Hypervariability in a minisatellite 3' of the apolipoprotein B gene in patients with coronary heart disease compared with normal controls

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Abstract Several recent reports have examined whether there is a correlation between the presence of some minor alleles of the highly polymorphic apolipoprotein B gene and atherosclerosis and premature heart disease. The present study extends this investigation. A high-resolution method was used to study the allele frequencies of a hypervariable minisatellite region close to the apolipoprotein B gene in 110 patients with severe coronary disease and in 117 normal controls. Alleles containing 38, 44, 46, or 48 hypervariable elements showed an association with coronary heart disease. These alleles were also associated with elevated serum levels of total cholesterol and apolipoprotein B among patients and with elevated serum levels of total triglycerides among controls. The hypervariable region showed strong linkage disequilibrium with a polymorphic *Eco*RI site in exon 29 and was in linkage equilibrium with a polymorphic *Msp*I site in exon 26. Two patients carried a base change at codon 3500 that results in an arginine-to-glutamine substitution; the base change was linked in both instances to the allele with 48 hypervariable elements. — **Friedl, W., E. H. Ludwig, B. Paulweber, F. Sandhofer, and B. J. McCarthy.** Hypervariability in a minisatellite 3' of the apolipoprotein B gene in patients with coronary heart disease compared with normal controls. *J. Lipid Res.* 1990. **31:** 659–665.

Supplementary key words LDL • apolipoprotein B gene • hypervariable region • heart disease

Elevated low density lipoprotein (LDL) cholesterol in the serum is an important risk factor for the development of premature coronary heart disease (1, 2). The protein moiety of LDL is apolipoprotein (apo) B-100, a large protein with a molecular mass of 550 kDa (3). Apolipoprotein B-100 mediates the uptake of LDL from the serum into liver and peripheral cells via the LDL receptor and therefore plays a central role in cholesterol homeostasis in humans (4). The gene coding for apoB-100 has been localized to chromosome 2, and its structure has been studied in great detail (5–7). A number of restriction fragment length polymorphisms (RFLPs) within and close to the apoB-100 gene have been identified (8–14). Several research groups have studied associations between RFLPs and lipid levels in coronary heart disease (15–17).

A high degree of heterozygosity is provided by hypervariable regions that are distributed throughout the human genome (18, 19). These regions consist of tandem repeats of short sequences of 10–50 base pairs (bp) and have been referred to as minisatellites. Allelic differences in the number of repeats give rise to polymorphism, providing markers that exhibit a high degree of heterozygosity. The present study focuses on a hypervariable region (HVR) located immediately downstream from the apoB-100 gene (20–22). It starts 73 bp 3' from the second putative polyadenylation signal, and the alleles vary in length over a range of about 400 bp. Using the polymerase chain reaction (PCR) and agarose gel electrophoresis, Boerwinkle et al. (23) defined 12 different alleles in a sample of 125 unrelated individuals. Ludwig, Friedl, and McCarthy (24) developed a method for rapid analysis of this region that provided an even higher degree of resolution among alleles of different sizes. By this method, 14 different alleles were defined. Most of the variation was due to the number of repeats of 15-bp hypervariable elements (HVE) with minor differences in sequence. In the present study this high-resolution method was used to type 110 patients who have survived a myocardial infarction and 117 matched controls who had non history of heart disease. All

Abbreviations: LDL, low density lipoprotein; apo, apolipoprotein; RFLP, restriction fragment length polymorphism; bp, base pairs; kb, kilobase; HVR, hypervariable region; PCR, polymerase chain reaction; HVE, hypervariable element.

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subjects were also typed for RFLPs detected with *MspI* and *Eco*RI near the 3' end of the gene. In addition, haplotype analysis was performed. All subjects were also screened for a recently described point mutation in the apoB-100 gene that creates an arginine-to-glutamine change in codon 3500. In previous studies, this mutation showed a strong association with LDL that was defective in binding to the LDL receptor and with elevated levels of plasma cholesterol (25–27).

