

Lack of association of a 9 bp insertion/deletion polymorphism within the bradykinin 2 receptor gene with myocardial infarction

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A B S T R A C T

The BK (bradykinin) B₂ receptor is the major cellular mediator of the effects of BK. A 9 bp deletion in the promoter of the receptor gene represents an allelic variant that is associated with enhanced mRNA expression levels. We tested whether this polymorphism is associated with the prevalence of MI (myocardial infarction) or with echocardiographically determined left ventricular function in post-MI patients. Patients with documented MI ($n=484$), matched controls and controls without evidence of coronary heart disease ($n=1363$) constituted cases and controls. MI patients and controls were carefully matched for age, gender and cardiovascular risk factors. Genotype distributions of the 9 bp insertion/deletion polymorphism were similar across the groups: $-9/-9$, $-9/+9$ and $+9/+9$ were 22.1, 49.5 and 28.5% in MI patients, and 23.0, 44.6 and 32.5% in matched control subjects respectively. The lack of association was also observed in selected subgroups, stratified by age, gender and cardiovascular risk factors. Furthermore, there was no relation between this polymorphism and left ventricular systolic function in post-MI patients. These findings indicate that the 9 bp insertion/deletion polymorphism of the BK B₂ receptor gene is neither related to the prevalence of MI nor to left ventricular function after MI.

INTRODUCTION

Bradykinin (BK) plays an important role in the cardiovascular system, affecting blood pressure regulation, cell proliferation and matrix synthesis by fibroblasts. Particularly, there is strong evidence that the kallikrein-kinin system is activated in acute MI (myocardial infarction) [1–7]. Moreover, the increase in plasma kallikrein-

kinin levels has been shown to be positively correlated with the early survival rate of MI patients [5]. Interestingly, it has been shown that kinins are released directly from the myocardium during ongoing MI [3,4] and contribute to the extent of ischaemic damage [8–13].

The biological action of kinins is mediated by activation of at least two subtypes of G-protein-coupled BK receptors, B₁ and B₂ [14–16]. The BK B₁ receptor is only

Key words: bradykinin receptor, genetic association study, myocardial infarction, polymorphism.

Abbreviations: BK, bradykinin; cESS, circumferential end-systolic wall stress; CVRF, cardiovascular risk factor; EF, ejection fraction; HbA1c, glycosylated haemoglobin; LDL, low-density lipoprotein; LVSD, left ventricular systolic dysfunction; MI, myocardial infarction; MONICA, Multinational Monitoring of Trends and Determinants in Cardiovascular Disease.

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weakly expressed under physiological conditions, but is induced by inflammatory stimuli [17]. In contrast, the BK B₂ receptor, which is constitutively expressed in most tissues, is considered to be the major mediator of the BK-induced effects in the cardiovascular system [16,18–20]. Protein and mRNA levels of both BK receptors are up-regulated in rat myocardium during the early phase following infarction, also suggesting that BK is involved in functional and structural alterations of the ischaemic myocardium [6,7]. The human BK B₂ receptor gene has been cloned and mapped to human chromosome 14q32 [21–23]. Notably, a significant linkage signal for MI has been recently identified on this site [24]. The gene is more than 25 kb in size and consists of three exons [25]. The absence of a 9 bp deletion in the gene encoding the BK B₂ receptor is associated with expression of higher concentrations of receptor mRNA [26,27], suggesting that this insertion/deletion polymorphism is functionally relevant. Recently, it has been reported that cardiovascular risk associated with hypertension amongst middle-aged men is influenced by this functional variation [28].

Given these encouraging results, we tested the hypothesis that there is association between MI and the +9/+9 genotype of the 9 bp insertion/deletion polymorphism within the BK B₂ receptor gene in a large population-based sample of MI patients and respective controls.

METHODS

Study populations

MI patients

In 1996–1997, MI patients from the population-based MONICA (Multinational Monitoring of Trends and Determinants in Cardiovascular Disease) MI registry (Augsburg) who had experienced their first MI before 60 years of age were examined in a study centre ($n = 609$). MI had been verified according to standard MONICA criteria [29]. Of these, 484 blood samples were available for genotyping in the present study.

