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Structure and Polymorphisms of the Human Natriuretic Peptide Receptor C Gene

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Natriuretic peptides (NPs) regulate cardiovascular homeostasis, including natriuresis, diuresis, vasodilation, regulation of endocrine secretion, and inhibition of cellular growth. Atrial natriuretic peptide receptor C (NPRC) has a short cytoplasmic domain that lacks guanylyl cyclase activity. We used information available for the cDNA of human NPRC to amplify products covering all genomic regions of the gene by long polymerase chain reaction (PCR) and thermal asymmetric interlaced (TAIL)-PCR. PCR products were sequenced directly after extraction and purification. The human NPRC gene spans >65 kb and contains eight exons and seven introns. All of the exon–intron junction sequences contain the GT/AG consensus junction sequence. We then used the PCR-single-strand conformation polymorphism (PCR-SSCP) to identify polymorphisms of the human NPRC gene. All eight exons and neighboring introns were analyzed by PCR-SSCP for 96 subjects, and migration variants were observed for intron 1, exon 2, and exon 5. Direct sequencing of these variants revealed the following sequence differences: a C to T transition in intron 1, an A to C transition in exon 2, and a C to T transition in exon 5. PCR-restriction fragment length polymorphism analysis (PCR-RFLP) was used to evaluate all three variations. We have determined the structural organization and identified polymorphic sites in the human NPRC gene. The results of this study will facilitate further genetic analyses of the human NPRC gene function.

Key Words: Natriuretic peptide receptor; type C gene structure.

Introduction

Natriuretic peptides (NPs) play important roles in natriuresis, diuresis, vasodilatation, regulation of endocrine secretion, and inhibitions of cellular growth, which all contribute to cardiovascular homeostasis *(1)*. There are three subtypes of natriuretic peptide receptor (NPRs): NPRA, NPRB, and NPRC. NPRA and NPRB are guanylyl cyclases, whereas the NPRC lacks this activity. NPRC has a single transmembrane domain and a short 37 amino acid cytoplasmic domain that has an inhibitory effect on adenylyl cyclase. NPRC is thought to act as a clearance receptor, although it may have additional functions. Nagase et al. *(2)* reported that expression of the NPRC gene was regulated by salt loading in a tissue-specific manner. A marked decrease in level of NPRC mRNA after salt loading was observed only in the kidneys of Dahl salt-sensitive rats, and this change may play an important role in the pathogenesis of salt-induced hypertension in this model, possibly due to impaired renal excretion of sodium. The roles of NPs in cardiovascular homeostasis have been well characterized. A recent study showed that mice lacking NPRC have excessive skeletal overgrowth, suggesting that these receptors may be involved in growth and differentiation of the growth plate during fetal development in the mouse *(3)*. The numbers of NPRC receptors on platelets are decreased in hypertensive patients and spontaneously hypertensive rats *(4)*.

Genetic dissection is a powerful tool for determining the causes of a complex trait such as cardiovascular disease (CD) *(5–7)*, and clarification of the relation between a gene and a disease may contribute to our understanding of the pathophysiological role of the protein encoded by the gene. An understanding of the genomic structure of a gene is essential for genetic studies; therefore, we recently determined the genomic structures of the human NPRA *(6)* and NPRB *(7)* genes. The human NPRC gene has been mapped to chromosome 5p13p14 *(8)*, and the cDNA and deduced amino acid sequence have been described *(9)*. In the present study, we determined the genomic structure and screened for polymorphisms in all exons and introns of the human NPRC gene in Japanese subjects.

Results

Structure of NPRC Gene

The structural organization of the human NPRC gene was determined by analysis of genomic clones and total genomic DNA by PCR amplification across introns followed by sequencing of the intron/exon junctions. In the present

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Table 1
Position and Size of Exon/Intron in Human NPRC Gene

Exon no.	Position in cDNA of exon	Exon size (bp)	Codons interrupted	Amino acid and position[a]	Intron no.	Intron size (kbp)
1	1–769	769	G/TG	Val_{57}	1	15
2	770–892	123	G/GA	Gly_{297}	2	>20
3	893–1059	167	TAC/GTT	Tyr_{353}-Val_{354}	3	>20
4	1060–1195	136	G/GT	Gly_{399}	4	2.1
5	1196–1290	95	GAG/GTT	Glu_{430}-Val_{431}	5	2.2
6	1291–1426	136	T/CA	Ser_{476}	6	1.8
7	1427–1514	88	AG/G	Arg_{505}	7	1.5
8	1515–1623	109				

[a]Amino acids are numbered consecutively beginning with the methionine of the start codon.

