An Erythropoietin Gene Polymorphism in the Hypoxia-Responsive Element at Position 3434 Is Possibly Associated with Hypertension

E.G. Schulz^a C.L. Neumann^a J. Menne^c V. Schettler^a C. Mackenbrock^a
A.F. Popov^f G.C. Hagenah^a J. Hinz^b M. Bargfeldt^a T. Gasser^e H. Haller^c
M. Hermann^d

^aCenter of Nephrology Göttingen, Hypertension Care and Research Unit, and ^bDepartment of Anaesthesiology, Emergency and Intensive Care Medicine, University of Göttingen, Göttingen, and ^cClinic for Nephrology, Hannover Medical School, Hannover, Germany; ^dDepartment of Cardiology, University Hospital Zurich, and ^eDepartment of Biostatistics, University of Zurich, Zurich, Switzerland; ^fDepartment of Cardiothoracic Transplantation and Mechanical Support, Royal Brompton and Harefield Hospitals, London, UK

Key Words

Hypertension • Erythropoietin polymorphism • Genetics • End organ damage • Hypoxia-responsive element • Ambulatory blood pressure monitoring

Abstract

Background/Aims: Several polymorphisms of vasoactive hormones have been implicated in hypertension. Erythropoietin (EPO) interacts with vasoactive substances, such as angiotensin II. Previously detected single nucleotide polymorphisms in the hypoxia-responsive element of EPO might be associated with hypertension and hypertensive end organ damages. **Methods:** 400 hypertensive patients and 200 age- and gender-matched normotensive controls were genotyped for an EPO polymorphism [cytosine (C)/thymine (T) single nucleotide polymorphism] at position 3434. Patients were grouped according to their genotype into the CC group (CC genotype) and the CT/TT group (CT and TT genotype). BP was measured by ambulatory BP monitoring. **Results:** The CC genotype was present in 87% of hypertensive pa-

tients and in 78.5% of controls ($p = 0.007$). In addition, patients with the CC genotype had higher BP levels compared with CT/TT genotypes (BP_{sys} 143.7 ± 20.4 vs. 136.1 ± 13.5 mm Hg, $p = 0.01$, and BP_{dias} 85.8 ± 11.6 vs. 82.4 ± 8.9 , $p = 0.043$) despite a nearly identical number of antihypertensive drugs (2.3 ± 1.5 vs. 2.3 ± 1.6 ; $p = 0.257$). 100% of the small number of patients with end-stage renal disease ($n = 15$) had the CC genotype. **Conclusion:** The CC genotype of the EPO gene at position 3434 is more frequently found in patients with hypertension and is associated with higher BP levels.

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Introduction

Arterial hypertension is one of the most important preventable risk factors for the development of cardiovascular and renal disease [1, 2].

The etiology of primary hypertension is complex and has not been dissected completely. The interaction of exogenous factors such as obesity, sodium intake, nicotine,

stress, socioeconomic conditions, and physical inactivity with vasoactive genes plays an important role in the pathophysiology of hypertension and development of hypertensive end organ disease [3].

Genome-wide screening and association analyses of single nucleotide polymorphisms (SNPs) detected several mutations and polymorphisms of vasoactive genes that are linked to hypertension and the development of hypertensive end organ damage [4].

Several studies demonstrated an interaction of erythropoietin (EPO) with vasoactive hormones [5] such as endothelin (ET) [6], nitric oxide (NO) [7], renin-angiotensin-aldosterone system [8] and prostaglandins. Indeed, EPO elicits vasoconstrictive, pro-aggregatory and proliferative effects, impairs endothelial function [9, 10], modulates ET-1-induced calcium mobilization and MAP-kinase activation in smooth muscle cells [11] and regulates the expression of ET-1 mRNA [12].

Transgenic EPO-overexpressing mice demonstrate an increased tubular expression of the prepro-ET-1 promoter sequence [13] and an activation of the tissue ET system [14]. On the other hand, these animals show increased endothelial NO synthase levels, NO-mediated endothelium-dependent relaxation and circulating and vascular tissue NO levels [7]. In addition, EPO enhances catecholamine sensibility of specific vessel receptors [5].

