Polymorphisms in the Genes Encoding for Human Kinin Receptors and the Risk of End-Stage Renal Failure: Results of Transmission/Disequilibrium Test

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Abstract. There is evidence that environmental factors and genetic predisposition affect the development of end-stage renal disease (ESRD). The role of kinin peptides in renal pathology has been also suggested, and a nephroprotective effect of kinins, mediated by B_1 and B_2 kinin receptors, has been postulated. Recently, two novel sequence differences in the B_1R gene were identified, and the C allele of the G→C substitution at position −699 in the promoter region of the B_1R gene was found to be less frequent among patients with ESRD compared with healthy control subjects. In this study, the association between B_1R and B_2R polymorphisms and ESRD was examined using a family-based study design: transmission/disequilibrium test. B_1R gene G→C substitution at position −699 in the promoter region and B_2R gene C→T transition at position 181 in exon 2 were genotyped in 247 family trios: offspring affected with ESRD and both parents. The less common alleles of both polymorphisms (B_1R C allele and B_2R T allele) were transmitted from heterozygous parents to offspring affected with ESRD less frequently than expected (37 and 36%, respectively; $P < 0.05$). In conclusion, results obtained in this study support a hypothesis of the protective role of bradykinin receptor gene polymorphisms in the development of ESRD.

End-stage renal disease (ESRD) is a complex phenotype, which results from the presence of underlying kidney disease, and superimposing inherited and environmental factors. Familial clustering of ESRD was demonstrated recently in different racial populations, including Caucasians (1–4). Data obtained in these studies suggest that a genetic susceptibility, independent of the etiologic factors of kidney disease, alters the risk of the development of end-stage renal failure.

Recently, the role of kallikrein-kinin system in the inflammatory process in the kidney has been stressed, and a protective effect of kinin peptides against the development of renal pathology (5–7) or hypertension (8–12) has been suggested. It has also been hypothesized that the nephroprotective effect of angiotensin-converting enzyme inhibitors may be at least in part a result of the inhibition of degradation of kinins (13–16). In a recent study by Bachvarov *et al.*, two sequence differences in the kinin B_1 receptor (B_1R) gene were found and were tested for association with ESRD (17), together with a previously identified polymorphic site in the B_2 kinin receptor (B_2R) gene (18). In that case-control study, the C allele of the G^{-699}→C transition in the promoter of the B_1R gene was found to be less frequent in patients with end-stage renal failure when compared with healthy control subjects, and the nephroprotective role of this polymorphism was postulated.

However, when using a case-control study design, "spurious" disease-marker association may arise, even in the absence of genetic linkage, as a result of population stratification, *i.e.*, when the study groups are drawn from genetically distinct subsets of population (19). To test the hypothesis of association between the B_1R and B_2R gene polymorphisms and the development of ESRD, and to avoid the potential bias of case-control studies, in the present study we applied the family-based study design, using the transmission/disequilibrium test (TDT). TDT, first introduced by Spielman *et al.* (20), is a test for linkage in the presence of allelic association, which evaluates the transmission of the associated allele from heterozygous parents to affected offspring (20,21). In this study, polymorphisms in the genes encoding for B_1R and B_2R were genotyped in a large group of family trios: children affected with ESRD and both parents.

Materials and Methods

Selection of Family Trios

Subjects for the study were recruited from 17 nephrology or dialysis centers. In total, 1657 Caucasian patients with a history of ESRD (creatinine clearance <30 ml/min) or submitted to chronic hemodialysis or peritoneal dialysis were identified. Etiologies of ESRD were: glomerulonephritis ($n = 729$), interstitial nephritis (including pyelo-

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nephritis) ($n = 263$), diabetic nephropathy ($n = 170$), hypertension ($n = 116$), polycystic kidney disease ($n = 40$), and other causes or unknown etiology ($n = 234$). For the family-based association study, we selected 247 patients: 120 with glomerulonephritis, 80 with interstitial nephritis (including chronic pyelonephritis), and 47 patients with ESRD due to diabetic nephropathy in type 1 diabetes, whose parents were alive and agreed to participate in the study. Families with ESRD attributed to polycystic kidney disease, and unknown or uncommon etiologies were excluded from the study. A detailed history of kidney disease was collected from all patients, and all parents provided basic epidemiologic data, according to our standardized questionnaire. All patients and parents gave written informed consent, and the study protocol was approved by the Ethics Committee of the Silesian School of Medicine.

