

Functional Deletion Mutation of the 5'-Flanking Region of Type A Human Natriuretic Peptide Receptor Gene and Its Association With Essential Hypertension and Left Ventricular Hypertrophy in the Japanese

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Abstract—The natriuretic peptide (NP) family is involved in the regulation of blood pressure and fluid volume. We isolated the 5'-flanking region of the type A human NP receptor gene and identified an insertion/deletion mutation in this region. We then assessed whether there is a genetic association between this mutation and essential hypertension (EH). The deletion allele lacks 8 nucleotides and alters binding sites for the activator protein-2 (AP-2) and Zeste transcriptional factors. We genotyped 200 EH and 200 normotensive (NT) individuals and found 9 subjects with the deletion (8 in the EH group and 1 in the NT group). All 9 individuals were heterozygous. The NT subject with the mutation had left ventricular hypertrophy without hypertension. Transcriptional activity of the deletion allele was <30% that of the wild-type allele. The plasma levels of brain NP in EH patients with the deleted allele were significantly higher than the levels in the EH patients with the wild-type allele, and plasma brain NP levels were significantly higher in subjects with the deleted allele than in subjects with the wild-type allele, despite comparable blood pressures. These findings suggest that in Japanese individuals, this deletion in the human NP receptor gene reduces receptor activity and may confer increased susceptibility to developing EH or left ventricular hypertrophy. (*Circ Res.* 2000;86:841-845.)

Key Words: natriuretic peptides ■ type A receptors ■ 5'-flanking region ■ essential hypertension

Three types of natriuretic peptides (NPs) have been isolated: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP), and C-type natriuretic peptide (CNP). The NP family elicits a number of vascular, renal, and endocrine effects that help maintain blood pressure and extracellular fluid volume.¹⁻³ These effects are mediated by specific binding of NP to cell surface receptors that have been characterized, purified, and cloned from the vasculature, kidney, adrenal gland, and brain.⁴⁻⁷ There are 3 subtypes of NP receptors: type A NP receptor (NPRA),⁴ type B NP receptor (NPRB),⁸ and type C NP receptor (NPRC).⁵ All 3 influence cellular second messengers. NPRA and NPRB are guanylyl cyclase receptors, and their activation increases cGMP levels.⁹ Activation of NPRC results in the inhibition of adenylyl cyclase activity.¹⁰ Human NPRA (hNPRA) has high structural homology with human NPRB (hNPRB) and also contains a highly conserved guanylyl cyclase domain.⁵ ANP and BNP bind primarily to NPRA, which exerts its effect on the vasculature, causing vasodilation and inhibition of the proliferation of vascular smooth muscle cells.¹¹

Essential hypertension (EH) is thought to be a multifactorial disorder, and there are only a few reports of candidate

genes associated with EH.¹² Mice with a targeted deletion of NPRA showed hypertension, cardiac hypertrophy, and sudden death.¹³ These findings suggest that NPRA may play key roles in vasodilation, maintenance of blood pressure, and cardiac remodeling and that lack of it may lead to hypertension and other cardiovascular diseases.

We recently described the exon/intron organization of the hNPRA gene.¹⁴ The aim of the present study was to isolate the 5'-flanking region of the hNPRA gene and any genetic variants of this region and to assess whether there is an association between the hNPRA gene and EH.

Materials and Methods

Isolation of the 5'-Flanking Region of hNPRA

To isolate the 5'-flanking region of the hNPRA gene, thermal asymmetric interlaced (TAIL)-polymerase chain reaction (PCR)¹⁵ was conducted. The amplification conditions and arbitrary degenerate primers were as previously described.¹⁶

Rapid Amplification of 5' cDNA Ends

Experiments involving rapid amplification of 5' cDNA ends were conducted to map the transcriptional start site as previously described.¹⁶ cDNA was synthesized with an antisense primer and human kidney poly(A)+ RNA.

