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Retrospective Analysis of Coagulation Factor II Receptor (F2R) Sequence Variation and Coronary Heart Disease in Hypertensive Patients

Bruna Gigante, Alessandro Bellis, Roberta Visconti, Marina Marino, Carmine Morisco, Valentina Trimarco, Gennaro Galasso, Federico Piscione, Nicola De Luca, Jonathan A. Prince, Ulf de Faire, Bruno Trimarco

Objectives—The purpose of this study was to evaluate the role of genetic variants within the coagulation factor II receptor (F2R) in the occurrence of coronary heart disease (CHD).

Methods and Results—Four SNPs (−1738 G/A, 2860 G/A, 2930 T/C, and 9113 C/A) and an ins/del polymorphism −506-/GGCCGCGGAAGC (D/I), replicating a consensus sequence for Ets-1 transcription factor, and their related haplotypes were tested for association to CHD in 1600 hypertensive patients divided in 2 groups according to presence (cases, n=559) and absence (controls, n=1041) of CHD. Allele I at −506 locus was associated with increased risk of CHD under additive, dominant, and recessive models of inheritance (all $P<0.01$). Three haplotypes carrying I allele were consistently associated with an increased risk of CHD (all $P<0.05$). Patients homozygous for the C allele at the 2930 locus also showed an increased risk of CHD ($P<0.05$). To test the functionality of −506 locus, nuclear extracts were incubated with −506D and −506I sequences by EMSA and F2R promoter activity (F2R-A) were assessed in HUVECs transfected with vectors carrying −506D and −506I sequences and exposed to hypoxia. Presence of the −506I sequence was associated with a 26% reduction of affinity binding to nuclear proteins and to blunted F2R-A in response to hypoxia as compared with the −506D sequence (all $P<0.05$).

Conclusions—F2R genetic variants may influence the natural history of CHD in patients at high risk of cardiovascular events. (*Arterioscler Thromb Vasc Biol.* 2007;27:1213-1219.)

Key Words: coronary heart disease ■ hypertension ■ F2R ■ haplotype ■ hypoxia

Coronary Heart Disease (CHD), a complex and heterogeneous disease with a heritability exceeding 50%,^{1,2} represents the leading cause of death among cardiovascular diseases, and almost half of the patients experiencing a first heart attack have high blood pressure levels.³ Individual susceptibility to CHD depends on multiple genetic and environmental factors that influence coronary artery lesion morphology and metabolism and therefore clinical presentation. Identification of genetic variants predisposing to CHD is essential to improve our knowledge of the mechanisms underlying CHD and, on a broader level, to ameliorate cardiovascular disease prevention and pharmacological treatment.

Recently, the Coagulation Factor II Receptor (F2R), the thrombin receptor expressed on human platelets and endothelial cells, has been identified as a powerful modulator of endothelial lining, and more generally, of vascular wall integrity and stability.^{4,5} In aortic⁶ and coronary artery rings⁷

thrombin-activated F2R promotes endothelial-dependent vasodilatation through a nitric oxide-dependent mechanism and infusion of an F2R agonist in mice results in rapid hypotension followed by sustained hypertension.⁸ In cultured endothelial cells prolonged F2R stimulation induces an inflammatory and angiogenic response through the enhanced expression of proinflammatory cytokines,⁹ vascular endothelial growth factor (VEGF) and VEGF receptors expression.¹⁰ Finally, experiments performed in animal model of vascular injury have suggested that inhibition of this receptor might be of clinical relevance in preventing acute arterial thrombosis¹¹ and vascular wall remodeling.¹² Taken together these observations strongly support F2R as an attractive candidate gene in the pathogenesis of CHD. A progressive loss of the endothelial lining anatomic and functional integrity represents, in fact, a hallmark of hypertension and a condition underlying atherosclerosis and strongly promoting CHD.

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To date the potential role of F2R sequence variation in the occurrence of cardiovascular diseases has only been investigated in 2 published case-control studies,^{13,14} both failing to find any association between F2R and the occurrence of myocardial infarction.

In the present study we investigated the potential role of genetic variants within the F2R gene in the occurrence of CHD in a population of patients at high risk of cardiovascular events through an integrated genetic and biological approach. We report the analysis of 5 genetic variants and related haplotypes with the occurrence of CHD and the characterization of the biological effect of -506I, the allelic variant associated to the highest risk of CHD.

Methods

Methods are described in details in the supplemental materials, available online at <http://atvb.ahajournals.org>.

Study Population

Study participants consisted of 1600 patients affected by essential hypertension retrospectively divided in 2 groups according to the presence (cases, n=559) or absence (controls, n=1041) of CHD. Chronic stable angina (SA) was diagnosed in 238 patients and a former acute coronary syndrome event (ACS) in 321 patients.

Single Nucleotide Polymorphisms Genotyping and Haplotype Generation

Six single nucleotide polymorphisms (SNPs) were identified from the HapMap database (public release#11, 09/02/2004; www.hapmap.org) on sequence with GenBank accession no. AF391809: rs2227744 (-1738 G/A, nucleotide [n]: 352), rs27135 (G/A n: 2860), rs27593 (T/C n: 2930), rs2227774 (C/A n: 9113), rs153311 (A/C n: 17841) and rs2227800 (C/T n: 19390). Two additional SNPs reported as polymorphic in the NCBI database (www.ncbi.nlm.nih.gov/), rs11267092 (-506D/I, -/GGCCGCGGGGAAGC, n:1631 to 1643) and rs2230849 (T/A n: 18627), were included in the analysis. Validation assays confirmed that -1738 G/A, -506D/I, 2860 G/A, 2930 T/C, and 9113 C/A were polymorphic in this population.

Haplotype counts and frequencies were inferred from original genotype data using the THESIAS program.¹⁵

Assessment of -506 Locus Biological Function

Biological properties of the two alleles at the -506 locus were assessed by electrophoretic mobility shift assay (EMSA) and by luciferase activity (LA) assay.