EPO is produced in the peritubular cells of the renal interstitium upon a decrease in oxygen saturation. Indeed, the inhibition of degradation leads to an accumulation of hypoxia-inducible factor-1 (HIF-1- α /- β) and increased binding to the hypoxia-responsive element (HRE), thus stimulating EPO gene expression [15, 16]. HRE and HIF-1 are able to modulate the expression of several other genes during hypoxia, such as plasminogen activator inhibitor-1 or leptin [17, 18].

While several studies demonstrated the prognostic relevance of the hormone for acute vascular events such as stroke [19] and myocardial infarction [20, 21], only one study showed a positive correlation between high normal hematocrit and hypertension in young males [22] while EPO-induced hypertension is widely recognized.

Four polymorphisms in the area of the HIF-1 and HNF-4 binding sites were detected in patients with erythrocytosis [23]. However, exact regulatory mechanisms and functional relevance remain elusive. DNA methylation of cytidyl-3'-5'-guanosine islands at the proximal promoter and the 3' enhancer and the 5'-untranslated region impairs the EPO gene expression by blocking HIF-1-DNA binding [24]. Interestingly, the hypoxia-inducible ET-1 expression was also mediated by HIF-1-DNA binding [25].

Recently, the C3434T polymorphism in the 3' HRE sequence of the EPO gene has been investigated in healthy, normotensive athletes [26]. However, this polymorphism had no influence on EPO levels in response to hypoxia. Thus, the aim of our study was to investigate the impact of the C3434T polymorphism at the HRE element on arterial hypertension, cardiovascular events and hypertensive end organ damage.

Methods

Study Population

The study was approved by the local institutional ethics committee of the Medical Faculty, University of Göttingen. After education and written informed consent, 400 Caucasian patients with essential hypertension and 200 normotensive Caucasian controls from a geographically defined area in lower Saxony were included, and detailed clinical data and medical history were assessed, blood samples were collected and DNA was extracted and frozen at -80°C . Baseline characteristics of all patients are shown in table 1. Hypertensive patients were recruited in the hypertension outpatient clinic, and age- and sex-matched normotensive controls in the Department of Nephrology and Rheumatology of the Medical University of Göttingen. Patients with rheumatoid arthritis were excluded.

Hypertension was defined as 24-hour mean blood pressure $>130/80$ mm Hg (ABPM; Space Labs 90207[®] blood pressure monitor) or normotension under treatment with at least 1 antihypertensive drug, according to the recent hypertension guidelines. Hypertension was categorized and graded according to ABPM blood pressure levels and number of antihypertensive drugs because most of the patients were pretreated at the time of inclusion [27]. Hypertension grade 1 (HTN 1) means hypertensive without/normotensive with 1 antihypertensive drugs (24 h mean $>130/80$ mm Hg/ $<130/80$ mm Hg, ABPM); 2 = hypertensive with 1/normotensive with 2 antihypertensive drugs; 3 = hypertensive with 2/normotensive with 3 antihypertensive drugs, and 4 = hypertensive with 3/normotensive with ≥ 3 antihypertensive drugs.

Renal damage was categorized as 0 = none; 1 = proteinuria [clinical proteinuria was diagnosed if (a) the urine albumin excretion was ≥ 300 mg/day or (b) the urine protein was ≥ 500 mg/day] and/or elevated serum creatinine (≥ 150 $\mu\text{mol/l}$)/decreased creatinine clearance (creatinine clearance, 15–89 ml/min per 1.73 m² of body surface area); 2 = end-stage renal failure because of arterial hypertension (patients who required continuous renal replacement therapy).

Vascular atherosclerotic damage (VD) was categorized as 0 = none, 1 = history of coronary artery disease (CAD) or occlusive artery disease or cerebrovascular affection, while vascular events were graded as absence (=0) or presence (=1) of stroke, myocardial infarction or amputation of extremities caused by hypoperfusion.