Control population

The control population for the group of MI patients was drawn as a subgroup from the third MONICA survey of the general population of the city of Augsburg in 1994–1995 that originated from a sex- and age-stratified cluster sample of all German residents of the Augsburg study area. This survey represents individuals from 25 to 74 years of age and approx. 300 subjects for each 10-year increment (total $n = 1674$) [30–33]. Of these, 1363 blood samples were available for genotyping in the present study.

All individuals were studied by a standardized interview, clinical examination, including echocardiography,

and biochemical [glucose, HbA1c (glycosylated haemoglobin), total cholesterol, HDL (high-density lipoprotein) cholesterol, LDL (low-density lipoprotein) cholesterol and triacylglycerols] and molecular analyses.

The study was approved by the local ethics committee, and all the participants gave written informed consent.

Echocardiography

Two-dimensional guided M-mode echocardiograms were performed by two expert sonographers using the Sonos 1500 (Hewlett Packard, Andover, MA, U.S.A.). M-mode tracings were recorded on strip chart paper at 50 mm/s. All M-mode tracings were analysed by a single cardiologist who was unaware of the clinical data. Measurements were made according to the Penn convention. EF (ejection fraction) was determined according to Simpson's rule [34]. cESS (circumferential end-systolic wall stress) at the midwall was calculated from the M-mode measurements, as described by Gaasch et al. [35].

Definitions

Arterial hypertension was defined as a blood pressure $\geq 140/90$ mmHg or by the use of antihypertensive medication. Only current smokers were considered as smokers. Diabetes mellitus was defined by the use of anti-diabetic medication, by clinical history or by elevated HbA1c ($\geq 6.5\%$). Hypercholesterolaemia was defined as a total cholesterol ≥ 250 mg/dL or an LDL cholesterol ≥ 130 mg/dL or the use of lipid-lowering drugs. Obesity was defined by a BMI (body mass index) ≥ 30 kg/m². LVSD (left ventricular systolic dysfunction) was defined as an EF of less than 40%.

Genotyping of the BK B₂ 9 bp insertion/deletion polymorphism

Genomic DNA was isolated from whole blood using the Gentra PureGene™ DNA extraction kit (BioZym, Hameln, Germany). After amplification using the primers BK2-F (5'-TCC AGC TCT GGC TTC TGG-3') and BK2-R (5'-AGT CGC TCC CTG GTA CTG C-3'; BioTeZ, Berlin, Germany), a PCR fragment of 89 bp was produced. Standard PCR was carried out in a 20 μ l volume containing 50 ng of genomic DNA, 10 pmol of each primer and 8 μ l of PCR Mastermix [*Taq* DNA polymerase (0.06 units/ μ l), 2.5 $\times$ *Taq* reaction buffer (125 mM KCl, 75 mM Tris/HCl, pH 8.3, 3.75 mM Mg²⁺ and 0.25 % Nonidet P40), 500 μ M dNTP and stabilizers; Eppendorf, Hamburg, Germany]. Samples were processed in a GeneAmp PCR System 9600 (PerkinElmer, Indianapolis, IN, U.S.A.). After an initial 5 min denaturation at 95 °C, 35 temperature cycles were carried out, comprising 20 s at 94 °C, 20 s at 60 °C and 20 s at 72 °C, followed by a final step of 10 min at 72 °C. PCR product (10 μ l) was subjected to electrophoresis in 3 % high-resolution agarose gels (Figure 1). Our method to

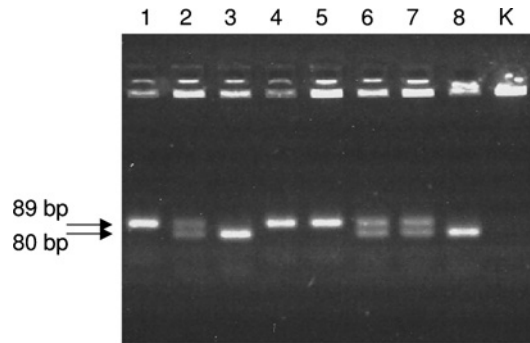


Figure 1 Gel electrophoresis of PCR fragments on an high-resolution 3% agarose gel to determine the genotypes of the 9 bp insertion/deletion polymorphism within the BK B₂ receptor gene

