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IDENTIFICATION OF POLYMORPHIC SITES OF THE HUMAN BRADYKININ B_2 RECEPTOR GENE*

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Summary The characterization of the genomic organization of the B_2 bradykinin receptor gene enabled us to systematically search for polymorphic markers in this gene in a South German cohort (N=179). We identified at least three polymorphic sites in each of the three exons existing: (i) in exon 1 next to the promoter region, a tandem repeat polymorphism consists of three common alleles, (ii) in exon 2 at nucleotide position 181 of the cDNA a C to T transition leads to an aminoacid substitution from arginine to cysteine in the receptor protein at position 14 (R14C), and (iii) a more complex repeat polymorphism, located in the 3′ not-translated region of exon 3, comprises at least two common alleles and two rare variants. These new genetic markers provide valuable tools to elucidate a potential role of a hereditary dysfunction of the B_2 bradykinin receptor gene in disorders such as hypertension or ischemic heart disease.

The nonapeptide bradykinin has been implicated in a variety of physiological and pathological processes including vasodilatation, increased vascular permeability, edema, pain, smooth muscle contraction, and cell proliferation. Other bradykinin-

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induced effects include bronchopulmonary contraction or increased sperm motility (1). Furthermore, increased levels of bradykinin may account for some of the cardioprotective effects of angiotensin converting enzyme inhibitors (2). Bradykinin is released by limited proteolysis from kininogen precursors in the circulation, in interstitial tissue fluids or from the surface of kininogen-binding cells. The large array of physiological activities is mainly mediated by specific receptors that pharmacologically classify as B_2 bradykinin receptors which exhibit high affinity for kallidin (Lys-bradykinin) and bradykinin. (3,4,5). Under pathological conditions B_1 bradykinin receptor-mediated effects may also play a major role (1,5). Depending on the different cell types and tissues, B_2 bradykinin receptors couple to G-proteins thereby triggering activation of phospholipase C and/or phospholipase A_2 accompanied by increased intracellular levels of Ca^{2+},NO, cGMP, and/or cAMP (3,4). The B_2 bradykinin receptor has been implicated as one of the candidate genes being involved in the complex genetic underpinnings of common chronic disorders such as hypertension or ischemic heart disease (6,7).

Recently, the human B_2 bradykinin receptor was cloned (8), and by homology search this receptor was grouped to the superfamily of the G-protein coupled seven transmembrane domain receptors. Studies on the genomic structure of the gene revealed that it consists of three exons (9,10). The knowledge on the genomic structure enabled us to systematically search for polymorphic markers in this gene that are necessary to elucidate the potential role of a hereditary dysfunction of B_2 bradykinin receptors in various disease.

MATERIALS AND METHODS

Genomic DNA. 179 individual genomic DNA samples of southern German origin were studied. The collection of samples and preparation of DNA has been described previously (11).

Amplification of exons bearing polymorphisms of the B_2 bradykinin receptor gene. The following forward (F) and reverse (R) primers were used for PCR amplifications: BE1F, 5'-GCCCTTGAAAGATGAGCTG-3' and BE1R, 5'-AACTCCC CACGACCACAG-3' for exon 1; BE2F2, 5'-CCATTTCTCCTCCCTGCTCGAG-3' and BE2R2, 5'-GGTGGGCACGGAGTCCTCTC-3' for a part of exon 2, B39F, 5´-GAAGGTGGCCCAGTATGAGC-3´ and B35R, 5´-GATTGGTCAGGATTTATGG-3´ for a part of exon 3. Primers were designed from the B_2 bradykinin receptor gene sequence (9). Primers BE2F and BE2R are mutated in the nucleotides underlined to create *Taq* I recognition sites. The total reaction volume was 50 µl and included 1 µg genomic DNA, 50 ng of each primer, 1.25 U *Taq* DNA polymerase (Boehringer-Mannheim, FRG), 200 µmol of each dNTP, and 1.5 mM $MgCl_2$. Cycling conditions for PCR of all three exons were initial 5 min at 94°C, followed by 1 min at 94°C, 1 min at 53°C, and 1 min at 72°C for 40 cycles and a final extension time of 5 min at 72°C.

Detection of exon 1 polymorphism. PCR products were subjected to single-strand conformation polymorphism (SSCP) electrophoresis. A 5µl portion of the PCR product was diluted with 15 µl formamide and denatured at 95 °C for 5 min. The SSCP gel, separating the polymorphic exon 1 alleles, contained 12% polyacrylamide buffered with 1xTBE (85mM TRIS, 90mM boric acid, 2 mM EDTA). Electrophoresis was carried out in 1xTBE buffer at room temperature at constant 120 V for 24h. After fixation in water:ethanol:acetic acid (895:100:5) for 15 min, the gels were stained in 0.1% silver nitrate for 30 min. Gels were developed in 300 ml of 375 mM NaOH solution containing 30 mg sodium borohydride and 1.2 ml formaldehyde for up to 1h.