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Subjects

The study group consisted of 200 patients (mean age 52.8 ± 9.6 years) with EH and 200 age-matched normotensive (NT) healthy subjects (mean age 52.5 ± 8.1 years). Both groups were selected according to the criteria as described previously.¹⁶ Subjects were recruited, and informed consent was obtained from each individual according to a protocol approved by the Ethics Committee of Nihon University.

Detection and Genotyping of a Deletion Mutation of hNPRA

To screen for mutations, 2 oligonucleotide primers (5'-TCTTACGAAGCGCTCACTCG-3' and 5'-CAGTACCACGGC-TACCGTCAGGTT-3') that recognize part of the 5'-untranslated region were designed. DNA was extracted from whole blood, and single-strand conformation polymorphism (SSCP) was then performed as described previously.¹⁷

To determine the genotypes, PCR was performed with the same primers and conditions as in the SSCP analysis. The PCR products were separated on sequencing gels and visualized by autoradiography.

Gel Mobility Shift Assay

To examine the DNA–nuclear protein complex formation, a gel mobility shift assay was performed with wild-type and mutant-type DNA fragments by using the Gel Shift Assay Core System kit (Promega), as previously described.¹⁸

Measurement of Transcriptional Activities of Wild-Type and Mutant-Type Alleles

Wild-type and mutant-type reporter constructs were made by subcloning the 1.3 kilobase pairs of the 5'-flanking region of the hNPRA gene into the luciferase reporter gene vectors. hNPRA promoter plasmids and a plasmid containing thymidine kinase–driven pRL (Toyo Ink) were cotransfected into human umbilical vein endothelial cells (HUVECs) by using liposome suspension (GIBCO-BRL). Luciferase activity was measured at least 3 times in duplicate with a double luciferase assay system (Toyo Ink). All data were normalized as relative light units per pRL–thymidine kinase activity.

Plasma Levels of ANP and BNP

Twenty-five subjects in the NT group and 25 patients in the EH group who had the wild-type allele were selected randomly. One subject in the NT group and 5 patients in the EH group who had the deleted allele were also selected. Plasma ANP and BNP were measured with each sensitive immunoradiometric assay.^{19–21}

Statistical Analysis

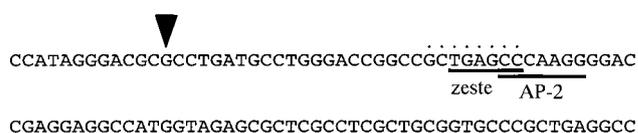
The distributions of the genotypes between EH patients and NT subjects were tested by a 2-sided Fisher exact test and multiple logistic regression analysis. Comparisons between plasma levels of ANP and BNP were analyzed by ANOVA. Correlations between plasma ANP and BNP levels and blood pressures were determined by linear regression analysis. ANCOVA was used for comparison of the slopes of regression lines. A value of $P < 0.05$ was considered statistically significant.

An expanded Materials and Methods section is available online at <http://www.circresaha.org>.

Results

Nucleotide Sequence of the 5'-Flanking Region

By use of TAIL-PCR, ≈ 1 kb of the 5'-flanking region was isolated. To isolate a region further upstream, a second TAIL-PCR was performed with 3 specific antisense primers (TAIL-prom R1 5'-CAA GTC TCC GGC TAT CCG GA-3', TAIL-prom R2 5'-CCA AGC TCC GAA CCC TCC CT-3', and TAIL-prom R3 5'-GGA TCT TTA ACT CCT TCC



ATG

Figure 1. Nucleotide sequence of hNPRA gene. ATG in bold type indicates the start codon, and the first nucleotide upstream from the ATG start codon is numbered -1 . *Cis* elements are underlined. The 8 nucleotides missing in the deletion-type allele are indicated by dots. The transcription initiation site is shown by a black arrowhead.

CCG-3') that allowed us to isolate a total of 2 kb of sequence from the 5'-flanking region of the hNPRA gene. More than 120 bp of the overlapping sequences were identical between the PCR products obtained in the first and second TAIL-PCRs. The 2-kb sequence of the 5'-flanking region of the hNPRA gene lacks a typical TATA box. However, several putative transcription factor binding sites, such as an inverted CCAAT box, stimulatory protein-1 (Sp1), activator protein-2 (AP-2), and Zeste binding sites, are present. The 2-kb sequence has been submitted to GenBank (accession No. AB012188). The transcriptional initiation site was identified by sequencing the concatenation product and is shown in Figure 1.