All HTN patients underwent echocardiography (Phillips Sonos 5500[®]) and chronic heart failure (CHF) was defined as 0 = preserved systolic function, 1 = impaired systolic function (LVEF $<50\%$), and left ventricular hypertrophy was graded as 0 = none

Table 1. Clinical data

	HTN				non-HTN				p value (HTN vs. non-HTN)
	(n = 400)	CC (n = 348)	CT + TT (n = 52)	p value (CC vs. CT + TT)	(n = 200)	CC (n = 157)	CT + TT (n = 43)	p value (CC vs. CT + TT)	
Age, years	54.3 ± 16.1	54.4 ± 15.7	53.8 ± 19.0	0.81	54.1 ± 16.2	53.9 ± 16.4	55.0 ± 15.3	0.697	0.917
Male	212 (53)	184 (52.9)	28 (53.8)	0.896	106 (53)	82 (52.3)	24 (55.8)	0.679	1
BMI	26.9 ± 4.4	27.0 ± 4.4	26.3 ± 4.1	0.923	23.5 ± 3.9	23.6 ± 3.7	23.2 ± 4.4	0.637	<0.0001
SBP, mm Hg/ABPM	142.7 ± 19.8	143.7 ± 20.4	136.1 ± 13.5	0.01	123.5 ± 7.4	123.6 ± 7.4	123.1 ± 7.5	0.699	<0.0001
DBP, mm Hg/ABPM	85.3 ± 11.3	85.8 ± 11.6	82.4 ± 8.9	0.043	75.3 ± 5.9	75.4 ± 5.8	74.8 ± 5.9	0.532	<0.0001
DM	63 (15.8)	55 (15.8)	8 (15.4)	0.932	3 (1.5)	3 (1.9)	0	0.083	<0.0001
HLP	145 (36.3)	122 (35.1)	23 (44.2)	0.205	4 (2)	2 (1.3)	2 (4.7)	0.321	<0.0001
Smoking	82 (20.5)	70 (20.1)	12 (23.1)	0.622	39 (19.5)	32 (20.4)	7 (16.3)	0.533	0.08
AH drugs	2.27 ± 1.50	2.26 ± 1.48	2.31 ± 1.64	0.257	0.05 ± 0.3	0.05 ± 0.27	0.02 ± 0.2	0.386	<0.0001
CCB	108 (27.0)	96 (27.6)	12 (23.1)	0.495	0	0	0		<0.0001
BB	203 (50.8)	177 (50.9)	26 (50)	0.908	5 (2.5)	4 (2.5)	1 (2.3)	0.933	<0.0001
ACEI/ARB	215 (53.8)	187 (53.7)	28 (53.8)	0.988	1 (0.5)	1 (0.06)	0 (0)	0.319	<0.0001
Diuretics	161 (40.3)	134 (38.5)	27 (51.9)	0.066	3 (1.5)	3 (1.9)	0 (0)	0.083	<0.0001
Statins	55 (13.8)	49 (14.1)	6 (9.6)	0.620	2 (1)	1 (0.06)	1 (2.3)	0.487	<0.0001
Age at onset of hypertension, years	42.89 ± 14.43	42.75 ± 13.94	43.9 ± 17.5	0.6					

AH = Antihypertensive drug; HTN = hypertensive patients; non-HTN = controls; BMI = body mass index; SBP = systolic blood pressure; DBP = diastolic blood pressure; ABPM = ambulatory blood pressure monitoring; HLP = hyperlipoproteinemia. Figures in parentheses indicate percentages.

or 1 = septum hypertrophy (>11 mm). Echocardiography was not performed in non-HTN patients.

The age at onset of hypertension was categorized as 0 = 18–38 years, 1 = 39–59 years, 2 = >60 years.

Genotyping

Genetic analysis was performed by persons blinded to clinical data. Whole blood was collected for DNA extraction from leukocytes by a standard method. For analysis of the C3434T SNP, the DNA fragment was amplified by polymerase chain reaction performed by the thermocycler GeneAmp® 2400 PCR System Thermal Cycler (Perkin Elmer) according to previously described procedures (PCR; protocol: 35 cycles of 1 min at 94°C, 1 min at 58°C and 1 min at 72°C; forward primer: 5'-GAA CTG AAA CCA CCA ATA TGA CTC-3'; reverse primer 5'-TTG GCA GCT GCC TTA CTG CGG TGA-3'; Invitrogen™) [23, 28].