Lanes 1, 4, and 5: PCR product of 89 bp from probands being homozygote for the 9 bp insertion; lanes 3 and 8: PCR product of 80 bp from probands being homozygote for the 9 bp deletion; lanes 2, 6 and 7: PCR product of 89 bp and 80 bp from probands being heterozygote for the 9 bp insertion/deletion polymorphism; lane K: negative control.

detect the 9 bp deletion provided sufficient resolution to distinguish between the two alleles in homozygote cases. The overall length of the PCR fragment was only 89 bp and the run-time of the agarose gel was selected to ensure maximum band separation. Ten percent of the genotypes were either re-run using the same method or separated on a 10% polyacrylamide gel. No mis-genotyping could be detected.

Statistical analysis

Using an automated, randomized selection of controls [developed by the Statistical Training and Technical Services department of SAS Institute (SAS Campus Drive, Cary, NC, U.S.A.), and M. Fischer, unpublished work], subjects were carefully matched by any potential confounding variables [age (± 5 years), gender, diabetes mellitus, arterial hypertension, cigarette smoking, hypercholesterolaemia and obesity) in order to avoid systematic differences in genetic composition between the two groups. Using this strategy, appropriate controls could be found for 94% of MI patients ($n=453$). Baseline characteristics were compared using the Fisher's exact test for discrete variables, and by the Student's *t* test for continuous variables. Frequency of genotypes were compared using the Cochran–Armitage test for trend. Multivariate logistic regression analyses were additionally performed to report adjusted odds ratios and their confidence limits. We hypothesized a disease prevalence rate of $K=0.01$, genotypic relative risks $\gamma_{(-9/+9)}=1.3$ and $\gamma_{(+9/+9)}=1.69$ (conservative estimates based on the findings of Dhamrait et al. [28], who found a coronary artery disease risk rate of 1.70 among normotensives heterozygous for the +9 alleles and 3.51

Table 1 Baseline clinical characteristics of patients with MI, matched control individuals and unmatched controls from the total study group

Age is expressed as the means $\pm$ S.D.

	MI patients ($n=453$)	Matched controls ($n=453$)	Total controls ($n=1363$)
Age (years)	59 ± 9	58 ± 8	52 ± 14
Female (%)	13.7	13.7	49.5
Arterial hypertension (%)	40.2	40.2	37.7
Diabetes mellitus (%)	11.3	11.3	3.6
Current smokers (%)	19.1	19.1	27.2
Hypercholesterolaemia (%)	63.3	63.3	61.9
Obesity (%)	30.2	30.2	19.6

among hypertensives carrying both +9 alleles), as well as a high-risk allele frequency of $P=0.5$. Assuming Hardy–Weinberg equilibrium and a controls per cases ratio of 1:1, we estimated that under a multiplicative model 451 cases and 451 controls are required to obtain 80% power and a type I error rate of 5%.

RESULTS

Table 1 shows the main baseline characteristics of patients with MI, matched control subjects and the unmatched controls of the total study group. MI patients and the matched controls were forced to be homogeneous with respect to age and gender, as well as the cardiovascular risk profile.

In total, 1847 individuals underwent genotyping for the BK B₂ receptor gene 9 bp insertion/deletion polymorphism. The distribution of genotypes was determined as $-9/-9=23.9\%$, $-9/+9=48.3\%$ and $+9/+9=27.8\%$, and the +9 allele frequency was calculated as 0.52. Genotype distributions and allele frequencies were compared between patients with MI, matched control subjects and the total control group. In essence, no obvious differences between the groups could be detected (Figure 2). The genotype distributions of the entire study population and the control and patient groups were consistent with those predicted with the Hardy–Weinberg equilibrium.