MATERIALS AND METHODS

Subjects

Two hundred twenty-seven unrelated male European Caucasians under the age of 55 were studied. One hundred ten subjects were patients who had severe coronary heart disease: 105 had suffered at least one myocardial infarction as diagnosed by EKG criteria and measurements of heart-specific serum enzymes, and the other 5 had >70% narrowing of at least one coronary artery as determined by coronary angiography. The patients were taken consecutively from a Cardiologic Care Unit at the Ladeskrankenanstalten Salzburg and a Rehabilitation Center for Heart Disease near Salzberg, Austria. Four patients were taking bezafibrate at the time of study. One hundred seventeen matching normal subjects were volunteers from the Austrian army and from various sports clubs. None had a history of heart disease, and all tested normal on electrocardiograms both during rest and physical stress. The mean age of the patients was 47.1 (± 5.7), and that of the controls was 49.7 (± 5.4). It was ascertained from questionnaires that all subjects in both the patient and control group were Middle European Caucasian.

DNA preparation, polymerase chain reaction, and separation of HVR alleles

Total human DNA was prepared from leukocytes by standard methods. The PCRs were performed with thermostable DNA polymerase from *Thermus aquaticus* (Cetus) in an automated Perkin-Elmer Cetus Thermal Cycler (28). Amplification conditons, labeling, separation, and size determination of the HVR alleles are described elsewhere (24). The positions of the oligonucleotide primers that were used to amplify the HVR alleles and the region containing the mutation in codon 3500 are shown in **Fig. 1**.

Genotyping for the *Eco*RI and *Msp*I RFLPs, and screening for the mutation at position 3500

Total genomic DNA was digested with the enzymes *Eco*RI and *Msp*I and analyzed by Southern blotting as previously described (17). The positions of the probes used are shown in Fig. 1. A 3.5-kb genomic fragment was used for *Msp*I (probe I), and a 0.5-kb cDNA was used for *Eco*RI (probe II). Labeling was performed with ^{32}P by using a random oligonucleotide primer method (29).

The PCR was used to amplify the region encompassing the codon for the arginine-to-glutamine mutation at position 3500. One microgram of the amplified DNA was applied to slot blots and hybridized to allele-specific oligonucleotides AS01 (wild type), AS02 (mutant), and AS03 (control). Probe labeling and hybridization conditions have been previously described (26).

Determination of serum lipid levels, and statistical analysis

Blood samples were drawn after a fasting period of 12–14 h and not less than 6 weeks after a myocardial in-

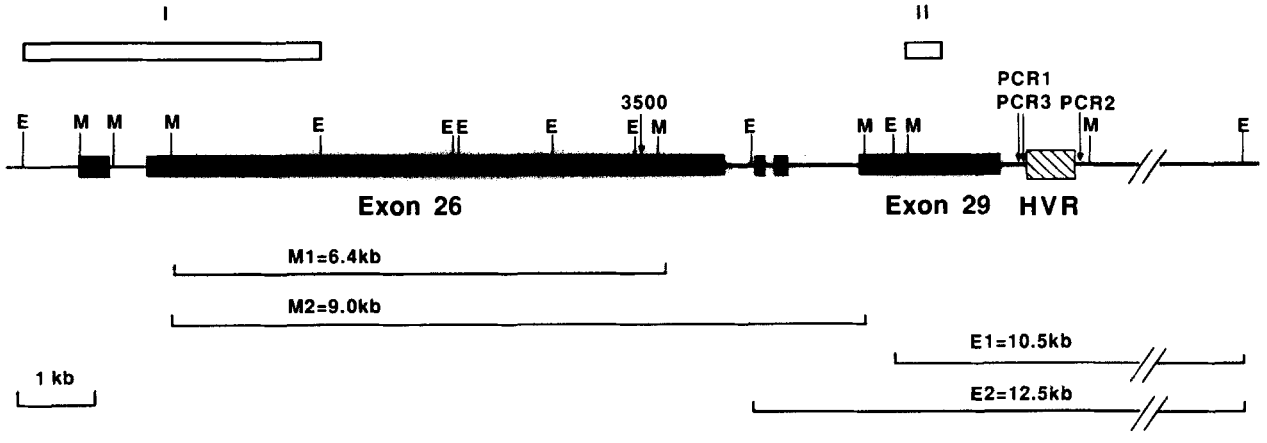

Fig. 1. Positions of the HVR, the *Eco*RI (E) and *Msp*I (M) RFLPs, and the point mutation in codon 3500 near the 3' end of the apoB-100 gene. Exons 25 through 29 (black boxes) with the interrupting intronic regions (lines) and the immediate region downstream of the gene are shown. The white bars in the upper part of the figure show the location of the probes used for Southern blotting. The horizontal brackets in the lower part indicate the sizes of RFLPs that were obtained with *Msp*I (M1 and M2) and *Eco*RI (E1 and E2). The positions of oligonucleotides used as amplification primers are indicated by arrows.