Detection of exon 2 polymorphism. 10 µl of the PCR product were digested with 4.0 U *TaqI* (Boehringer-Mannheim, FRG) at 65°C for 1 hour. Electrophoretic separation was done by 2,5% MetaPhor-Agarose gels (Biozym, Hameln, FRG) with 1xTBE buffer (85mM TRIS, 90mM boric acid, 2 mM EDTA) containing 35 µg/ml ethidium bromide. at 100 V for 2 hours. The separation was directly visualized by UV fluorescence.

Detection of exon 3 polymorphism. Exon 3 specific PCR products (10 µl) were directly loaded on a 1% agarose gel (Biozym, Hameln, FRG). Electrophoretic separation was done with 1xTBE buffer (85mM TRIS, 90mM boric acid, 2 mM EDTA) containing 35 µg/ml ethidium bromide. at 100 V for 2 hours. The separation was directly visualized by UV fluorescence.

RESULTS AND DISCUSSION

A tandem repeat polymorphism (named BE1) was detected in exon 1 starting at nucleotide position 12 backward the transcription start site. In 179 samples of unrelated individuals from southern Germany three different common alleles could clearly be differentiated by SSCP electrophoresis (fig. 1). DNA sequencing revealed

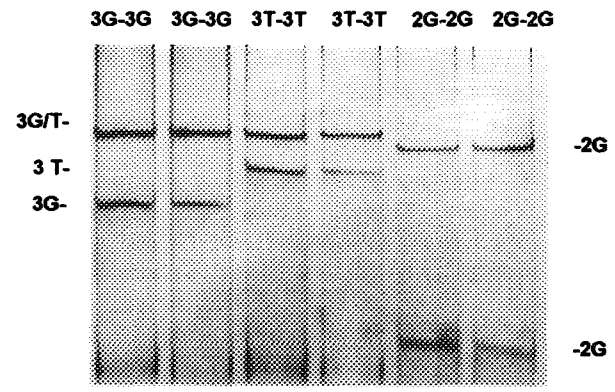

Fig.1. **SSCP analysis of the exon 1 polymorphism (BE1).** Silver stained 12% polyacrylamide gel disclosing the homozygous single strand conformational pattern of the three different alleles 2G, 3T, and 3G located in exon 1 as indicated at the top. At the left margin the specific bands for the 3T and 3G alleles and at the right margin the specific bands for the 2G allele are indicated. The SSCP pattern was highly reproducible and initially confirmed by DNA sequence analysis.

that allele 2G consists of two and the allele 3G of three repeat units (GGTGGGGAC), respectively. In the allele 3T a G to T transversion (GGTGGTGAC) occurs in the intermediate one of the repeats. Thus, exon 1 exists at least in two different length, i.e. 102 or 93 bp, respectively. The frequencies of the alleles and genotypes in comparison with the values predicted by the assumption of Hardy-Weinberg equilibrium in the population are shown in table 1.

Initially exon 2 was screened for polymorphisms by SSCP electrophoresis and a variant pattern was detected. Subsequent sequence analysis showed at nucleotide position 181 (according to ref. 8) a C to T transition (named BE2). This mutation in the coding part of the receptor gene (9) leads to a arginine to cysteine substitution at amino acid position 14 (R14C). Large scale screening for this polymorphism in exon 2 was done by PCR using mutated primers, because of higher efficiency and reproducibility as compared to the SSCP method performed with the originally designed primers (9). The sense primer (BE2F2) contains a C:G mismatch introducing an invariant *Taq*I recognition site in the PCR product which serves as an internal control of the enzyme cleavage efficiency. The antisense primer (BE2R2) introduces a T:A mismatch and creates a *Taq*I site for the case that a C is present at position 181. The amplified fragment had a length of 184 bp. After digestion with *Taq*I we obtained a 145 bp fragment for the C-allele and a 165 bp fragment for the T-allele (data not shown). Distribution of genotypes and allelic frequencies are given in Table 2.

In exon 3 we detected a more complex polymorphism (named BE3) that consists of multiple repeat units of 15 or 16 bp in length, respectively, and that are characterized by the following consensus sequence: TGGA(A)GGGCTAGAACC. The polymorphism is located in the 3´ not-translated region of this exon. The two most common alleles consist of 48 or 35 repeat units (named R48 and R35),

Table 1. Distribution of the exon 1 genotypes and allelic frequencies (BE1) in a sample of 179 unrelated individuals of southern Germany

Exon 1 - Polymorphism						
Genotypes	2G-2G	2G-3T	2G-3G	3T-3T	3T-3G	3G-3G
numbers observed	48	52	42	9	18	10
numbers expected	50,4	46,7	42,5	10,8	19,7	8,9
Alleles	Frequency		Statistical analysis			
BE1-2G BE1-3T BE1-3G	0,5307 0,2458 0,2235		χ^2-value exact p-value*		1,293 0,730694	

* according to the method of Müller and Clerget-Darpoux (12).