Selected subgroups of the total study group were examined (Table 2): male and female study participants, hypertensives, normotensives, subjects without CVRFs (cardiovascular risk factors) and individuals younger than 55 years of age. The results did not show any significant difference of genotype distributions and allele frequencies between control subjects and patients with MI.

Finally, with multiple logistic regression analysis adjusted for age and gender, we assessed whether or not lack of association between MI and the presence of the +9/+9 genotype persisted in the matched study participants, as well as in the selected subgroups (Figure 3).

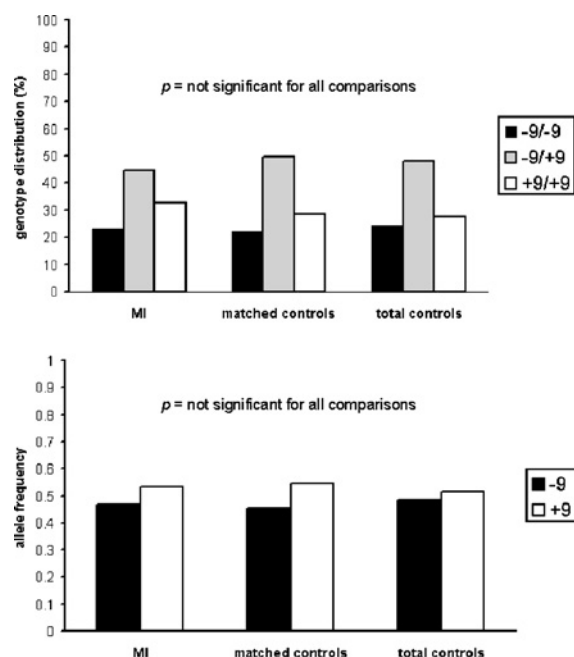


Figure 2 Genotype distribution (upper panel) and allele frequencies (lower panel) of the 9 bp insertion/deletion polymorphism of MI patients, matched controls and the total control sample

Genotypes are shown as percentages. Alleles are presented as relative frequencies.

The odds ratio was not significantly different from 1 in any of the subgroups. The same was true when we tested for the presence of the +9 allele (results not shown). Thus we found no relationship between the 9 bp insertion/deletion polymorphism and the prevalence of MI.

Since there is evidence that BK exerts cardioprotective effects after induction of myocardial ischaemia, we studied whether the 9 bp insertion/deletion polymorphism correlated with LVSD in post MI patients.

Notably, MI patients with the -9/-9, the -9/+9 and the +9/+9 genotypes were comparable regarding the CVRF distribution and anthropometric characteristics, as well as the use of ACE (angiotensin-converting enzyme) inhibitors and β -blockers (results not shown). However, for genotypes or for alleles, an association of the 9 bp insertion/deletion polymorphism could not be detected with the EF or cESS, as well as with the prevalence of LVSD (Figure 4).

DISCUSSION

In the present study, genotype distributions and allele frequencies of the 9 bp insertion/deletion polymorphism, located in exon 1 of the BK B₂ receptor gene, were determined in patients with MI and in a large control group. In essence, the results did not reveal any allele-

Table 2 BK B₂ receptor gene 9 bp insertion/deletion polymorphism genotype distributions and allele frequencies in selected subgroups of MI patients and controls

Genotypes are shown as percentages. Alleles are presented as relative frequencies and shown in *italic*. The *P* values are not significant for all comparisons between subgroups of MI patients and controls. The first *P* value refers to the comparison of the -9/-9, -9/+9 and +9/+9 genotypes within each subgroup, whereas the lower *P* value (in *italics*) in each subgroup refers to the comparison of the +9 allele only.