farction. Total serum cholesterol and triglyceride levels were measured enzymatically using a commercial kit (Boehringer GmbH, Mannheim, FRG) (17). Serum apoB levels were determined with a radial immunodiffusion kit (Immuno Austria). Body mass index was calculated from the formula weight (kg)/height (m^2). The mean values for age, body mass index, and lipid parameters among patients and controls were analyzed using a Student's *t*-test. A Bonferroni correction for multiple analyses was applied.

The allele frequencies among patients and controls were analyzed by using a chi-square test. Variance of lipid levels in relation to genotype groups was determined with a one-way analysis of variance by using the Minitab statistical computing system (State College, PA). The standardized linkage disequilibrium statistic (Δ) was used pairwise between *Eco*RI and HVR and between *Eco*RI and *Msp*I to obtain an estimate of linkage disequilibrium (30).

RESULTS

HVR alleles in patients and controls

A high-resolution method was used to type a hypervariable minisatellite region that begins 73 bp downstream from the second putative polyadenylation signal of the gene. Among the 227 individuals studied, 14 different alleles were observed, as previously described (24). The alleles differed in the number of repeats of HVE that they contained: from 25 to 52 (HVE 25–HVE 52). The distribution of the alleles among patients and controls is displayed graphically in **Fig. 2**. The relative frequency of the most common allele (HVE 36) was almost the same in both groups. Most alleles smaller than HVE 36, i.e., HVE 30, HVE 32, HVE 34, and HVE 35, were more frequent in the control group. On the other hand, most alleles larger than HVE 36, i.e., HVE 38, HVE 44, HVE 46, and HVE 48, were more common among patients. The rarer alleles HVE 25, HVE 40, HVE 42, HVE 50, and HVE 52 did not follow this pattern: one allele, HVE 52, occurred only in the patient group, and allele HVE 42 was evident only in the control group. Frequencies for HVR alleles among patients and controls are compared in **Table 1**.

Genotyping for *Msp*I and *Eco*RI, and linkage analysis with the HVR locus

All subjects were also typed for two RFLPs within the apoB-100 gene: a variable *Msp*I site in exon 26 in codon 3611, and a variable *Eco*RI site in exon 29 in codon 4154. Genotypes E2/E2 and E1/E2 displayed an association with coronary heart disease, as previously reported (17). No significant association was observed between the *Msp*I polymorphism and coronary heart disease (data not shown). Pairwise haplotypes were determined for *Eco*RI/HVR and for *Eco*RI/*Msp*I. In the absence of family data, haplotypes could only be determined for subjects who were homozygous for at least one of the markers. Because of the high variability of the HVR, 68 double heterozygotes were observed (**Table 2**). From the patients and controls who were E2/E2 or E1/E1 homozygotes, 318 individual haplotypes could be deduced (Table 2). To test the hypothesis that the *Eco*RI alleles are randomly associated with HVR alleles, the HVR alleles were grouped according to the number of repeats (HVE 25–36 vs. HVE 38–52) and a standardized linkage disequilibrium statistic (Δ) was calculated (30); Δ was 0.684, indicating linkage disequilibrium between *Eco*RI and HVR (**Table 3**).