Association of the Deletion Mutation With EH

We used SSCP to search for mutations or polymorphisms in the 5'-flanking region of the hNPRA gene in patients with EH, and we identified an insertion/deletion mutation. This deletion removes 8 nucleotides and affects the Zeste and AP-2 binding sites (Figure 1).

We genotyped 200 EH and 200 NT individuals and found 9 subjects with the deletion: 8 in the EH group and 1 in the NT group (Table). All were heterozygous. The overall distribution of the genotype differed significantly between the EH patients and the NT group (2-sided Fisher exact value, $P = 0.037$). Logistic linear regression analysis adjusted for age and sex demonstrated the deleted allele to be associated significantly with EH (odds ratio 8.8, 95% CI 1.1 to 72.5). The NT subject who carried the deleted allele had LVH but not hypertension.

Gel Mobility Shift Assay

As shown in Figure 2, 300 ng/lane of human recombinant AP-2 formed a complex with the wild-type probe, which decreased in a dependent manner with the addition of 50- to 100-fold molar excesses of an unlabeled AP-2 binding site fragment. The complex was not changed by the addition of a 100-fold molar excess of the nonspecific fragment. However, the mutant-type probe formed no complex with human recombinant AP-2 in the gel shift assay (Figure 2).

Transcriptional Activities

To study transcriptional activity, we transfected HUVECs with promoter constructs of the hNPRA gene. The promoter activity of the deletion allele was 33% that of the insertion/wild-type allele (Figure 3).

Patients With Deletion-Type Allele

Patient	BMI	Age at Onset, y	Blood Pressure, mm Hg	LVH	Optic Fundus	Urinary Protein
EH group (n=8)						
72-year-old man	27.8	50	174/108	+	ND	+
58-year-old man	26.3	28	182/92	+	KW I	-
58-year-old man	24.8	55	160/100	+	KW O	-
56-year-old woman	21.8	53	170/96	+	KW I	-
55-year-old man	23.0	55	182/120	-	ND	+
53-year-old man	22.4	51	152/96	-	KW O	+/-
40-year-old woman	26.8	39	180/110	-	KW I	-
39-year-old man	33.0	34	171/118	-	ND	-
NT group (n=1)						
57-year-old man	23.8	...	125/77	+	ND	-

BMI indicates body mass index; ND, not determined.

Plasma Levels of ANP and BNP

Figure 4 shows plasma ANP and BNP the levels in NT subjects and in EH patients with and without the deleted allele. Plasma ANP and BNP levels were significantly higher in EH patients with the wild-type allele than in NT subjects with the wild-type allele. The plasma BNP levels in EH patients with the deleted allele were significantly higher than levels in NT subjects without the deleted allele and levels in EH patients without the deleted allele. The relationship between ANP and mean blood pressure is shown in Figure 5. Scatterplots show that the slopes of the line characterizing the plasma ANP levels in subjects with and without the deleted allele were not significantly different. However, the slope of the line characterizing the plasma BNP levels in subjects with the deleted allele was steeper than that in subjects without the deleted allele ($P=0.011$).

Discussion

Three receptors for the NP family have been identified: NPRA, NPRB, and NPRC. NPRA binds ANP and BNP. The cDNA of hNPRA and the deduced amino acid sequence have been described,² and the hNPRA gene has been mapped to human chromosome 1q21-q22.²² We recently characterized

the exon/intron structure of the hNPRA gene.¹⁴ In the present study, we examined the sequence of the 5'-flanking region of the hNPRA gene and found that it lacks a TATA box but contains 3 potential Sp1 binding sites and an inverted CCAAT box. We also found the core sequence of the shear stress responsive element (SSRE) twice in the promoter region. SSREs have been found in the promoters of several genes involved in blood pressure regulation or vessel proliferation.²³ These SSREs are found not only in humans but also in other mammals. Interestingly, SSRE in the promoter region of the NPRA gene has been found only in humans.²⁴ Thus, blood pressure regulation may differ between species.