Nuclear extracts from a human cervical cancer cell line (Hela) (Santa Cruz) were incubated with ³²P- labeled probes encoding -506D, -506I, and an Ets-1 consensus sequence. Complexes were run on 8% polyacrylamide gel and visualized by autoradiography.

Pooled human umbilical vein endothelial cells (HUVECs; Cambrex) were transiently transfected with empty, p-506D and p-506I vectors and stimulated for 4, 6, and 8 hours with Angiotensin II (ATI, 10⁻⁶ mol/L) isoproterenol (Is, 10⁻⁷ M), or incubated in hypoxic conditions and left to recover overnight in normoxic conditions. LA, normalized by β galactosidase light units in the same sample, was expressed as fold increases in relative LA versus p-506D baseline values.

Statistical Analysis

Differences in genotypic, allelic, and haplotype frequencies among cases and controls were calculated by χ^2 test. Haplotype counts and frequencies were inferred from genotypes. Unadjusted odds ratios (ORs) with 95% confidence interval (CI) were estimated for -506 D/I and 2930 T/C variations and for haplotype configurations. Age, male sex, and diabetes were identified as independent predictors of CHD and treatment with angiotensin receptor blockers (ARBs), diuretics, and dihydropyridine Ca⁺⁺ antagonists (DHP Ca⁺⁺ ant) as

potential confounders and were adjusted for in a multivariable logistic regression analysis.

In vitro assays were performed 3 times in duplicate. Results are expressed as mean \pm SEM. Differences among groups were evaluated using the Kruskal-Wallis H test.

Results

Demographic, anthropometric, biochemical, and therapeutic data collected in the present study population are outlined in Table 1. Family history of hypertension was more common among controls, whereas history of CHD was more frequent in cases. At diagnosis, diastolic blood pressure (DBP) was higher in controls ($P<0.0001$). However, when blood pressure values were obtained throughout the course of years, both groups showed comparable systolic blood pressure (SBP) and DBP values. Compared with controls, cases were older ($P<0.0001$), predominantly of male sex ($P<0.0001$), and current smoking ($P<0.05$) and overt diabetes mellitus ($P<0.0001$) were more frequently observed. No differences were noted in the occurrence of hypercholesterolemia between the 2 groups of patients. However, among cases, hypercholesterolemia was more frequent in SA patients as compared with patients who had suffered ACS ($\chi^2_{2DF}:6.53$ $P=0.03$).

The following risk factors were identified as independent predictors of CHD (OR, (95%CI)): age 1.02 (1.01 to 1.03), male sex 1.81 (1.37 to 2.39), and diabetes mellitus 3.98 (2.78 to 5.69) and pharmacological treatment with ARBs 0.54 (0.44 to 0.67), DHP Ca⁺⁺ ant 0.55 (0.44 to 0.68), and diuretics 0.55 (0.45 to 0.67) were identified as potential confounders in genotype/phenotype association study and adjusted for in a multivariable logistic regression analysis.

F2R SNPs and Haplotypes and the Risk of CHD

Genotype and allele frequencies at -1738 G/A, -506D/I, 2860 G/A, 2930 T/C, and 9113 C/A loci are reported in Table 2. No significant deviation from Hardy-Weinberg equilibrium was observed in controls or cases at any of the 5 loci. LD data are reported in supplemental Table II.

At -1738 G/A, 2860 G/A, and 9113 C/A, no significant differences were found in genotype and allele frequencies among cases and controls. At -506D/I locus, genotype II was more frequent in cases (0.11 versus 0.05 in controls, $P<0.0001$) with allele I having a frequency of 0.31 in cases and 0.22 in controls ($P<0.0001$) and at 2930T/C locus frequency of C allele was higher in cases (0.25 versus 0.22, $P=0.02$). We tested the hypothesis of allele I and C being associated with an increased OR of CHD. As shown in Table 3, presence of the I allele at -506 locus was associated with an increased risk, OR (95%CI), of CHD assuming an additive (unadjusted 1.24 [1.11 to 1.39]; adjusted 1.25 [1.10 to 1.42]), dominant (unadjusted 1.68 [1.36 to 2.09]; adjusted 1.72 [1.35 to 2.19]), and recessive (unadjusted 2.40 [1.61 to 3.58]; adjusted 2.63 [1.67 to 4.13]) models of inheritance. At the 2930 locus, patients homozygous for the C allele were at increased risk of CHD at both unadjusted 1.62 (1.04 to 2.53) and adjusted 1.65 (1.00 to 2.73) logistic regression analyses, whereas no significant association was found under additive or dominant models of inheritance.

TABLE 1. Study Population: Relevant Demographic, Anthropometric, Biochemical, and Therapeutical Data

	Controls (n=1041)	Cases (n=559)	P
Age, y \pm SEM (range)	57 \pm 0.3 (20–92)	59 \pm 0.4 (28–82)	0.00004
Sex, M/F	626/420	429/135	<0.00000001
Follow up, y \pm SEM	11 \pm 0.2	11 \pm 0.5	
Risk factors, n (%)			
FH Hypertension	839 (80)	307 (54)	<0.00000001
FH CHD	336 (32)	255 (45)	<0.00000001
Diabetes	66 (6)	142 (25)	<0.00000001
Hypercholesterolemia	661 (63)	334 (59)	0.49
Hypertriglyceridemia	326 (32)	187 (33)	0.43
Obesity	230 (22)	84 (20)	0.57
Metabolic syndrome	276 (26)	169 (30)	0.13
Smoke	555 (53)	336 (60)	0.01
Anthropometry, mean \pm SEM			
SBP at diagnosis	160 \pm 0.6	158 \pm 1	0.78
DBP at diagnosis	100 \pm 0.3	95 \pm 0.6	<0.00000001
SBP during treatment	128 \pm 0.3	128 \pm 0.4	0.18
DBP during treatment	78 \pm 0.3	77 \pm 0.2	0.14
BMI, kg/m ²	27 \pm 0.1	28 \pm 0.2	0.21
Biochemistry, mg/dl (mean \pm SEM)			
Glicemia	97 \pm 0.8	114 \pm 1.8	<0.00000001
Total cholesterol	212 \pm 1.3	210 \pm 1.7	0.32
LDL cholesterol	136 \pm 1.2	132 \pm 2.2	0.15
HDL cholesterol M/F	45 \pm 0.4/53 \pm 0.6	45 \pm 0.6/50 \pm 0.2	0.26/0.65
Triglycerides	138 \pm 2.5	144 \pm 3.2	0.14
Therapy 1 st /2 nd /3 rd line, n			
ACE-I	490/97/30	273/28/4	0.0004
ARBs	184/75/61	37/17/9	<0.00000001
β -blockers	367/92/36	204/14/7	0.0000003
DHP Ca ²⁺ ant	332/82/39	110/10/9	<0.00000001
N-DHP Ca ²⁺ ant	64/50/26	52/9/6	0.0003
Diuretics	389/117/43	111/23/7	<0.00000001
Lipid lowering	377	216	0.36