The grouping of the HVEs into two categories and the exclusion of the double heterozygotes might lead to some overestimation of the linkage disequilibrium. Although

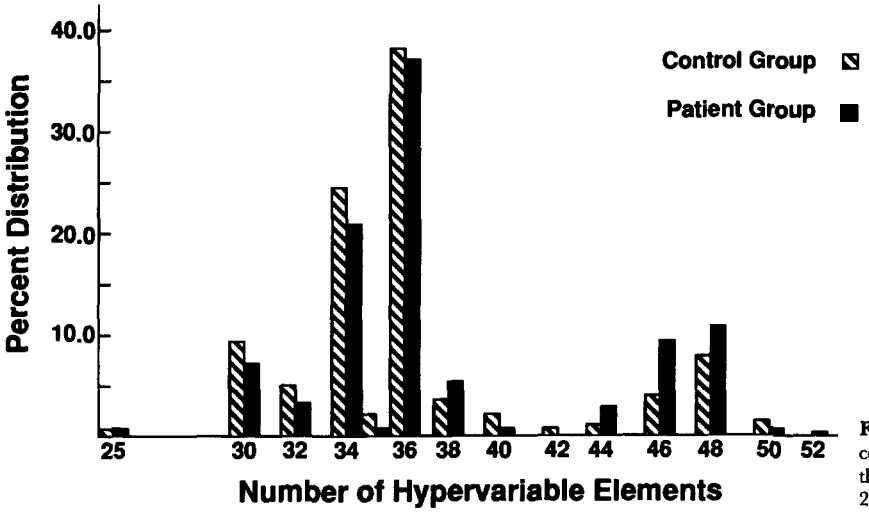

Fig. 2. Frequencies of HVR alleles in patients and controls. The frequencies are given as the percentage of the total number of alleles: 220 in the patient group and 234 in the control group (see Table 1 also).

TABLE 1. Relative allele frequencies for hypervariable elements, the variable loci for *Msp*I and *Eco*RI, and an arginine-to-glutamine substitution at position 3500 in patients and controls

Genotypes	Total Alleles (n = 454)	Alleles of Patients (n = 220)	Frequencies	Alleles of Controls (n = 234)	Frequencies	*P*
HVEs			%		%	
25	2	1	0.46	1	0.43	
30	40	17	7.73	23	9.83	
32	20	7	3.18	13	5.56	
34	102	43	19.55	59	25.21	
35	7	2	0.91	5	2.14	
36	169	83	36.75	86	37.73	
38	22	13	5.91	9	3.85	
40	5	1	0.46	4	1.71	
42	1	0	0	1	0.43	
44	8	6	2.73	2	0.86	
46	28	19	8.64	9	3.85	
48	43	25	11.36	18	7.69	
50	6	2	0.91	4	1.71	
52	1	1	0.46	0	0	
HVE groups[a]						
25-35 (<36)	171	70	51.09	101	68.24	$P = 0.025$
38-52 (>36)	114	67	48.91	47	31.76	$P = 0.01$
Substitution						
Arg to Gln at 3500		2	0.40	0	0	

[a]The *P* values are shown in the last column. Results that were considered statistically significant at a $P < 0.05$ are listed.

we could not deduce the haplotypes in the double heterozygotes, a nonrandom association seems likely, since the majority of the double heterozygotes have one "short" and one "long" HVR allele. Linkage analysis for *Eco*RI with *Msp*I indicated linkage equilibrium between the two sites ($\Delta = 0.124$).

Correlation of lipid parameters with HVR alleles

A comparison of lipid parameters of patients and controls is given in the upper section of **Table 4**. Mean body mass index and age distribution are also shown. The subjects were subdivided into three groups on the basis of HVR genotypes (shown in the lower section of Table 4). The first group contained subjects who were homozygous for a low number of HVE (HVE 25-36/HVE 25-36), the second group comprised subjects who carried one allele with a low HVE number and one allele with a high HVE number (HVE 25-36/HVE 38-52), and the third group comprised subjects who were homozygous for a high HVE number (HVE 38-52/HVE 38-52). Some association was observed between the HVE 38-52 alleles and higher levels of cholesterol, triglycerides, and apoB. Although this association did not reach statistical significance, the same trend was observed in both patient and control groups.