Table 2
Sequences of the Exon/Intron Junctions of the Human NPRC Gene[a]

5' Boundary	Exon No.	3' Boundary
	.... 1 .... AGTGAGAGAG	gtgagcaggtgcgcgtcccgggccccgggc
gcctcatgtgtgttgtttttcctcccctag	TGGTGATCAT .... 2 .... TCTTCCTATG	gtaactctgcttctctttccccctcctctgc
ggaattataaatatttttattcttccatag	GAGATGGCTC .... 3 .... GGAGGATTAC	gtaagtgctgattatgagcctagaccttta
tttggttcacccatctgggtttcttttcag	GTTAACATGT .... 4 .... ACATTTGAAG	gtggggattccatctataaggcaattacat
aacgttgattcttcgcttctggtcctgtag	GTATCGCCGG .... 5 .... CACCCAGGAG	gtgagccgtgagactctgcacagttgctcc
tttgtctatttgtttttgcctctatatag	GTTATTGGTG .... 6 .... TGCAAATCAT	gtaagtctggagacttaatttgctatgtat
aaccctgattatccatgcttctttttcaag	CAGGTGGCCT .... 7 .... ACTTTTTCAG	gtgaggaggtttgtaaggtacattcactct
ttattaccacattcactttcctttacccag	GAAGAAATAC 8	

[a]Exon sequences are indicated in capital letters, and intron sequences are indicated in lowercase letters. The amino acid codons interrupted by introns are listed in Table 3. The nucleotide sequence has been submitted to the GenBank/EMBL/DDBJ Data bank.

Table 3
TAIL Primers and Primers for Sequencing
Analysis of the Human NPRC Gene Exon/Intron Junctions[a]

Primer no.	Position in cDNA and 5' Sequences 3'	Location of exon and intron 2	
1	CCAGTGGAGACTACGCCTTCTTC	869-891	exon 2
2	XGTCGASWGAXAWGAA	intron 2 (D1)	
3	GCTTCAAAGTCGTGTTTGTCTCCTC	911-935	exon 3
4	AG(AT)G(AGCT)AG(AT)A(AGCT)CA	intron 2(D5)	
5	GAGAAGTTTTCCATGGAGGTG	997-1017	exon 3
6	(AGCT)TC GA(GC) T(AT)T (GC)G(AT) GTT	intron 3 (D8)	
7	GAGGAGGATGGCATCGTGGAATCC	1078-1101	exon 4
8	TC(GC)T5CG(AGCT)AC5T(AT)GGA	intron 3 (D7)	

[a]Position in the forward primer is identical to at of the cDNA (ref. *7*); position in the reverse primer is complementary to the cDNA sequence.

study, we determined the genomic structure of the human NPRC gene by direct sequencing of the LA-PCR products, not by screening a genomic library. LA-PCR can be used to amplify lengths of DNA greater than 20 kbp. Confirmation that the PCR products were derived from the NPRC gene was done by sequencing of at least 40 nucleotides within 5' and 3' portions of the exon. The sizes and positions of the exon and the codons and corresponding amino acids interrupted by the introns are listed in Table 1. The NPRC gene is approximately >65 kbp in length, and contains seven introns of variable size. The exons range in size from 88 bp (exon 7) to 769 bp (ATG-exon 1), and the introns range in size from 1.5 kbp (intron 7) to 6.5 kbp (introns 2, 3). All of the intron–exon junction sequences contained the GT-AG consensus sequence and are shown in Table 2. TAIL-PCR with the AD1, AD5, AD7, and AD8 primer yielded a single clear band in each reaction (Table 3). These bands were all approximately 5 kbp, and the sequence was