Y indicates years; FH, Familiar History; ACE-I, ACE-Inhibitors; N-DHP Ca²⁺ ant, non-dihydropyridine Ca²⁺ antagonists.

Neither of these two loci showed a significant association with the presence of any of the cardiovascular risk factors identified in this population and no epistatic interactions were found.

Overall, 32 different haplotypes were inferred from the original genotype data. Table 4 lists counts and frequencies of the haplotypes observed in cases and controls with a frequency higher than 0.01 as well as the OR (95%CI) associated to each haplotype. Haplotypes bearing the I allele at –506 locus were more frequent in cases. The effect of the I allele was associated with an increased risk of CHD in patients carrying Hap2 GIGTA, Hap4 AIATC, and Hap8 GIGCC in both un- and adjusted logistic regression analyses (all $P < 0.05$). The I allele, as compared with D allele, was

associated with an OR 1.77 (1.19 to 2.62) $P = 0.004$ on the G-GTA background; to an OR of 1.95 (1.28 to 2.97) $P = 0.001$ on A-ATC background; and to an OR of 2.79 (1.4 to 5.58) $P = 0.003$ on G-GCC background. No apparent increase in the risk of CHD was associated with the presence of allele I in any of the other haplotypic configurations in this population. Hap8 represents the haplotype bearing I and C allele with the highest frequency, all the other haplotypes having a frequency ranging from 0.0003 to 0.003.

Consistently, haplotypes carrying the discordant allele at –506 locus Hap0 ADATC, Hap3 GDGTA, and Hap6 GDGTC and Hap9 ADATA were more frequently observed in controls. At multivariable analysis Hap6 was associated with a reduced risk of CHD (OR: 0.41 [0.21 to 0.80]). At the

TABLE 2. Genotypic and Allelic Frequencies in Controls and Cases at -1738G/A, -506D/I, 2860G/A, 2930T/C, and 9113C/A Loci

	Controls (n=1041)	Cases (n=559)	P
-1738 G/A			
GG	267 (0.29)	146 (0.28)	
GA	445 (0.49)	268 (0.52)	
AA	203 (0.22)	102 (0.20)	0.42
Allele A	0.46	0.46	0.72
-506D/I			
DD	592 (0.60)	250 (0.48)	
DI	338 (0.35)	217 (0.41)	
II	48 (0.05)	58 (0.11)	<0.0001
Allele I	0.22	0.31	<0.0001
2860 G/A			
GG	242 (0.26)	125 (0.25)	
GA	444 (0.49)	256 (0.51)	
AA	227 (0.25)	116 (0.23)	0.58
Allele A	0.49	0.49	0.98
2930 T/C			
TT	607 (0.62)	297 (0.58)	
TC	323 (0.33)	176 (0.34)	
CC	46 (0.05)	38 (0.07)	0.06
Allele C	0.22	0.25	0.02
9113 C/A			
CC	536 (0.59)	301 (0.57)	
CA	310 (0.34)	191 (0.36)	
AA	60 (0.07)	35 (0.07)	0.72
Allele A	0.24	0.25	0.46

Genotype counts (frequency) at each of the 5 loci are reported. Because of rounding not all frequencies sum up to 1.

analysis of the haplotypic background, however, the protective effect seemed to be related to the presence of T allele on a GDG-C background with an OR 0.48 (0.30 to 0.76) $P=0.005$; presence of either D or I allele did not change, in fact, the OR associated to the G-GTC haplotype (OR:1.21 [0.52 to 2.80] $P=0.65$).

Assessment of -506 Locus Biological Function

Given -506I being the allelic variant showing the strongest effect on CHD at the genotype/phenotype association analyses, we assessed the functionality of -506D and -506I sequences by means of EMSA. Thus, we tested the hypothesis that sequence variation at the -506 locus might influence F2R-A in response to ATII, Is, and hypoxia, 3 stimuli mimicking a microenvironment that favors development and progression of CHD.

The -506I sequence (Figure 1, L5) showed a 26% reduction in the affinity binding to nuclear proteins as compared with -506D (Figure 1, L3; $ADU \pm SEM$; 88804 ± 4354 versus 119717 ± 4975 , $P<0.05$). The Ets-1 consensus sequence was used as positive control (L1). Specificity of the reaction was controlled by adding a molar excess of the unlabeled Ets-1 and -506D probes (L6 and L7).