A mutation at amino acid position 3500

All subjects were also screened for a specific mutation in codon 3500, which is associated with familial defective apolipoprotein B-100 (25-27). Two patients carried the mutation. Both had severe coronary heart disease and had suffered myocardial infarctions. In both cases, it was possible to determine their haplotypes unequivocally: the first patient carried alleles M1-E2-HVE44 and M1-E2-HVE48, and the second patient carried alleles M1-E2-HVE46 and M1-E2-HVE48. Family studies revealed that in both cases the mutation occurred on an M1-E2-HVE48 allele (data not shown). The mutation did not occur in the control group.

TABLE 2. Haplotypes observed with *Eco*RI and HVR in patients and controls

Unambiguous Haplotypes (318)[a]		Double Heterozygotes (68)	
*Eco*RI	HVE	*Eco*RI	HVE
1	25 (1)	1/2	25/48 (1)
1	30 (34)	1/2	30/40 (1)
1	32 (18)	1/2	30/46 (3)
1	34 (81)	1/2	30/48 (2)
1	35 (6)	1/2	32/46 (1)
1	36(137)	1/2	32/48 (1)
1	38 (11)	1/2	34/36 (1)
1	40 (4)	1/2	34/38 (4)
1	44 (1)	1/2	34/44 (1)
1	46 (5)	1/2	34/46 (6)
2	38 (1)	1/2	34/48 (6)
2	44 (3)	1/2	34/50 (3)
2	46 (5)	1/2	35/48 (1)
2	48 (10)	1/2	36/38 (2)
2	52 (1)	1/2	36/44 (2)
		1/2	36/46 (7)
		1/2	36/48(19)
		1/2	36/50 (1)
		1/2	36/52 (1)
		1/2	38/44 (1)
		1/2	38/48 (2)
		1/2	38/50 (1)
		1/2	42/48 (1)

[a]Number of observations is given in parentheses.

TABLE 3. Pairwise linkage analyses of *Eco*RI with HVR and *Msp*I in patients and controls

	HVE 25-36	HVE 38-52	M1	M2
E1	277(0.78)[a]	21(0.06)	317(0.73)	38(0.087)
E2	0(0)	20(0.06)	76(0.17)	1(0.002)
	n = 318 Δ^b = 0.684[c]		n = 432 Δ^b = 0.1244	

[a]Haplotype frequencies are given in parentheses.
[b]Δ values were calculated as described in ref. 30.
[c]Significantly different from zero.

DISCUSSION

The point of departure for the present study was the proposition that some minor alleles of the highly polymorphic apoB gene act as a risk factor for atherosclerosis and premature heart disease. Previous tests of this hypothesis were based on attempts to correlate the presence of various RFLPs or polymorphisms defined by antibodies with lipid, lipoprotein, or apoprotein levels and with coronary heart disease (21, 31-36). Although some correlations have been reported, other results have been contradictory (16). For example, one report revealed no association between RFLPs and lipid data but did demonstrate an association of some markers with coronary disease (15). Nevertheless, it does seem clear that some apoB alleles lead to higher levels of apoB and LDL in the plasma (31, 35). Another recent study concluded that some apoB RFLPs and haplotypes derived from them were correlated with elevated serum cholesterol levels, coronary heart disease, and obesity (36). The disagreement among these various studies is perhaps not surprising and may be accounted for by the small number and ethnic diversity of the subjects studied and the choice of uninformative RFLP markers.

In our investigation, we chose to study a relatively homogenous Austrian population of coronary heart disease and control subjects that has been described earlier (17). As a marker to distinguish among different alleles, we chose a hypervariable region just 3' of the apoB gene. At least 14 different alleles are distinguishable by the method employed (24), and the average level of heterozygosity was 78%. This variability provides high resolution,

TABLE 4. Clinical data for patients and controls and mean lipid levels of HVE genotype groups in patients and controls

