcriptional activity than is seen with other (CT)*n* alleles. In our population, we found no correlation

between alleles in which (CT)*n*=9, 10, 11 or 12 and essential hypertension, which is in good agreement with earlier observations (*20*, *21*). The (CT)*n* polymorphism within the promoter region of the GC-A gene thus appears to be a potent susceptibility marker for hypertension.

In addition to hypertension, mice lacking GC-A exhibit marked cardiac hypertrophy (*10*). Indeed, the natriuretic peptide/GC-A system has been shown to exert protective effects against both cardiac hypertrophy and fibrosis (*22*), and our preliminary data indicate that the incidences of left ventricular hypertrophy tend to be higher in patients carrying the (CT)*n*=6 allele than in those carrying other alleles (unpublished observation). It is thus plausible that the (CT)*n*=6 allele is also associated with the susceptibility to cardiac hypertrophy. One recent report has shown that (CT)*n*=11/12 polymorphism is associated with significantly higher values for septal wall thickness and left ventricular mass index than other genotypes (*21*), though the (CT)*n*=6 allele was not detected in that population. Consequently, further investigation will be necessary to evaluate the contribution of (CT)*n* repeat polymorphism to cardiac hypertrophy.

It has been reported that plasma BNP levels are significantly higher in subjects with other GC-A mutation (*13*). In the present study, plasma ANP and BNP levels did not differ between subjects with and those without (CT)*n*=6. In addition, among subjects with (CT)*n*=6, plasma levels of ANP and BNP did not differ significantly between the hypertensive group and the normotensive group. Since ANP or BNP levels are affected by several cardiac (*e.g.*, diastolic function) and extra-cardiac (*e.g.*, renal function) factors, the large deviation in those factors in the hypertension group might have impeded the statistical significance of the differences in ANP and BNP.

Our observation that the CT6-containing GC-A promoter drives less transcription in human AoSMCs than the CT10-containing promoter is in contrast to the findings of Knowles *et al*., who reported that, in mouse AoSMCs, the transcriptional activity of the human CT6-containing promoter was significantly greater than the activities of the CT10- and CT11-containing promoters (*15*). One possible explanation for this discrepancy is that they used a promoter fragment extending from −770 bp to the ATG start codon to assess transcriptional activity, whereas we used a fragment extending from position −1425 to −41 bp. This suggests the possibility that mechanisms other than the direct transcriptional control of GC-A gene expression also contribute to the association of (CT)*n*=6 with essential hypertension. One earlier report demonstrated an association between the insertion/deletion variants (8 bp deletion) within the GC-A promoter region and essential hypertension (*13*). By contrast, we found no correlation between the 8 bp insertion/deletion variants and essential hypertension in our subjects. Further studies may be necessary to resolve these discrepancies.

Earlier reports also showed that the (CT)*n*=6 allele is linked to an A at position −77 in the GC-A promoter region and that the (CT)*n*=10 and 11 alleles are linked to a G at that position (*15*). We confirmed this linkage and found that the (CT)*n*=9 and 12 alleles also are linked to a G at position −77. Apparently, only the (CT)*n*=6 allele is linked to A at position −77; all the others are linked to G. We therefore hypothesized that the A at position −77 is responsible for the reduced transcriptional activity of the GC-A promoter in the (CT)*n*=6 allele. However, an A→G substitution at position −77 in the (CT)*n*=6 allele resulted in only an insignificant increase in GC-A promoter activity. In addition, an A→G substitution in the (CT)*n*=10 allele had no effect on promoter activity. It thus appears that the 6 CT repeats at position −293 and the A at position −77 act synergistically to downregulate transcriptional activity of the GC-A promoter.

In summary, our results show that there is a significant association between the presence of only 6 CT repeats at position −293 of the GC-A gene and essential hypertension and that the (CT)*n*=6 allele may be functionally involved in the pathogenesis of essential hypertension through the downregulation of GC-A gene transcription. This study thus defines the (CT)*n* polymorphism in the 5′-flanking region of the GC-A gene as a potent and novel susceptibility marker for hypertension.

Acknowledgements

We wish to thank Dr. Kenji Mori for his help with the data presentation and Ms. Komaki Okazaki for her excellent secretarial work. We also thank Professor Nobuyo Maeda of the University of North Carolina for discussion about the polymorphisms of the human GC-A gene.

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