TABLE 3. Relative Risk Associated With CHD According to Genotype at Each Locus After an Additive, Dominant, and Recessive Inheritance Model*

	Unadjusted		Adjusted*	
	OR (95% CI)	P Value	OR (95% CI)	P Value
-1738 G/A				
Additive	1.05 (0.93–1.20)	0.37	1.04 (0.90–1.22)	0.54
Dominant	1.04 (0.82–1.32)	0.72	1.03 (0.76–1.30)	0.98
Recessive	0.86 (0.66–1.12)	0.28	0.83 (0.61–1.11)	0.21
-506D/I				
Additive	1.24 (1.11–1.39)	0.0001	1.25 (1.10–1.42)	0.01
Dominant	1.68 (1.36–2.09)	0.000002	1.72 (1.35–2.19)	0.00001
Recessive	2.40 (1.61–3.58)	0.00002	2.63 (1.67–4.13)	0.00003
2860 G/A				
Additive	1.06 (0.93–1.21)	0.36	1.05 (0.98–1.22)	0.44
Dominant	1.07 (0.83–1.37)	0.58	1.06 (0.81–1.41)	0.63
Recessive	0.92 (0.71–1.18)	0.52	0.92 (0.69–1.23)	0.61
2930 T/C				
Additive	1.06 (0.94–1.19)	0.28	1.08 (0.95–1.22)	0.23
Dominant	1.18 (0.95–1.47)	0.12	1.21 (0.95–1.55)	0.12
Recessive	1.62 (1.04–2.53)	0.03	1.65 (1.00–2.73)	0.04
9113 C/A				
Additive	1.04 (0.93–1.17)	0.42	1.02 (0.89–1.16)	0.75
Dominant	1.08 (0.87–1.35)	0.44	1.04 (0.82–1.33)	0.71
Recessive	1.00 (0.65–1.54)	0.98	1.07 (0.66–1.72)	0.77

*Adjusted for age, sex, diabetes, and treatment with ARBs, DHP Ca^{++} ant, and diuretics.

F2R-A was selectively increased in HUVECs transfected with p-506D (4.44 ± 0.82 LA fold increase versus baseline, $P<0.05$) after 8 hours of hypoxia, a change that reverted after exposure to normoxic conditions (Figure 2C, gray bars). In contrast, cells transfected with p-506I showed a blunted response of F2R-A (Figure 2C, black bars). No differences in F2R-A were observed among HUVECs transfected with p-506D or p-506I in response to ATII or Is stimulation (Figure 2A and 2B, respectively).

Discussion

In the present study we have explored the potential role of genetic variants within F2R gene in the occurrence of CHD in a population of hypertensive patients. Our findings demonstrate that 2 SNPs, mapping in the F2R promoter region at locus -506, and in the intron at position 2930, and 3 related haplotypes are associated with an increased risk of CHD. Presence of the I allele at the -506 locus was the strongest genetic predictor of CHD in this cohort. Within the 13 base pairs replicated in the insertion lies a consensus sequence for Ets-1 transcription factor.¹⁶ Ets-1 is a strong activator of endothelial cell specific promoters, and it has been recently described as one of the major regulators of hypoxia/ischemia-driven transcription.¹⁷ This polymorphism occurs in a conserved region of the promoter, and there is evidence for at least one expressed sequence tag (EST) mapping directly over this position, indicating that it could regulate alternative

TABLE 4. Haplotypes Counts (Frequencies) and Relative Risk of CHD in Controls and Cases

Index	Haplotype	Controls	Cases	Unadjusted		Adjusted*	
				OR (95% CI)	P Value	OR (95% CI)	P Value
Hap0	ADATC	757 (0.377)	366 (0.335)	1		1	
Hap1	GDGCC	332 (0.166)	185 (0.169)	1.09 (0.86–1.39)	0.44	1.10 (0.83–1.45)	0.49
Hap2	GIGTA	228 (0.114)	161 (0.148)	1.42 (1.09–1.85)	0.009	1.43 (1.05–1.96)	0.02
Hap3	GDGTA	164 (0.081)	60 (0.055)	0.80 (0.57–1.11)	0.18	0.86 (0.59–1.24)	0.43
Hap4	AIATC	62 (0.031)	69 (0.063)	1.95 (1.28–2.97)	0.001	2.29 (1.37–3.82)	0.001
Hap5	GDATC	80 (0.040)	48 (0.044)	1.22 (0.78–1.88)	0.35	1.48 (0.91–2.38)	0.09
Hap6	GDGTC	90 (0.045)	25 (0.023)	0.58 (0.32–1.07)	0.08	0.41 (0.21–0.80)	0.009
Hap7	GIGTC	90 (0.044)	32 (0.029)	0.71 (0.42–1.21)	0.22	0.80 (0.46–1.35)	0.40
Hap8	GIGCC	34 (0.017)	55 (0.050)	3.07 (1.62–5.79)	0.0005	2.62 (1.25–5.48)	0.01
Hap9	ADATA	65 (0.032)	23 (0.021)	0.73 (0.38–1.41)	0.35	0.50 (0.23–1.06)	0.07

PHASE permutation test $P=0.01$. Hap0 was taken as reference category. *Adjusted for age, sex, diabetes, and treatment with ARBs, DHP-Ca²⁺ ant, and diuretics.

promoter usage. To explore the biological relevance of this polymorphism we have tested if the alternative alleles at the –506 locus might display different biological properties. Our results show that presence of the I allele at –506 locus reduces binding affinity to nuclear proteins and is associated with a blunted response to hypoxia.