DNA Analysis

From all patients and parents, DNA was isolated from peripheral blood leukocytes.

B_1R Gene $G^{-699}\rightarrow C$ Substitution in the Promoter Region. A total of 191 bp of the B_1R promoter was PCR-amplified with primers described in the study by Bachvarov *et al.* (17). Five microliters of the PCR product was digested at 37°C, overnight, with 10 U of *Aci*I (New England Biolabs, Beverly, MA) and electrophoresed on 3% DNA Typing Grade Agarose (Life Technologies, Grand Island, NY). The C allele produced bands at 135 and 56 bp, and the G allele remained as an undigested 191-bp band.

The B_2R Gene $C^{181}\rightarrow T$ Transition in Exon 2. PCR with primers flanking the polymorphic site in exon 2 (18) was performed, resulting in a 184-bp product. Five microliters of PCR product was cleaved at 65°C for 2 h with 4 U of *Taq*I (New England Biolabs) and electrophoresed on 3% DNA Typing Grade Agarose (Life Technologies). The C allele was presented as a 145-bp band, and the T allele was presented as a 165-bp band.

The B_1R gene exon 3 $A^{1098}\rightarrow G$ transition was determined with the method described in Bachvarov *et al.* (17). Briefly, 486 nucleotides from a terminal fragment of the coding region and 3′ flanking region of the B_1R gene were PCR-amplified, digested with 10 U of *Taq*I at 65°C for 2 h, and electrophoresed on 2% agarose gel.

For the examined polymorphisms, PCR was performed from 100 to 200 ng of genomic DNA, in 25-μl reaction volume containing 10 pmol of the specific primers, 0.5 U of DynaZyme DNA polymerase (Biometra, Tampa, FL), 1.5 mmol/L $MgCl_2$, and 2.5 mmol/L of each dNTP, in one of the following cyclers: UNO II (Biometra) or Mastercycler Gradient (Eppendorf, Hamburg, Germany). After 5 min of initial denaturation at 95°C, 35 cycles of 1 min at 95°C, 1 min at 60°C (B_1R promoter and B_2R exon 2), 1 min at 65°C (B_1R exon 3), and 1 min at 72°C were performed, followed by 10 min of final extension at 72°C.

Genotyping was repeated for all trios, in which the examined heterozygous parent did not transmit the less common allele of one of the examined polymorphisms.

Statistical Analyses

In the descriptive statistics of the study group, data are presented as mean and SD for normally distributed continuous variables, whereas for variables not distributed normally, median and quartiles are presented. In the TDT, the observed transmission of alleles from heterozygous parents to affected offspring was compared with an expected proportion of 50% transmission for an allele not associated with the phenotype, and McNemar's test was used for the comparisons. $P < 0.05$ (two-tailed) was considered statistically significant. All calculations were performed using Excel 97 spreadsheet (Microsoft).

Results

Among 247 cases included in the study, 157 patients were submitted to renal replacement therapy (144 hemodialysis and 13 peritoneal dialysis), with median duration of dialysis of 2.0 yr (0.0 to 8.0 yr). Ninety patients were in the predialysis period and had a median creatinine clearance (calculated according to the Cockcroft-Gault formula [22]) of 14.5 ml/min (8.4 to 27.5) for women and 17.3 ml/min (12.6 to 27.7) for men. Mean age, body mass index, and median serum creatinine of the examined patients, as well as the proportion of genders in our study group, are presented in Table 1.