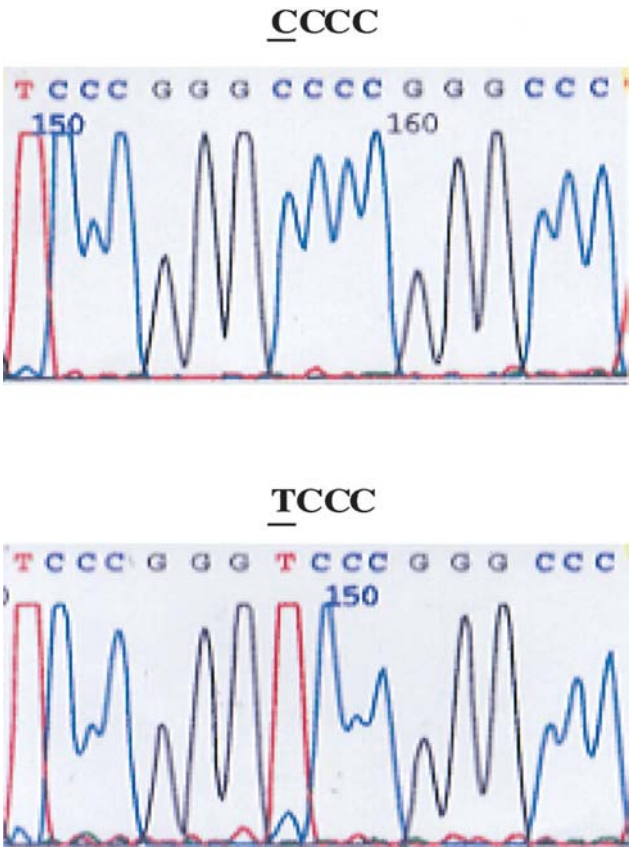

Fig. 1. Nucleotide sequence of the polymorphic region of intron 1 of human NPRC. The C to T transition is shown on the left.

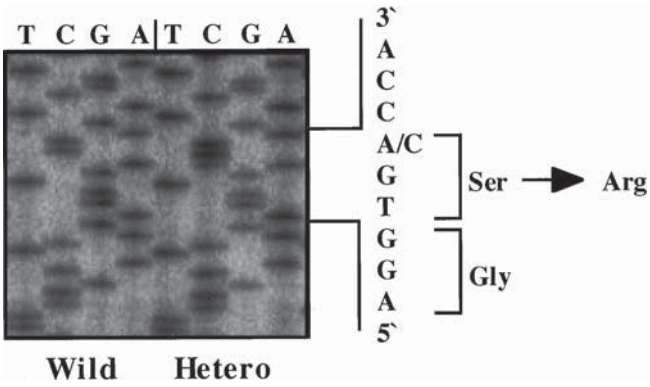

Fig. 2. Sequence of the polymorphic region of exon 2 of human NPRC. The wild-type and heterozygous sequences are shown. This mutation alters the relevant amino acid sequence from i.c. a serine to an arginine.

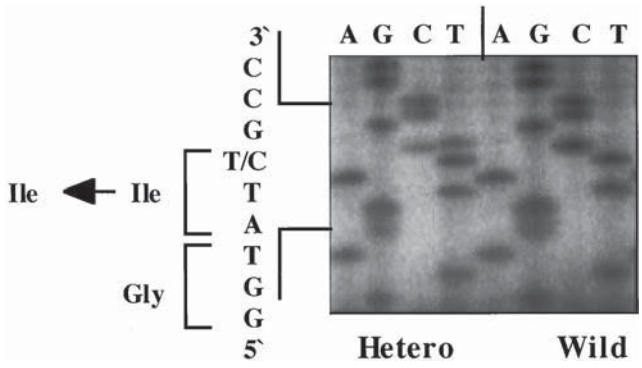

Fig. 3. Sequence of the polymorphic region of exon 5 of human NPRC. The wild-type and heterozygous sequences are shown.

determined after purification. Approximately 250 bp of each fragment was sequenced (Table 3).

Characterization of Polymorphisms

SSCP analysis revealed abnormally migrating bands for intron 1, exon 2, and exon 5. Direct sequencing of these DNA fragments revealed the following sequence alterations: a C to T transition in intron 1 (Fig. 1), an A to C missense mutation is exon 2 at nucleotide 841, which causes a serine to arginine change (Fig. 2), and a C to T transition at nucleotide 1200 in exon 5, which does not alter the amino acid sequence (Fig. 3). PCR- RFLP assays were developed to detect the C to T transition in intron 1 and the A841C variations.

Table 4
Allele Frequencies
for the C to T Polymorphism in Intron 1 and the A841C
Polymorphism of the NPRC Gene in Japanese Subjects

	C to T			A841C		
Genotype	CC	CT	TT	AA	AC	CC
n = 105	0.87	0.12	0.01	0.99	0.01	0
f (C/T)	(0.93/0.07)		f (A/C)	(0.995/0.005)		

Frequency of Novel Polymorphisms in the Human NPRB Gene

The relation between the C to T transition in intron 1, the A841C missense mutation, and C200T transition is shown in Table 4. These polymorphisms were not linked completely.