A crucial role of hypertension in the occurrence of CHD has been highlighted in epidemiological as well as clinical studies.¹⁸ However, differences in the individual response to blood pressure lowering drugs and copresence of other risk factors determine major differences in the rate of occurrence of CHD among hypertensive patients. Hypertension represents a major confounding factor in observational genetic studies as well as in the analysis of the mechanisms regulat-

ing vascular wall homeostasis. The analysis of the potential role of candidate genes in the occurrence of complex disease in patients selected for an intermediate phenotype may represent a suitable approach to reduce the distance between genes and complex phenotypes.^{19,20}

In this population, age, male sex, and diabetes were identified as independent predictors of CHD, whereas hypercholesterolemia did not show a significant association with CHD. This apparent paradox has already been observed in other studies²¹ and related to the use of drugs that lower serum cholesterol levels making the discrimination between exposed and nonexposed individuals more difficult to establish. In our population, the percentage of patients taking lipid lowering drugs, as well as the serum lipid profiles, were comparable in cases and controls, but hypercholesterolemia was more frequently observed in the subgroup of patients affected by SA. We cannot exclude that, given the complexity and heterogeneity of CHD, serum cholesterol levels may differently influence various forms of CHD. Likewise, serum lipid profiles differentially influence the occurrence of various stroke subtypes.²²

The F2R gene spans 22 Kb on the long arm on human chromosome 5 in position 5q1.3. It is composed of an upstream regulatory region and 2 exons separated by a large intron. The analysis of 5 SNPs within F2R gene in our study population has identified two loci, –506 and 2930, whose allelic frequencies were significantly different among cases and controls. The 2930T/C polymorphism influenced the risk of CHD only in patients carrying variant C in homozygosity, whereas allele I was consistently associated with an increased risk of CHD in hypertensive patients both in genotype and haplotype analyses. Patients carrying haplotypes identified as Hap2, Hap4, and Hap8 were exposed to an increased risk of CHD and we cannot exclude that in a longitudinal observational study these patients might exhibit differences with regard to prognosis or to response to pharmacological treatment.

An analysis of the potential role of the –506D/I polymorphism in the occurrence of myocardial infarction did not show any significant association in a French population.¹³

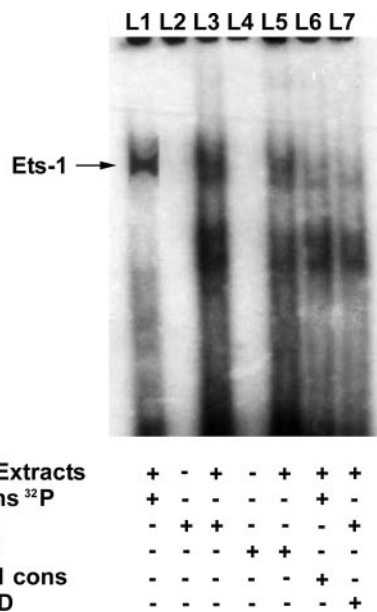


Figure 1. Representative EMSA. Nuclear extracts were challenged with ³²P Ets-1 consensus (cons) (L1), –506D (L3), and –506I (L5) sequences. –506D and –506I were run in the absence of nuclear extracts (L2, L4). Ets-1 and –506D binding was displaced by an excess of unlabeled Ets-1 and –506D sequences (L6, L7).

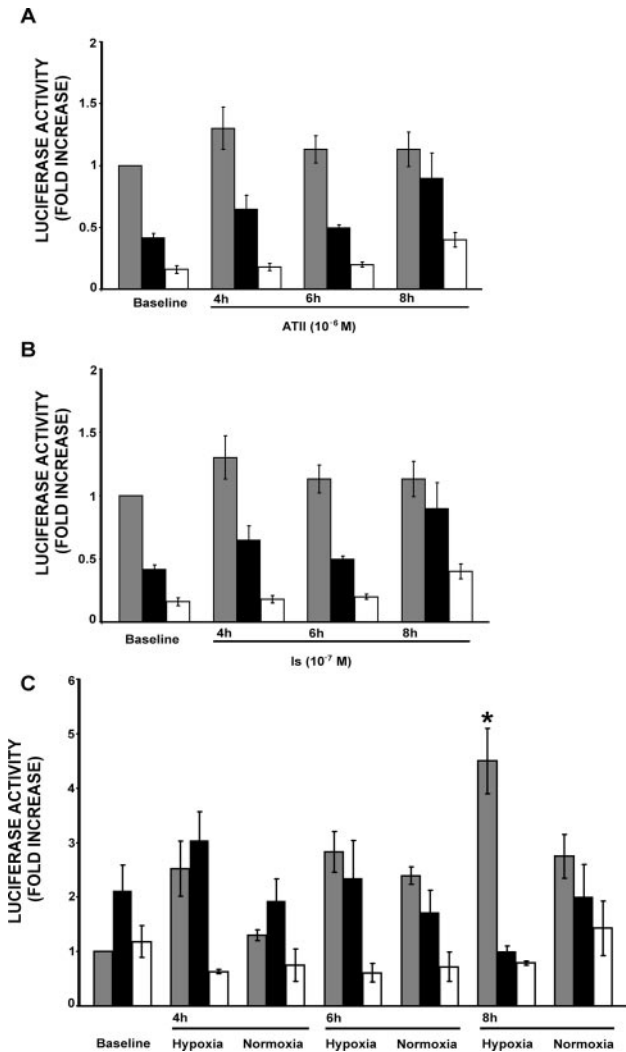


Figure 2. Bar graphs showing F2R-A in HUVECs transfected with empty (white bars), p-506D (gray bars), and p-506I (black bars) vectors and stimulated for 4, 6, and 8 hours with ATII (A), Is (B), hypoxia, and overnight recovery (Normoxia; C). * $P < 0.05$ vs baseline and p-506I vector at 8 hours.

This discrepancy may be related to the fact that we performed our study in a population selected for hypertension, and we did not perform association analysis in the 2 subgroups of cases, SA or ACS, to avoid small sample size bias. On the contrary, the $-506II$ genotype was associated with a reduced risk of deep vein thrombosis in male patients.²³ In our population, incidence of deep vein thrombosis and pulmonary embolism was extremely low (2 patients in controls and 1 patient in cases), and we could not perform any analysis to replicate this finding. It has been suggested that human thrombotic diathesis shows a focal nature and that the same genetic variant may differentially affect predisposition to deep vein and arterial thrombosis.²⁴ With regard to F2R this hypothesis is supported by the observation that F2R elicits vasorelaxation in arteries and vascular wall cell proliferation and vasoconstriction in veins,²⁵ and therefore the same genetic variation may differentially affect the development of thrombosis in different vascular beds.