The determination of the genotype was performed by cycle-sequencing with the ABI PRISM® 310 Genetic Analyzer (Applied Biosystems) [29].

Statistics

Univariate analyses (χ^2 and t test) were used to compare the characteristics of the patient groups. Comparisons of different variables among the different genotypes were performed by χ^2 analysis or ANOVA. Data were checked for normality before statistical analysis. Allele frequencies were estimated by counting, and Hardy-Weinberg's equilibrium was checked by χ^2 test. Significance was considered at $p < 0.05$. All statistical analyses were computed by SPSS for Windows (SPSS Inc., Chicago, Ill., USA). For statistical investigation of the allele distribution, all patients were divided into 2 groups of genotypes (CC and CT + TT).

Results

Baseline Characteristics

Clinical data of the investigated population are shown in table 1.

There were no significant differences in age, gender, and smoking status between hypertensives and controls. As expected, systolic and diastolic blood pressure values were higher in hypertensives than in controls. Diabetes mellitus ($p < 0.0001$) and hypercholesterolemia ($p < 0.0001$) were more present in hypertensives, and BMI was higher compared to controls.

Blood Pressure

A different frequency of the C allele was observed. Indeed, the CC genotype was more frequently present in hypertensive patients (87%, $n = 348$) than in non-hypertensive controls (78.5%, $n = 157$; $p = 0.007$). CT/TT frequency was 13% ($n = 52$) in hypertensive patients and 21.5% ($n = 43$) in controls.

In a second step, the different subgroups of hypertensive patients were examined (table 2). However, no association was detected for different grades of hypertension or age at onset of disease (table 3). The mean age at the onset of the disease in all patients was 42.9 ± 14.4 .

Table 2. EPO C3434T polymorphism genotypes of hypertensive patients and controls according to end-organ damage

End organ damage	HTN				non-HTN (n = 200)
	(n = 400)	CC (n = 348)	CT + TT (n = 52)	CC (TT + CT) p value	
Renal disease	106 (26.75)	91 (26.2)	15 (28.9)	0.826	1 (0.5)
Vascular disease	114 (28.5)	100 (28.7)	14 (26.9)	0.758	0
CAD	94 (23.5)	84 (24.1)	10 (19.2)	0.659	0
Left ventricular hypertrophy	132 (33)	115 (33.0)	17 (32.7)	0.960	not investigated
CHF	36 (9)	33 (9.5)	3 (5.8)	0.383	not investigated
Vascular events	62 (15.5)	54 (15.5)	8 (15.4)	0.758	3 (1.5)

Figures in parentheses indicate percentages.

Table 3. EPO genotypes of hypertension subgroups according to hypertension severity, stages of renal damage and age at onset of the disease

Subgroups	CC (n = 348)	CT + TT (n = 52)	CC (CT + TT) p value
<i>Hypertension grade</i>			
HTN 1, n = 91 (22.75)	75 (21.5)	16 (30.8)	0.476
HTN 2, n = 91 (22.75)	80 (23.0)	11 (21.2)	
HTN 3, n = 81 (20.25)	73 (21.0)	8 (15.4)	
HTN 4, n = 137 (34.25)	120 (34.5)	17 (32.6)	
<i>Renal damage</i>			
RD 0, n = 294 (73.5)	257 (73.8)	37 (71.2)	0.196
RD 1, n = 91 (22.75)	76 (21.9)	15 (28.8)	
RD 2, n = 15 (3.75)	15 (4.3)	0 (0)	
<i>Age at onset of hypertension</i>			
AOH 1, n = 143 (35.75)	125 (35.9)	18 (34.6)	0.62
AOH 2, n = 206 (51.5)	183 (52.6)	23 (44.2)	
AOH 3, n = 51 (12.75)	40 (11.5)	11 (21.2)	

Figures in parentheses indicate percentages.

However, hypertensive patients with the CC genotype had higher blood pressure levels compared to CT/TT patients, despite a comparable number of antihypertensive drugs (table 1).

Renal Function

No difference in renal function was observed between both groups (CC vs. CT/TT).