Genotype distribution and allele frequency			
Genotype	MI patients	Controls	<i>P</i> value
Men	<i>n</i> = 419	<i>n</i> = 688	
- 9/- 9	22.2	25.0	0.57
- 9/+ 9	49.4	47.4	
+ 9/+ 9	28.4	27.6	
+ 9 allele	0.54	0.51	0.08
Women	<i>n</i> = 59	<i>n</i> = 675	
- 9/- 9	28.8	23.6	0.63
- 9/+ 9	47.5	48.9	
+ 9/+ 9	23.7	27.6	
+ 9 allele	0.47	0.52	0.36
Hypertensives	<i>n</i> = 183	<i>n</i> = 514	
- 9/- 9	25.7	23.2	0.57
- 9/+ 9	51.4	50.2	
+ 9/+ 9	23.0	26.7	
+ 9 allele	0.49	0.52	0.49
Normotensives	<i>n</i> = 295	<i>n</i> = 849	
- 9/- 9	21.4	25.0	0.41
- 9/+ 9	47.8	46.9	
+ 9/+ 9	30.9	28.2	
+ 9 allele	0.55	0.52	0.21
Without CVRF*	<i>n</i> = 149	<i>n</i> = 476	
- 9/- 9	18.1	23.9	0.28
- 9/+ 9	50.3	44.5	
+ 9/+ 9	31.5	31.5	
+ 9 allele	0.57	0.54	0.14
Young subjects†	<i>n</i> = 148	<i>n</i> = 740	
- 9/- 9	23.7	24.2	0.75
9/+ 9	47.3	49.7	
+ 9/+ 9	29.1	26.1	
+ 9 allele	0.53	0.51	0.89

* Without the traditional CVRFs: arterial hypertension, diabetes mellitus, smoking, hypercholesterolaemia and obesity.

† Individuals younger than 55 years of age.

specific or genotype-specific relation of this common variant with MI. Such a lack of association was also found for selected subgroups of patients with MI.

There are several reasons for considering the 9 bp insertion/deletion polymorphism within the BK B₂ receptor gene in a genetic association study. First, the

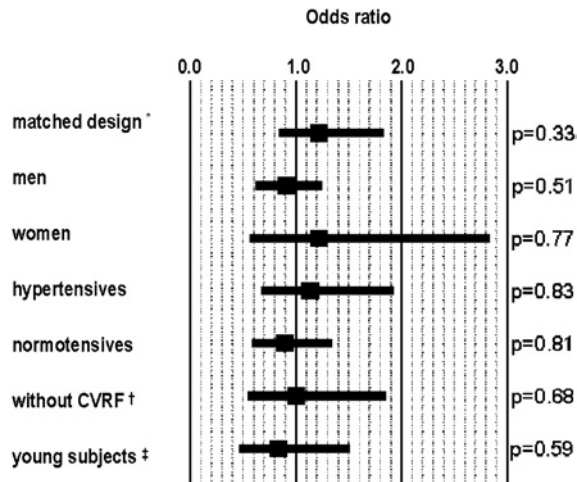


Figure 3 Odds ratios with the 95% confidence limits, adjusted for age and gender, of having the +9/+9 genotype in MI patients and controls in selected subgroups

*Cases versus matched controls; †without the traditional CVRFs: arterial hypertension, diabetes mellitus, smoking, hypercholesterolaemia and obesity; ‡individuals younger than 55 years of age.

activation of the BK B₂ receptors may play a role in the modulation of atherosclerotic risk through coronary vasodilation [17], promotion of micro-angiogenesis [36], increased nitric oxide synthase [37], anti-thrombotic actions [38] and inhibition of vascular smooth muscle cell growth [39]. Second, there is sufficient evidence from clinical and experimental investigations that the kallikrein-kinin system is activated after acute MI and contributes to the impact of ischaemic damage [1-7]. Furthermore, the stimulation of BK B₂ receptors implicates powerful cardioprotective mechanisms [10-13].