One major caveat in observational genetic studies is that positive association findings may represent spurious results

because of the presence of population stratification.¹⁶ According to NCBI database (www.ncbi.nlm.nih.gov/), the I allele frequency in the European panel is 25%; however, in our genotype data 3 of the 5 SNPs examined, $-1738 G/A$, $2860 G/A$, and $9113 C/A$, did not show any difference in allelic frequency between cases and controls speaking against the presence of stratification in this population.

To test the biological relevance of sequence variation at the -506 locus we have explored the possibility that duplication of the Ets-1 binding site might change the functionality of this locus. In our experimental conditions, presence of the I sequence was associated with reduced nuclear binding affinity and blunted response to prolonged hypoxia as compared with the D sequence, suggesting that duplication of the Ets-1 consensus sequence might be responsible for a reduced Ets-1 binding and a blunted response to hypoxia. An abnormal response of gene machinery to a hypoxic stimulus might be detrimental for endothelial and vascular wall metabolism and can therefore be a factor promoting coronary artery lesion development and growth. Endothelial responses to the same stimulus, however, are different in each vascular bed and depend on the local microenvironment and further studies in atherosclerotic as well as healthy coronary arteries are needed to conclusively demonstrate this biological phenomenon.

Several limitations of the present study need to be acknowledged. The study design is based on a case-control retrospective approach and is limited to hypertensive patients. To reach more general conclusions with regard to the role of F2R on the occurrence of CHD, a prospective longitudinal study as well as the replication in a population not selected for hypertension are warranted. Genetic variation at a specific locus will vary among different populations^{26,27} and will change as more extensive knowledge of human genetic variability is gained. In the current (January 2007) HapMap release 3 more tSNPs for this locus, 2 mapping in the intron and 1 in the mRNA untranslated region, have been reported, therefore 63% of the total genetic variation at this locus has been explored in the present study and the haplotypes identified may well be implemented and extended in future studies. Finally, although our results suggest that changes in Ets-1 binding affinity are involved in the selective response of $-506D$ and $-506I$ to hypoxia, new experiments are needed to investigate whether the conformational change within the promoter sequence actually reduces Ets-1 binding or enhances binding of other transcription factors with opposite effects.

In conclusion, our results demonstrate that F2R sequence variation influences the occurrences of CHD. The $-506I$ allele, which throughout the study exhibited the strongest effect, has peculiar biological properties, suggesting that it may play a role in the onset and progression of coronary artery lesions modulating F2R activity.

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Disclosures

None.

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DATA SUPPLEMENT

METHODS

Study population

Study participants are patients at high risk of cardiovascular events affected by essential hypertension who have been referred for follow up and further assessment of cardiovascular function to the outpatient Unit for Treatment and Prevention of Hypertension and CHD of our Department since 1985. For the present study 1600 patients, who had come for a regular visit between January 2000 and December 2003 and gave their informed consent to participate in the study, have been retrospectively divided in two groups according to the presence (cases, n=559) or absence (controls, n=1041) of CHD. Hypertension diagnosis was confirmed if systolic (SBP) and diastolic blood (DBP) pressure were higher than 140 and 90 mmHg, respectively, in repeated measurements or if patients were already taking anti-hypertensive medications. Criteria for diagnosis were updated according to published guidelines¹. Diagnosis of CHD was based on clinical findings, non invasive tests and/or coronary angiograms according to the ACC/AHA guidelines: chronic stable angina (SA)² was diagnosed in 238 patients and a former acute coronary syndrome event (ACS) in 321 patients^{3,4}. Presence of common risk factors for cardiovascular diseases was assessed for each patient. A positive familiar history for hypertension and/or CHD was defined as having at least one close relative (biological mother, father or siblings) diagnosed with hypertension and/or CHD before age 65. Diabetes mellitus, serum lipid profile abnormalities, obesity and metabolic syndrome were classified according to criteria from published guidelines⁵⁻⁷. Diabetes mellitus type II was defined in presence of repeated fasting blood glucose levels >7.0 mmol/L (126 mg/dl) or clinical history of drug, insulin treatment and/or specific diet to control glucose level⁵. Serum lipid profile was assessed through the evaluation of total, LDL and HDL cholesterol and triglyceride levels. Serum cholesterol and/or LDL-cholesterol levels were defined

high if > 5.2 mmol/L (200 mg/dl) and 3.4 mmol/L (130 mg/dl), respectively. HDL cholesterol levels were considered a risk factor if < 1 mmol/L (40 mg/dl) in male and < 1.3 mmol/L (50 mg/dl) in female. Serum triglyceride levels were defined high if >1.7mmol/L (150 mg/dl)⁶. Obesity was diagnosed when body mass index (BMI) was higher than 30 kg/m²⁷. Metabolic syndrome was diagnosed when at least two of the following risk factors affected the same patient: impaired fasting glucose levels, hyper-triglyceridemia, low HDL-cholesterol serum levels and obesity⁶. Smoking habit was considered current if the patient was still smoking and former if the patient had stopped smoking at least two months before coming to our Department. A detailed history of antihypertensive medication used as first, second and third line to treat hypertension has been collected and recorded for each patient.

Single Nucleotide Polymorphisms (SNPs) genotyping

A blood sample was obtained from each patient and genomic DNA was extracted using a commercially available kit (Qiagen). SNPs selection was based on HapMap (public release#11, 09/02/2004) (www.hapmap.org) that identified 6 SNPs, on sequence with GenBank accession n. AF391809, one SNP in the upstream regulatory region rs2227744 (-1738 G/A, nucleotide (n): 352) four intronic SNPs rs27135 (G/A n: 2860); rs27593 (T/C n: 2930), rs2227774 (C/A n: 9113) and rs153311 (A/C n:17841) and one SNP mapping in the mRNA untranslated region rs2227800 (C/T n:19390). Because of their potential importance we also genotyped two additional SNPs reported as polymorphic in the NCBI database (www.ncbi.nlm.nih.gov/), one in the promoter region rs11267092 (-506D/I, -/GGCCGCGGGAAGC, n:1631-1643) formerly associated with a decreased risk of deep vein thrombosis⁸ and a second SNP in exon 2 rs2230849 (T/A n: 18627). A validation assay has been performed and the following SNPs: -1738 G/A, -506D/I, 2860 G/A, 2930 T/C and 9113 C/A were confirmed to be polymorphic in our population.