All patients with end-stage renal failure because of arterial hypertension (n = 15) showed the CC genotype.

Cardiovascular Disease

The prevalence of CAD (CC: 24.1%; CT/TT: 19.2%) was comparable (p = 0.488). Impaired left ventricular function was present in 33 (9.5%) patients in the CC group compared to 3 patients (5.8%) in the CT/TT group (p = 0.06). Left ventricular hypertrophy was equally detected in both groups (CC: 33.0%; CT/TT: 32.7%; table 2).

Cardiovascular Events (Stroke, Myocardial Infarction, Amputation of Extremities) and Cardiovascular Damage (CAD, Cerebrovascular Damage, Occlusive Disease)

No difference was observed for cardiovascular events (CC: 15.5% and CT/TT: 15.4%; p = 1.0) or cardiovascular damage (CC: 28.7 and CT/TT: 26.9%; p = 0.87; table 2).

Homozygous TT Genotype

The unique TT homozygous genotype was only seen in 1 patient (HTN III, 39 years old) with an early onset of hypertension and severe end organ damage including 2-vessel CAD with previous myocardial infarction, impaired left ventricular function (EF 35%), left ventricular hypertrophy, and impaired renal function.

Discussion

Over the last years, several polymorphisms in the HRE of the EPO gene have been identified. Although EPO plays an important role in the vascular homeostasis, all previously identified polymorphisms in the HRE have not been associated with essential hypertension, the course of disease, cardiovascular complications or hypertensive end organ damage especially in the renal vascular

bed [23]. Therefore, we are the first to describe an association of an EPO/HRE C3434T polymorphism with arterial hypertension in a well characterized study population. Indeed, hypertensive patients show a higher prevalence of the CC genotype compared to matched normotensive controls. Furthermore, hypertensives subjects with the CC genotype have higher blood pressure levels than patients with the CT and TT genotype.

Despite the impact on blood pressure levels, the C3434T polymorphism might act differentially in different vascular beds. Indeed, all patients with ESRD in our cohort showed the CC genotype, while the only patient with the TT genotype had an early onset of severe hypertension with multiple end organ damage, including 2-vessel CAD with previous myocardial infarction, impaired left ventricular function (LVEF 35%), left ventricular hypertrophy and impaired renal function.

Interestingly, the analysis of associated cardiovascular diseases or cardiovascular damage revealed no impact of the polymorphism. The incidence of left ventricular hypertrophy, myocardial infarction, CHF or stroke was comparable in all groups and independent of the underlying genotype.

The functional relevance of the investigated polymorphism remains highly speculative. The C3434T polymorphism could be related to different gene expression and promotor activity as described by Wenger et al. [30]. Increased EPO levels can negatively affect vascular function by several mechanisms, such as inhibition of cyclooxygenase-dependent vasorelaxation [10], while decreased EPO levels could be associated with worse outcome after acute vascular events, such as stroke or myocardial infarction [19, 20].

The functional relevance of the described polymorphism should be investigated in specific cell culture con-

ditions and in cardiovascular high-risk patients such as patients after acute myocardial infarction. With a closer look at our data, patients with CHF would be of specific interest. Indeed, although not significant, 9.5% of all hypertensive patients with the CC genotype had an impaired systolic left ventricular function compared to only 5.8% of hypertensive patients with the CT/TT genotype. However, until now, no association of EPO polymorphism and CHF has been described.

Furthermore, the small hypertensive subgroup of patients with hypertensive end-stage renal failure should be separately investigated in a larger study.

A limitation of this study is the fact that data about functional changes of the EPO pathway are not available for the C3434T polymorphism. It is not known whether the CC genotype is associated with higher or lower EPO levels with increased or impaired activity of the EPO/HRE. Unfortunately, EPO levels were not measured in our study. However, it is known that an SNP in the EPO promotor region significantly increased the transcriptional activity [31].

In conclusion, we demonstrated that the CC genotype at position 3434 of the HRE of the EPO gene is widely prevalent in the general population with an even higher prevalence in hypertensive patients. Furthermore, hypertensive patients with the CC genotype have higher blood pressure levels compared to hypertensive subjects with the CT/TT genotype.

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