All family trios with at least one parent heterozygous for a given polymorphism were selected for the TDT. In total, 73 and 53 parents were heterozygous for the B_1R promoter $G^{-699}\rightarrow C$ polymorphism, and B_2R exon 2 $C^{181}\rightarrow T$ polymorphism, respectively, and thus were suitable for the determination of allele transmission to affected offspring (Table 2). For these polymorphisms, allele transmission was significantly different from the proportion of 50%/50% expected for no association. The less common alleles—B_1R C allele and B_2R T allele—were transmitted from heterozygous parents to affected offspring less frequently than expected; transmission of B_1R C allele was 37% ($P = 0.03$) and transmission of B_2R T allele was 36% ($P = 0.04$). $A^{1098}\rightarrow G$ transition in exon 3 of the B_1R gene (17) was also tested in a random sample of approximately one-half of our available trios (139 trios; 56%). Among them, 12 families (8.6%) were informative for the evaluation of allele transmission, which did not deviate from the proportion of 50:50 (A and G allele transmission was 5:7, respectively).

Table 1. Selected clinical data of the affected offspring analyzed in the TDT test[a]

Characteristic	Total	GN	IN	DN
n	247	120	80	47
Female/male	111/136	53/67	36/44	22/25
Age (yr)	26.7 ± 12.1	27.5 ± 10.9	19.3 ± 9.8	37.2 ± 9.8
BMI (kg/m^2)	20.9 ± 3.9	21.1 ± 3.6	19.3 ± 4.1	23.4 ± 3.3
Serum creatinine (mg/dl)	8.2 (5.5 to 9.9)	9.0 (7.4 to 10.1)	7.1 (3.9 to 9.1)	7.7 (4.0 to 9.2)

[a] TDT, transmission/disequilibrium test; GN, glomerulonephritis; IN, interstitial nephritis; DN, diabetic nephropathy; BMI, body mass index.

Table 2. Transmission of the B_1R promoter and B_2R exon 2 alleles from heterozygous parents to offspring with ESRD[a]

Category	B_1R Promoter $G^{-699} \rightarrow C$		B_2R Exon 2 $C^{181} \rightarrow T$	
	G Allele Transmitted	C Allele Transmitted	C Allele Transmitted	T Allele Transmitted
Total	46 (63%)	27 (37%)[b]	34 (64%)	19 (36%)[c]
GN	22 (65%)	12 (35%)	18 (69%)	8 (31%)[d]
IN	16 (62%)	10 (38%)	12 (63%)	7 (37%)
DN	8 (62%)	5 (38%)	4 (50%)	4 (50%)

[a] Results are given as *n* (%). ESRD, end-stage renal disease. Other abbreviations as in Table 1.
[b] $P = 0.03$.
[c] $P = 0.04$.
[d] $P = 0.05$.

Discussion

In this study, we have examined the relationship between polymorphisms in the genes encoding for human kinin receptors with the presence of ESRD in patients with chronic glomerulonephritis, interstitial nephritis (including pyelonephritis), or diabetic nephropathy. Using a family-based study design with a TDT, we have demonstrated significantly diminished transmission of the B_1R promoter C allele, as well as the B_2R exon 2 T allele, from heterozygous parents to affected offspring.

In the original report of Bachvarov *et al.* (17), the C allele of the polymorphism in the B_1R promoter was found to be less frequent among 287 cases with end-stage renal failure, compared to 102 control subjects, selected from a healthy population. In the same study, PCR fragments of the polymorphic promoter were subcloned in front of the reporter gene, and the presence of the C allele was found to be associated with higher promoter activity, compared with the G allele. The position of the $G^{-699} \rightarrow C$ polymorphic site in the distal part of the regulatory region, in the proximity of enhancer and silencer sequences (-604 to -448 bp, and -682 to -604 bp upstream from the transcription initiation site, respectively) (23), could suggest a direct impact of this sequence difference on the transcriptional regulation of the B_1R expression, which might in turn promote the nephroprotective effect of endogenous kinins. On the other hand, overexpression of the B_1 receptor induced by inflammatory mediators (interleukin-1β, tumor necrosis factor-α, and bacterial lipopolysaccharide) was found to be mediated by a nuclear factor κB-like site in the proximal part of the promoter (-64 to -55 bp) (24). Interestingly, a deficit of the B_1R promoter C allele was also found among patients with inflammatory bowel disease (25). Taken together, we may suspect that this sequence difference determines a common mechanism modifying the inflammatory response in various tissues, either as a pathogenic mutation itself, or as a marker remaining in linkage disequilibrium with another functionally important polymorphic site.