The transcription initiation site of the hNPRA gene was identified at base/nucleotide -88 with respect to the ATG start codon; thus, plasmids that contained the 1300 bp upstream from the start codon were used for luciferase reporter gene assays. We proved that the promoter activity of the deletion allele was lower than that of the wild-type allele in HUVECs; this suggests that the promoter activity of the deletion allele is lower in other cells or tissue expressing the hNPRA gene, because transcription factors AP-2 and Zeste are widely distributed in tissues and involve in embryonic morphogenesis and cell development.^{25,26} Using a gel shift assay, we demonstrated that the AP-2 protein could not bind the deletion-type allele. Transcription factor AP-2 is thought to be a constitutive regulator of several genes that have

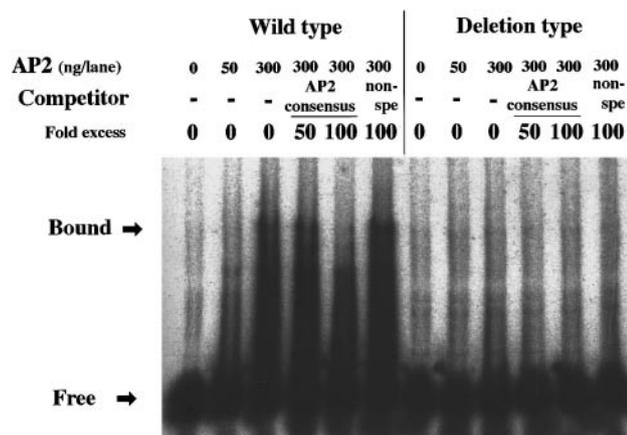


Figure 2. Gel mobility shift assay. Complexes between the DNA probes and human recombinant AP-2 are indicated as bound. Nonspecific competitors are indicated as non-spe.

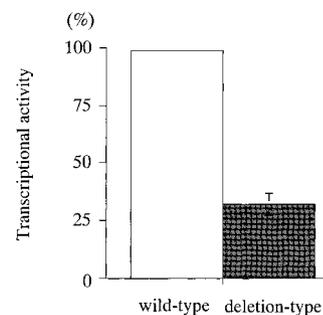


Figure 3. Transcriptional activities of reporter constructs. The activities of the wild-type constructs were considered as 100%. Values are averages of 3 independent experiments. Data are mean \pm SEM.

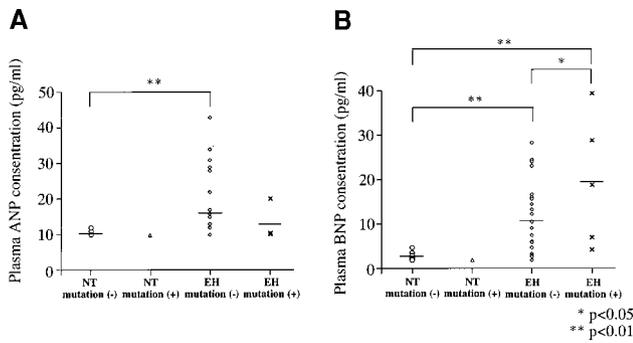


Figure 4. Comparison of plasma ANP and BNP levels in each group. Plasma ANP levels (A) and plasma BNP levels (B) are shown for NT subjects without (–) and with (+) the deleted allele and EH patients without (–) and with (+) the deleted allele.

fundamental roles during development.^{27,28} This is consistent with our findings that a plasmid containing the deletion-type allele has reduced transcriptional activity.