Table 2. Distribution of the exon 2 genotypes and allelic frequencies (BE2) in a sample of 179 unrelated individuals of southern Germany

Exon 2 - Polymorphism			
Genotypes	CC	CT	TT
numbers observed numbers expected	140 142,1	39 34,8	0 2,1
Alleles	Frequency	Statistical analysis	
BE2-C BE2-T	0,8911 0,1089	χ^2-value exact p-value*	2,675 0,12

* according to the method of Müller and Clerget-Darpoux (12).

respectively. This length difference can clearly be seen by electrophoretic separation in 1% agarose gels (fig. 2). Examination of a panel of human fibroblast cell lines by PCR and RT-PCR revealed that the polymorphism is present at the genomic level as well as at the level of mRNA (data not shown). Thus, the possibility of an additional small intron in exon 3, being present in some individuals, is excluded. Therefore, exon 3 mainly exists in two different lengths, i.e. 3839 bp for R48 or 3642 bp for R35, respectively. Among the 179 individual DNA samples we also observed two variant alleles with an intermediate number of repeat units (RV1 genotypes and allelic frequencies are given in Table 3. A homology search showed that this repeat does not exist in the rat and mouse B_2 receptor cDNA. However, by PCR amplification followed by sequencing we were able to detect a homologous

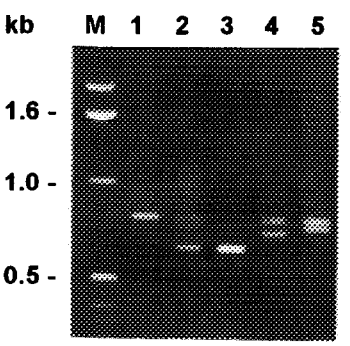

Fig.2. **Analysis of the exon 3 polymorphism (BE3).** Ethidium bromide stained 1% agarose gel disclosing the various genotypes of the polymorphic alleles located in exon 3. lane M) DNA length standard (1 kb ladder, BRL-Gibco), lane 1) genotype homozygous R48-R48, lane 2) genotype heterozygous R48-R35, lane 3) genotype homozygous R35-R35, lane 4) genotype heterozygous R48-RV1, lane 5) genotype heterozygous R48-RV2. The alleles R48 and R35 were confirmed by DNA sequence analysis. A heteroduplex band is seen in the heterozygous genotype R48-R35 (lane 2).

Table 3. Distribution of the exon 3 genotypes and allelic frequencies (BE3) in a sample of 179 unrelated individuals of southern Germany

Exon 3 - Polymorphism										
Genotypes	R48 R48	R48 R35	R48 RV1	R48 RV2	R35 R35	R35 RV1	R35 RV2	RV1 RV1	RV1 RV2	RV2 RV2
numbers observed	127	40	5	2	5	0	0	0	0	0
numbers expected	126,5	42,0	4,2	1,7	3,5	0,7	0,3	0,0	0,0	0,0

Alleles	Frequency	Statistical analysis	
BE3-R48	0,8409		
BE3-R35	0,1397	χ^2-value	2,009
BE3-RV1	0,0197	exact p-value*	0,9189
BE3-RV2	0,0059		

* according to the method of Müller and Clerget-Darpoux (12).

region with comparable length in the B_2 receptor gene of the gorilla. It is noteworthy that this multiple repeat sequence was not included in the EMBL/Genbank data library.

With respect to the polymorphisms in all exons the statistics (tables 1, 2, and 3) revealed that the various genotypes in our population study are in equilibrium according to the Hardy-Weinberg principle, and the proportions, therefore, might remain constant from one generation to another. A more detailed population genetics including haplotype analysis of these polymorphic markers would require the use of both larger and different ethnic groups.

Nevertheless, the allelic distribution in our population study shows that all of these loci are clearly polymorphic. Therefore, they could be utilized as new experimental tools in genetic studies evaluating the B_2 bradykinin receptor gene as a candidate disease gene e.g. in the pathogenesis of common cardiovascular disorders with a genetic determinism. The hypotheses (7) are largely delineated from the strong evidence demonstrating beneficial effects of angiotensin converting enzyme (ACE, Kininase II) inhibitors in the treatment of heart failure, arterial hypertension or myocardial infarction (2,13,14). These cardioprotective effects may in part be explained by inhibition of bradykinin degradation thereby increasing local concentrations of bradykinin (2). It is assumed that these effects of bradykinin are mediated by B_2 bradykinin receptors. In this context it is also interesting to note that an ACE gene polymorphism (DD-genotype) - leading to reduced local bradykinin concentrations - is reported to be associated with an elevated risk of left ventricular hypertrophy (15) or of ischemic heart disease. (16,17). B_2 bradykinin receptor polymorphisms might eventually result in similar biological consequences as induced by variations in the ACE gene.

The present study, however, does not yet allow any conclusions whether or not these B_2 bradykinin receptor gene polymorphisms represent biologically relevant mutations. This will require ongoing further functional studies in transfected cells and/or large case-control (association) and linkage studies in subjects with or without defined disease conditions.

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