Third, consideration of the genomic location of the BK B₂ receptor gene (14q32) has also caught our attention for this gene, since published reports of linkage signals for MI are located in the immediate vicinity [24]. Finally, a common functional variant has recently been described in exon 1 of the gene for the BK B₂ receptor, in which the presence (+9), rather than the absence (-9), of a 9-bp repeat sequence is associated with lower gene transcriptional activity [26] and lower mRNA expression [27], resulting in an excess of prospectively studied cardiovascular risk [28]. In addition, hypertensive left ventricular hypertrophy is an independent CVRF [40], and reduced kinin receptor activity is associated with left ventricular hypertrophic responses in animals [41] and humans [42]. Thus reduced kinin receptor activity may bridge hypertrophic responses and risk of coronary artery disease. In fact, Dhamrait et al. [28] found a remarkable increase of coronary risk (hazard ratio, 3.51; 95% confidence interval, 1.69-7.28; $P < 0.001$) attributable to systolic hypertension in +9/+9 homozygotes, further provoking the present investigation.

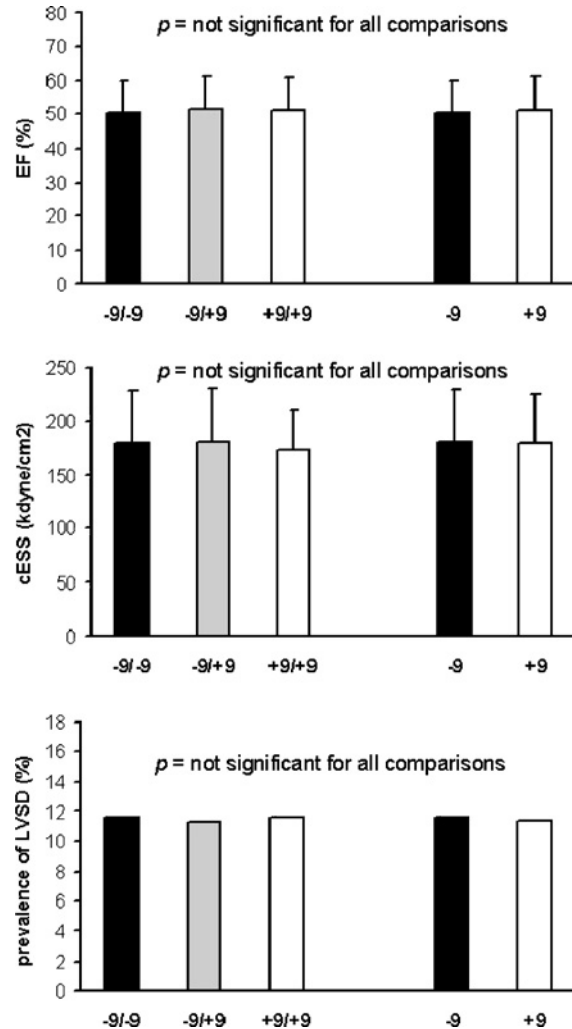


Figure 4 Left ventricular EF (upper panel), cESS (middle panel) and prevalence of LVSD (lower panel) in post MI patients with the -9/-9 genotype ($n = 94$), -9/+9 genotype ($n = 212$) and the +9/+9 genotype ($n = 112$), as well as in post MI patients carrying the -9 and the +9 allele

The present study is the first reporting findings of a genetic association analysis of a BK B₂ receptor gene polymorphism specifically with MI in Caucasians. Our results clearly argue against a relationship between the 9 bp insertion/deletion polymorphism within the BK B₂ receptor gene and MI. With the findings of above mentioned reports in mind, we think that our investigation had sufficient power to exclude any clinically relevant associations in the population under investigation, although we cannot exclude a small risk increase. Moreover, we cannot exclude that other variants within the BK B₂ receptor gene may increase susceptibility to MI.

In this respect, in a Japanese population, another polymorphism in the promoter region of the BK B₂ receptor gene has been found to be correlated with MI patients with arterial hypertension, but not with MI

patients without hypertension [43]. However, this association was weak and the study sample small, implicating a high risk of an alpha error.

Some limitations of the present study should be mentioned. First, investigation of a single ethnic group implicates that the results may not be valid for other populations. Furthermore, approx. 30 % of MI patients die before reaching the hospital. Thus a considerable number of potentially eligible MI patients could not be included, resulting in an under-representation of a potential risk allele for sudden cardiac death in our study population. Thus prospective studies may be needed to address this topic.

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