The NP family is involved in the regulation of blood pressure and fluid volume. Thus, it is possible that abnormalities in NP are associated with cardiovascular diseases such as EH. Several reports showed that plasma ANP and BNP levels are higher in individuals with EH than in NT individuals.^{29,30} Transgenic mice with prolonged overexpression of ANP or BNP have lower blood pressures than do their nontransgenic littermates.^{31,32} These findings suggest that abnormalities in ANP, BNP, or NPRA may be related to the pathophysiology of hypertension. In the present study, plasma ANP and BNP levels were measured, and they were higher in EH patients than in NT subjects, which is consistent with previous reports.^{29,30} Although plasma ANP levels are not significantly different between subjects without the deleted allele and subjects with the deleted allele, the levels of plasma BNP in EH patients with the deleted allele are significantly higher than those in EH patients without the deleted allele. Furthermore, scatterplots between BNP and mean blood pressure show that plasma BNP levels are significantly higher in subjects with the deleted allele than levels in subjects without the deleted allele, despite having comparable blood pressures. These findings suggest that the compensatory elevation of plasma BNP is caused by decreased NPRA resulting from a deletion in the hNPRA gene. Eight patients with a deleted NPRA allele were found in the EH group, and their hypertension may be caused by decreased hNPRA activity. Differences in plasma levels of ANP may not have been observed because of the limited sensitivity of the technique for measuring ANP concentrations.

Nishikimi et al²⁰ reported that plasma ANP and BNP levels are increased in EH patients with LVH and that BNP secretion is elevated in concentric hypertrophy. In animal models, suprarenal aortic coarctation leads to progressive hypertension, resulting in pressure-overload LVH and progressive increases in plasma ANP and BNP.³³ Furthermore, an elevated plasma BNP has been shown to be a better marker of LVH than ANP in patients with suspected cardiac disease independent of blood pressure.³⁴ Some reports have suggested that infusion of ANP or BNP improves left ventricular function in patients with congestive heart failure.^{35,36} In

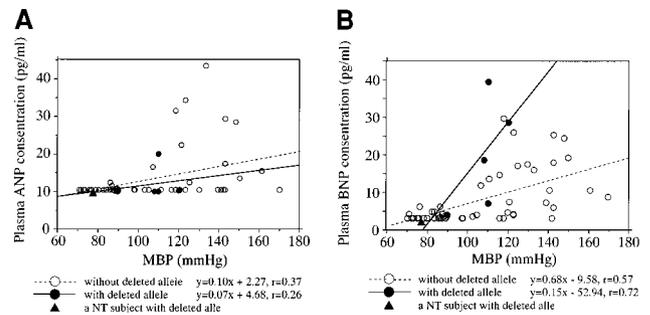


Figure 5. Scatterplots show the relationships between plasma ANP levels (A), BNP levels (B), and mean blood pressure (MBP) in subjects with deleted allele and without the deleted allele. Slopes of the plasma ANP levels in subjects without and with the deleted allele were not significantly different. The slope of the plasma BNP levels in subjects with the deleted allele was steeper than that of subjects without the deleted allele ($P = 0.011$).

cardiac hypertrophy induced by endothelin and phenylephrine, ANP and BNP expression levels are significantly high.³⁷ Thus, hemodynamic overload and cardiac hypertrophy may be related directly to increases in plasma BNP and ANP levels. These findings suggest that the NP system plays a protective role in the prevention of LVH. We found 1 subject with the deleted allele who has LVH despite having normal blood pressure. His LVH may be caused by decreased hNPRA activity. The 4 EH patients with the deleted allele had no LVH. This may be due to the short periods of time that these patients have had hypertension; all have been diagnosed within the last 5 years.

Mice with targeted deletion of NPRA have experienced hypertension, cardiac hypertrophy, and sudden death,¹³ suggesting that NPRA may play a key role in vasodilation, maintenance of blood pressure, and cardiac remodeling. Abnormal NPRA levels or function may lead to hypertension and other cardiovascular diseases. Thus, it is likely that hypertension or LVH in individuals with the deleted allele is caused by reduced hNPRA activity.

The structure of the 5'-flanking region of the hNPRA gene may provide insight into the transcriptional regulation of this gene. This is the first report that associates a mutation in the 5'-flanking region of the hNPRA gene with transcriptional activity. This insertion/deletion mutation of the hNPRA gene may confer increased susceptibility to EH or LVH in Japanese individuals.

Acknowledgments

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