-506D/I variation consisted in an insertion (I) replicating the preceding 13 bp (GGCCGCGGGAAGC). The replicated sequence contains a putative binding site for the

transcription factor E twenty-six (Ets) [GGA(A/T), in bold]. Sense and anti-sense primer sequences correspond to bases 1565-1584 (5' TCCTGGCCGGGGCGTCCACT3') and to bases 1881-1905 (5' CAGAGACTCTCACTGCACGCCGGA3'), respectively. Amplification reaction was carried out using 25pmol of each primer and 3U of Thermal ACE polymerase (Invitrogen). Thermal cycling conditions consisted of 3 minutes at 98°C followed by 30 cycles with a 30 seconds (sec) denaturation at 98°C, 30 sec annealing (65°C) and 50 sec extension (72°C). PCR products were resolved on 20% polyacrilamide gels.

-1738 G/A, 2860 G/A, 2930 T/C and 9113 C/A were genotyped by means of Dynamic Allele Specific Hybridization (DASH) using custom DASH machinery (ThermoHybaid, Middlesex, UK)^{9,10}. Primers and probes utilized for the current experiments are summarized in Table I.

Random DNA samples were genotyped twice to check for concordance of genotyping. The percentage (%) of drop out for each of the five SNPs was: 10, -1738 G/A; 6.0, -506D/I; 11, 2860 G/A; 7.0, 2930 T/C and 10, 9113 C/A.

Haplotype Generation

Haplotype counts and frequencies were inferred from original genotype data using THESIAS program¹¹. THESIAS takes into account the ambiguity of haplotype assignment in unrelated individuals. However, only haplotypes where at least three loci were successfully genotyped (Controls n=1003; cases n=547) were considered in the analysis.

Electromobility Shift Analysis (EMSA)

Nuclear extracts (10µg) obtained from an immortalized human cervical cancer cell line (Hela) (Santa Cruz) were incubated 30' room temperature with 10ng of the following ³²P- labelled probes-506D 5'CGGCCGCGGGAAGCAGCCTGCGAGCCGTGC'3, -506I 5'CGGCCGCGGGAAGCGGCCGCGGGAAGCAGC'3 and Ets-1 consensus 5'GATCTCGAGCAGGAAGTTCGA'3, (Santa Cruz) in presence of 10 mM MgCl₂, 20 mM Hepes pH 7.9, 20% glycerol, 100 mM KCl, 0,2 mM EDTA, 0,5 mM DTT; Poly dI:dC (2 µg/µl)

(Amersham) and BSA 20 µg . Band specificity was evaluated adding a 20-fold molar excess of unlabeled Ets-1 consensus and -506D sequences to the binding mixture. Probes were end labelled in presence of T4 polynucleotide kinase (NE Biolabs) with ³²P-γ ATP (250 µCi) (Amersham). A single nuclear extracts and probe concentration was tested throughout the experiments. Samples were loaded on Novex 8% polyacrylamide gels (Invitrogen) and visualized by autoradiography. A densitometric analysis was performed by Image Quant software and value expressed as arbitrary densitometric units (ADU).

Vector Construction and Luciferase Assay

-506D and -506I alleles were amplified from genomic DNA of homozygous, DD and II, patients using the following primer pairs: sense 5'**GAAGATCTTATAAATCCCCACGTTACAAAAGCAG**'3 and anti-sense 5'**CCCAAGCTTCCTCTCTCCTGACTTCTG**'3. Sense primer includes *BglIII* recognition sequence (AGATCT) and TATA box (in bold) and corresponds to bases 1435-1452; anti-sense primer includes *HindIII* recognition sequence (AAGCTT) (in bold) and corresponds to bases 2061-2086. This promoter region has been previously characterized and shows an activity comparable to the complete promoter sequence ¹². Amplification reaction was carried out using 25pmol of each primer and 4.5U of Thermal ACE polymerase (Invitrogen). Thermal cycling conditions consisted of 3 minutes at 98°C followed by 30 cycles with a 30 sec denaturation (98°C), 30 sec annealing (60°C) and 50 sec extension (72°C). PCR products were ligated and cloned in pGL3-Basic vector (Promega). Correct insertion of the two PCR products was confirmed by direct sequencing.

Pooled human umbilical vein endothelial cells (HUVECs) (Cambrex), a primary endothelial cell line usually used to study the regulation of human endothelial cells genes, were used to test the effect of -506D/I polymorphism on F2R activity (F2R-A) in three different experimental settings: stimulation with Angiotensin II (ATII), the main effector of circulating and tissutal renin angiotensin system, stimulation with Isoprotenerol (Is), a adrenergic β-receptor agonist, and finally

following prolonged exposure to hypoxia, to mimick three different conditions all correlated to the onset and progression of CHD.

HUVECs at 2nd - 3rd passage were grown in Endothelial Cell Growth medium with 2% serum and the addition of growth culture medium factors following manufacturer' instructions. Duplicate 24-wells plates with 50.000 cells/well were transiently transfected using Lipofectamine 2000 (Gibco) with 0.6µg pGL3-Basic empty vector+0.2µg CMV-β galactosidase vector (Empty), 0.6µg pGL3-506D +0.2µg CMV- β galactosidase (p-506D) and 0.6µg pGL3-506I+0.2µg CMV- β galactosidase (p-506I). Six hours after transfection, medium was replaced and the cells incubated again for 36 hours. Cells transfected with the empty, p-506D and p-506I vectors have been stimulated for 4, 6 and 8 hours with ATII, (10^{-6} M), Is (10^{-7} M), or incubated in hypoxic conditions (95% N₂, 5%CO₂) in a culture medium containing NaCl 116 mM, KCl 54mM, Mg₂SO₄ 800 µM, NaHCO₃ 26.2 mM, NaH₂PO₄ 1 mM, CaCl₂ 1.8 mM, Glycine 10mM and Phenol Red 0.001%, pH 7.3 saturated in a mixture containing 95% N₂ and 5%CO₂ and left to recover overnight in normoxic conditions. Cells lysates were analyzed for luciferase and β galactosidase activities with Dual Light Kit (Tropix) on a Sirius luminometer (Berthold Detection Systems). Luciferase activity, normalized by β-galactosidase light units in the same sample, is expressed as fold increases in relative luciferase activity. In all three experimental settings baseline luciferase activity levels observed in cells transfected with p-506D vector have been taken as reference value.