B_2R gene $C^{181} \rightarrow T$ polymorphism in exon 2 was also tested in the study of Bachvarov *et al.* (17), and carriers of the T allele were found to be less frequent among ESRD cases compared with healthy subjects (12.9% *versus* 19.4%), but the difference did not reach statistical significance. In our study, the magnitude of the effect was bigger, with the difference in the transmission of the two B_2R alleles being significant in McNemar's test ($P = 0.04$). That can most likely be explained by the following issues. The first is study design. TDT may be more sensitive in detecting allelic association than a conventional case-control approach, as shown by two association studies of angiotensinogen M235T polymorphism and diabetic nephropathy in insulin-dependent diabetes mellitus patients: case-control (26) and TDT (27). The second may be a difference in the structure of etiologies of ESRD between our study and the original report (17). A group of patients with ESRD analyzed in our study could be slightly more homogeneous, because we excluded patients with uncommon or unknown etiologies of ESRD, as well as patients with polycystic kidney disease, where the predisposition to end-stage renal failure may depend on the genetic background of the disease itself. A C to T transition in coding exon 2 of B_2R gene leads to arginine to cysteine substitution at residue 14 in the N-terminal extracellular domain of the receptor. Whether this substitution itself alters the properties of the receptor or is a marker remaining in linkage disequilibrium with another functionally important sequence difference located nearby (*e.g.*, in the promoter region of the gene) remains to be determined.

However, both our study and the original report (17) have certain limitations. Analysis of patients affected with ESRD and a control group selected from a healthy population, as well as TDT based on the analysis of affected offspring, may detect association with the genetic *locus* implicated in the predisposition to underlying kidney disease, not with the genetic background of the disease complication. To exclude such a possibility, one should examine the prevalence, or transmission, of B_1R C and B_2R T alleles in patients with preserved renal function despite long duration of kidney disease. However, similar effects observed in patients with different etiologies of ESRD in both our study and that of Bachvarov *et al.* (17) argue against the association between kinin receptor gene polymorphisms and specific etiologic mechanisms leading to kidney disease. It is also unlikely that the kallikrein-kinin system is involved in the pathogenesis of glomerular or interstitial nephritis.

In our study, the impact of population stratification was excluded by the family-based study design, but other forms of

selection bias, *e.g.*, associated with patients' survival, could have been introduced. Thus, it should be considered whether unequal transmission of the examined alleles results from worse survival of carriers of a given allele in the course of ESRD (*e.g.*, during renal replacement therapy). In the study of Bachvarov *et al.* (17), all examined patients were submitted to hemodialysis or had undergone renal graft. Among our affected offspring, 90 patients (36%) were in the predialysis stage, with creatinine clearance <30 ml/min. In these subjects, a similar effect of diminished transmission of B_1R C and B_2R T alleles was observed, but no significance was reached, most likely due to reduced sample size (data not shown).

It would also be plausible to speculate that polymorphisms of kinin receptor genes reduce the risk of ESRD in diabetic patients. Earlier studies in diabetic rat models suggested that the renal kallikrein-kinin system might contribute to adaptation of the kidney to diabetic state (reviewed in reference (28). By contrast, recent experiments on diabetic animals provided some evidence that the amelioration of glomerular structure due to angiotensin-converting enzyme inhibitors does not result from their ability to increase kinin activity (29). On the basis of our study design and the small number of patients with ESRD due to diabetic nephropathy in our sample, we cannot conclude whether kinin receptor polymorphisms have any impact on the course of diabetic kidney disease. Association studies with a larger number of type 1 and type 2 diabetic patients would be required to establish which stage of diabetic nephropathy (*i.e.*, initiation of microalbuminuria or progression to more advanced stages), if any, is influenced by these polymorphisms.

In summary, our family-based study provided further evidence that nucleotide substitution in the promoter of the B_1R gene represents a molecular, nephroprotective mechanism decreasing the risk of the development of ESRD. Results of our study may also suggest the impact of the B_2R gene on the development of end-stage renal failure; however, additional association studies, as well as functional experiments, should be performed to clarify this issue.

Appendix

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