Parameters	Patients (n = 110)	Controls (n = 117)	P*[a]
Mean age (years)	47.1 (± 5.7)	49.7 (± 5.4)	0.0025
Body mass index (kg/m^2)	26.3 (± 3.3)	25.2 (± 2.7)	0.0275
Serum cholesterol (mg/dl)	220.5 (± 34.9)	206.2 (± 37.8)	0.0175
Serum triglycerides (mg/dl)	144.7 (± 74.1)	124.9 (± 73.7)	0.0225
Serum apoB (mg/dl)	107.5 (± 22.0)	98.0 (± 24.5)	0.0125
HVR genotype groups			
A. Cholesterol			
HVE 25-36/HVE 25-36	218.9 (± 34.9) [n = 55]	207.5 (± 39.6) [n = 73]	
HVE 25-36/HVE 38-52	220.3 (± 32.6) [n = 43]	200.2 (± 34.4) [n = 41]	
HVE 38-52/HVE 38-52	234. 7 (± 44.9) [n = 12]	224.0 (± 40.6) [n = 3]	
	P = 0.365	P = 0.427	
B. Triglycerides			
HVE 25-36/HVE 25-36	139.2 (± 78.7)	112.5 (± 60.3)	
HVE 25-36/HVE 38-52	147.1 (± 67.6)	137.6 (± 83.5)	
HVE 38-52/HVE 38-52	168.2 (± 83.9)	186.3 (± 167.1)	
		P = 0.068	
C. Apolipoprotein B			
HVE 25-36/HVE 25-36	106.5 (± 22.1)	98.8 (± 21.8)	
HVE 25-36/HVE 38-52	107.1 (± 22.1)	94.2 (± 27.0)	
HVE 38-52/HVE 38-52	122.9 (± 29.2)	111.3 (± 53.5)	
	P = 0.074	P = 0.387	

[a]P*, P after Bonferroni correction.

but the degree of heterozygosity is not so high as to suspect excessive germ-line instability that characterizes the most hypervariable minisatellite loci (37). In none of the families studied have alleles of a new length appeared that are inconsistent with normal Mendelian inheritance (21–24). The linkage disequilibrium between the two groups of HVEs and the *Eco*RI polymorphism also attests to this stability. In the present study we found a correlation between HVR alleles HVE38, HVE44, HVE46, and HVE48 and severe coronary heart disease. A previous study by Hegele et al. (15) demonstrated a strong correlation between the apoB 3' HVR polymorphism and myocardial infarction. These investigators used restriction digests of total genomic DNA along with *Msp*I and Southern analysis to analyze this polymorphism. An exact size determination of the fragments was not made; the fragments were simply divided into two size groups (ID1 and ID2). ID1 contained fragments larger than or equal to 2.5 kb, and ID2 contained fragments smaller than 2.5 kb. Since the frequency distribution of HVE alleles is bimodal (23, 24), it is probable that ID1 corresponds to HVE 44–52 and ID2 to HVE 25–36. The ID1 group of alleles proved to be more frequent in coronary heart disease in patients than in controls. The present study confirms and extends this previously reported correlation of HVR alleles with coronary heart disease. The fact that similar correlations were found in two totally different populations suggests that this finding may have some generality and that the hyperallelism of the 3' HVR may be a useful marker for minor apoB alleles that are deleterious.

To test the hypothesis that HVE alleles were associated with serum levels of cholesterol, triglycerides, or apoB, we grouped the HVR genotypes as described under Results. Serum levels in subjects carrying groups of alleles that showed an association with coronary heart disease (HVE 38–52) were compared with those that failed to show any such association (HVE 25–26). In both patient groups, the larger HVR alleles were associated with elevated levels of serum cholesterol and serum apoB. But none of these differences were statistically significant.

Two patients carried a point mutation that creates an arginine-to-glutamine change at position 3500 of the apoB gene (26). The mutation was not found in the control group. Interestingly, a second degree relative of one of the patients with the mutation died from a heart attack at the age of 39. From family studies it could be inferred that he also was a carrier of this mutation (data not shown). The precise frequency of the mutation in the population has not yet been established, and its role in the development of premature coronary heart disease remains to be elucidated, although its presence does correlate with elevated LDL cholesterol (27, 38).

In conclusion, the hyperallelism displayed by the 3' HVR provides a high resolution method to distinguish multiple apoB alleles. The present study has provided evidence for unequal distribution between patient and control groups. However, the fact that so many alleles are distinguishable means that large numbers of subjects must be studied to obtain statistically significant data concerning the association of individual alleles with disease. Another line of investigation in which the HVE marker may also prove useful concerns the origin, evolution, and population distribution of mutations within the apoB gene that are associated with disease, such as the mutation in the codon for amino acid 3500.

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