Statistical Analysis

Continuous variables were expressed as mean±SEM. Differences in the distribution of quantitative traits and categorical variables were tested by one-way ANOVA and by χ² test, respectively. Concordance to the Hardy-Weimberg equilibrium was tested by χ² test with 1DF. Differences in genotypic and allelic frequencies among case and control groups were calculated by χ² test. Unadjusted odds ratio (OR) with 95% confidence interval (CI) were estimated for each

individual SNP under the assumption of an additive (assigning a score of 0, 1 and 2 for homozygous –1738 GG, -506DD, 2860GG, 2930TT, 9113CC; heterozygous –1738 GA, -506DI, 2860GA, 2930TC, 9113CA and homozygous –1738 AA, -506II, 2860AA, 2930CC, 9113AA, respectively), dominant (assigning a score of 0 for homozygous –1738 GG, -506DD, 2860GG, 2930TT, 9113CC and 1 for combined homozygous/heterozygous –1738 AA/GA, -506II/DI, 2860AA/GA, 2930CC/TC, 9113AA/CA) and recessive (assigning a score of 0 for combined homozygous/heterozygous –1738 GG/ GA, -506DD/DI, 2860GG/GA, 2930TT/TC, 9113CC/CA and a score of 1 for homozygous –1738 AA, -506II, 2860AA, 2930CC, 9113AA, respectively) model of inheritance.

Pairwise linkage disequilibrium (LD) between markers was estimated calculating D' values with Arlequin software¹³ and the square root of the correlation coefficient between two loci, r^2 metric¹⁴.

Unadjusted odds ratio (OR) with 95% CI were estimated for each haplotype by THESIAS taking the more commonly observed haplotype in this population, Hap0, as reference category. THESIAS also calculates the OR and 95% CI associated with each allele according to the haplotypic background. In this setting the OR corresponds to the exponential of the difference between the OR observed with each of the two alleles in the context of a specific haplotype. PHASE v2.0 program¹⁵ was used to perform a permutation analysis to test the hypothesis that haplotypes observed in cases and controls are extracted from a common set of haplotypes present in the population and that differences observed are related to the case or control status and not to a chance finding.

Two different stepwise logistic regression analysis models, backward stepwise and likelihood ratio forward stepwise, identified age, male sex and diabetes as independent predictors of CHD and treatment with ARBs, diuretics and DHP Ca^{++} antagonists as potential confounders and were adjusted for in a multivariable logistic regression analysis at both single SNP and haplotype analysis.

One potential problem regarding analyses of associations of several SNPs within a gene and a phenotype is the need for corrections for multiple testing. Upon completion of the collection of HapMap Project, it has been proposed to use a gene-wide significance level (P) that takes into account the overall genetic variation in the genomic region under investigation¹⁶. This approach corrects the actual p value for the ratio (r) SNPs tested/all SNPs validated within that specific region and applying the formula $P=1-(1-0.05)^r$. Up to date 20 SNPs have been validated within the F2R region giving a corrected P value of 0.012. Even using this more conservative P value our findings at the -506 locus and the results of haplotype analyses still yield significant associations.

Experiments performed in *in vitro* were done three times in duplicate. Results are expressed as mean±SEM and differences among different groups were evaluated using the Kruskal-Wallis H-test.

All calculations were carried out using SPSS statistical package (vers12.1, Chicago, IL).

RESULTS

Pairwise LD values among the five SNPs, represented by D' and r^2 metric values, are reported in Table 2.

Table I. Assays design for Dynamic Allele Specific Hybridization

<i>SNP</i>	<i>Sequence</i>	<i>Genomic sequence</i>	<i>MRNA orientation</i>	<i>Position</i>	<i>Biotin labelled primer</i>	<i>Probe sequence</i>	<i>Probe orientation</i>
-1738 G/A	AACTTCT [G/A]TGTACAACA	AF391809	Plus	352	Sense	GTTGTACA C AGAAGTTG	reverse
2860 G/A	GGGACATG[G/A]AGAGGATT	AF391809	Plus	2860	Sense	GGGACATG G AGAGGATT	forward
2930 T/C	CCCCAGGA[T/C]GGGCTCC	AF391809	Plus	2930	Sense	GGGAGCCC G GTCCTGGG	forward
9113 C/A	TTGGTGAC[C/A]ATGCAAAG	AF391809	Plus	9113	Antisense	TTGGTGAC C ATGCAAAG	forward

Table II. Pairwise LD values between SNPs within F2R gene

	-1738G/A	-506 D/I	2860G/A	2930T/C	9113C/A
-1738G/A	*	0.68	0.92	0.81	0.73
-506 D/I	<i>0.13</i>	*	0.67	0.28	0.45
2860G/A	<i>0.70</i>	<i>0.13</i>	*	0.89	0.73
2930T/C	<i>0.18</i>	<i>0.008</i>	<i>0.22</i>	*	0.81
9113C/A	<i>0.14</i>	<i>0.19</i>	<i>0.13</i>	<i>0.06</i>	*

Values reported on the upper right side of the table represent D' values. Values on the lower left hand side of the table (*italics*) represent r^2 values.

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