Discussion

In the present study, the genomic structure of the human NPRC gene was determined by analysis of total genomic DNA by PCR amplification across the intron/exon boundaries followed by sequencing. This method is advantageous because radioisotopes and screening of genomic libraries are not necessary. However, LA-PCR cannot amplify sequences more than 20 or 30 kbp long, which may include some introns. We then used TAIL-PCR to amplify long DNA fragments that could not otherwise be amplified. The genomic structure of NPRC is very similar to that of the bovine gene. Nucleotide sequence identity between the human NPRC cDNA and bovine NPRC cDNA is 89.1% *(12)*. Comparison of the predicted amino acid sequences of human NPRC, NPRA, and NPRB revealed that the extracellular domain of NPRC has 34.3% amino acid identity with that of NPRA and 29.5% identity with that of NPRB, whereas NPRA domain has 43.3% identity with the same region of NPRB. The deduced amino acid sequence of NPRC is very similar to that reported for the bovine protein with differences in only 27 of 496 amino acids (~5%) *(9)*. The human and bovine NPRC genes are also very similar in their exon–intron organizations. Previously, we used PCR-SSCP to identify mutations in the human NPRA, NPRB, and prostacyclin synthase genes, and found correlations with cardiovascular diseases *(7,13,14)*. The exon/intron organization and polymorphic sites data for the human NPRC gene from the present study will assist in further research into the relation between the human NPRC gene and diseases.

Materials and Methods

Examination of Genomic Sequences Including Protein-Coding Region

Genomic DNA was extracted from peripheral blood leukocytes by standard methods. We used available information for the human NPRC cDNA sequences to design oligonucleotide primers; specific primers were designed to amplify the intronic regions. More than 25 primers were designed and synthesized (Sawaday Technology, Tokyo, Japan) to cover the genomic sequence from the start codon to the termination codon. Long and accurate polymerase chain reaction (LA-PCR) *(10)* was performed with these primers, and a single PCR product was generated for each primer pair. Genomic DNA from several individuals was used as a template to confirm identity with the structure of the PCR products. PCR consisted of an initial denaturation step of 3 min at 94ºC followed by 30 cycles of denaturation for 25 s at 98ºC, annealing and extension for 20 s at 68ºC, and a final extension at 72ºC for 10 min. LA-PCR was performed according to the manufacturer's instructions (LA-PCR Kit Ver. #2, Takara Syuzo, Tokyo, Japan). LA-PCR products were separated by electrophoresis on 0.4 % agarose gels. For sequencing, the bands were excised from the gel and purified over a column (Microcon 100, Amicon Inc., Beverly, MA).

Isolation of Uncharacterized Regions

To isolate uncharacterized regions of the NPRC gene, thermal asymmetric interlaced (TAIL)-PCR was used. PCR conditions were described previously *(11)* except that the annealing temperature was modified to 68ºC. Five-hundred nanograms of genomic DNA was used as the template in the primary PCR reaction. Four arbitrary degenerate (AD) primers were used: AD1, AD5, AD7, and AD8. Four specific primers complementary to the NPRC gene were synthesized (Table 5), and the PCR products were separated by electrophoresis on 1.5% agarose gels. For sequencing, bands were excised from the gel and purified as described above.

Sequencing

PCR products were sequenced directly with a Thermo Sequenase Kit (Amersham LifeScience Corp., Cleveland, OH) and ABI (PE Biosystems) autosequencer. At least 40 nucleotides of the coding region of the NPRC gene were sequenced together with the intron–exon boundaries to confirm that the products were derived from NPRC genes.

Subjects for PCR Single-Strand Conformation Polymorphism (PCR-SSCP) Analysis and Genotyping

Genomic DNAs from peripheral blood leukocytes of 96 healthy subjects were used for SSCP analysis. DNAs from a different group of 105 healthy subjects were used for genotyping of the C to T transition on intron 1 and C841T polymorphisms.

DNA Analysis for Detection of Polymorphisms by PCR-SSCP

We designed oligonucleotide primers from the sequence information for the introns of the human NPRC gene (Table 4). Cycling conditions were an initial denaturation at 94ºC for 3 min followed by 25 cycles of 94ºC for 30 s, 65ºC for 1 min, and 72ºC for 1 min. Each reaction was completed by a final extension at 72ºC for 10 min. PCR products were

Table 5
Primers for PCR-SSCP Analysis of the NPRC Gene[a]

Position in cDNA and Primer Product (bp)	Length of the PCR 5' Sequences 3'	Location of exon	
F1-1	GGGAACCTTGGAGAGAAGAG	exon 1	312
R1-1	CCCGGGTGAGTGAAAACAAGTACG	exon 1	
F1-2	GATCGAGGTGCTGGTGTTACTGCC	exon 1	267
R1-2	GCTGCTGCATACTCGCACACTGG	exon 1	
F1-3	TGTGGGAACCGTGCGCTCTTCAGC	exon 1	188
R1-3	TCAGAGTCCTTGTGCTGGAAGCCAGC	exon 1	
F1-4	GCTTGCATCGCACTGGGACCTGC	exon 1	265
R1-4	GATGGACGTGTGCAAACCCTCCTC	exon 1	
F1-5	GACAAGCTGGAGCGGAACTGCTAC	exon 1	308
R1-5	GTCTGCAGAGTGGACGAGTGTGC	exon 1	
F2	TCAGCATGCTCGAAGTGCTCTGC	exon 2	248
R2	CATGGGCATCTGACAACCTCTCG	exon 2	
F3	CAGTTGTGAAGGGAGAATGGCCTC	exon 3	274
R3	CAGGATGCTAAAGGTCTAGGCTC	exon 3	
F4	CCTGGCATGAGTCACTTGGTGTTTGG	exon 4	280
R4	GAAAGGCCCCACAGCTGCAACTGAG	exon 4	
F5	CCAACGTTGATTCTTCGCTTCTGGTCC	exon 5	216
R5	TGGAGACAGCTCTTTGGCCACCAG	exon 5	
F6	GGGGAATAGCATGTGGAAGGCTGC	exon 6	270
R6	CACTGAGGTTAGAGGTGATGAC	exon 6	
F7	GCCTCCAATGGGAAATCGAGGAAC	exon 7	293
R7	CCTGGGTGAAAGAGGGATGTGGAC	exon 7	
F8	GAAACCCAGATGTCCCTTGAGGGCAC	exon 8	188
R8	GGGATCTGATGGAATCTTCCCG	exon 8	

[a]Forward primer sequences are identical to those of introns. Reverse primers are complementary to cDNA sequences. Oligonucleotide primers based on sequence information for the introns surrounding each exon of the NPRC gene.

placed on ice after denaturation at 100ºC for 5 min, and 2.5 µL aliquots were loaded onto nondenaturing 6% acrylamide gels. Each sample was separated by electrophoresis is 5% glycerol at room temperature for 10 h at 15 W constant power.

Direct Sequencing of Electrophoretic Variants

DNAs from a subject with a variant electrophoretic pattern and from controls were reamplified by PCR performed under the above conditions (unlabeled primers). PCR products were purified with a Microcon 100 column (Amicon Inc.). Direct sequencing was performed with [γ-32P]dATP end-labeled primers from SSCP with a Thermo Sequenase kit (Amersham Life Science Corp.) and ABI autosequencing kit (PE Biosystems). The C to T polymorphism in intron 1 was evaluated with *HaeIII*, the A841C polymorphism in exon 2 was evaluated with *BrsI* restriction fragment-length polymorphism (RFLP), and the C1200T polymorphism in exon 5 did not contain a variant restriction site. The primers for intron 1 were sense primer 5'-AAGATGGGCGAGATG ATGCTCGCCC-3' and antisense primer 5'-ACACTCGTC CACTCTGCAGAC-3'. PCR products were loaded onto 13% polyacrylamide gels the *HaeIII* digestion. The exon 2 A841C polymorphism was amplified with sense primer 5'-TCAGC ATGCTCGAAGTGCTCTGC-3' and antisense primer 5'-CGAGAGGTTGTCAGATGCCCATG -3'. The PCR product was digested with *BrsI* and separated by electrophoresis on 1.5% agarose gels. It was then visualized by ethidium bromide staining and UV illumination. The exon 5 polymorphism was amplified with sense primer 5'-CCAACGT TGATTCTTCGCTTCTGGTCC-3' and antisense primer 5'-CTGGTGGCCAAAAGACTGTCTCCA-3' with the PCR conditions described above. A 248-bp fragment containing all of exon 2 and the 5'- and 3'-flanking intronic regions was amplified.

Statistical Analysis

Data are presented as mean ± SD. Allele frequencies were calculated from the genotypes of all subjects. Hardy-Weinberg equilibrium was assessed by chi-square (χ^2) analysis, p value <0.05 for significance.

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