The apolipoprotein B R3531C mutation: characteristics of 24 subjects from 9 kindreds

Clive R. Pullinger,[1,2,*] Dairena Gaffney,[1,] Monica M. Gutierrez,[††] Mary J. Malloy,[*,†] Verne N. Schumaker,[††] Chris J. Packard,[**] and John P. Kane**[*,§]

Cardiovascular Research Institute, Department of Medicine,* Department of Pediatrics,† and Department of Biochemistry and Biophysics,§ University of California, San Francisco, CA 94143-0130; Institute of Pathological Biochemistry,** Glasgow Royal Infirmary, Glasgow, Scotland; and Department of Chemistry and Biochemistry,†† University of California, Los Angeles, CA 90095

Abstract Familial ligand-defective apolipoprotein B (apoB) is a group of disorders caused by mutations in the apoB gene. In this report the R3531C mutation is characterized further using a monoclonal antibody MB19/dynamic laser light scattering technique to measure ratios of Cys[3531] to normal low density lipoprotein (LDL) particles. All six subjects studied showed a preferential accumulation of particles carrying the defective apoB allotype. We determined binding properties of LDL from R3531C heterozygotes by measurement of high-affinity binding to LDL receptors on fibroblasts and its ability promote growth of U937 cells. LDL from R3531C heterozygotes, compared to normal LDL, had 49.3% of the binding affinity and was 74% as effective in a U937 cell proliferation assay. To identify new probands, we screened 2570 subjects for the R3531C mutation. Nine probands were found with 15 affected relatives. Of the seven haplotypes we uncovered, two were novel, while five were identical to one initially reported as associated with Cys[3531]. Three silent mutations were detected also: T3540T, N3542N and T3552T. Analysis of lipid profiles of R3531C families showed, as with the R3500Q mutation, variable expression of the phenotype, modulated by environmental and other genetic factors. Both mutations tend to produce lower plasma levels of LDL in affected subjects than do defects of the LDL receptor (familial hypercholesterolemia, FH). This study shows that the Cys[3531] LDL particles are not only defective at binding to the LDL receptor, as determined by two separate methods, but that in all cases they accumulate preferentially compared to the normal allotype.— Pullinger, C. R., D. Gaffney, M. M. Gutierrez, M. J. Malloy, V. N. Schumaker, C. J. Packard, and J. P. Kane. **Apolipoprotein B R3531C mutation: characteristics of 24 subjects from 9 kindreds.** *J. Lipid Res.* **1999.** 40: **318–327.**

Supplementary key words apolipoprotein B • atherosclerosis • hypercholesterolemia • haplotypes • genetic mutation

Familial ligand-defective apolipoprotein B (apoB) is a group of autosomal-dominant inherited disorders caused by mutations of the low density lipoprotein (LDL) receptor ligand domain in apoB (1). A somewhat less severe form of hypercholesterolemia is associated with these disorders than seen with mutations of the LDL receptor in familial hypercholesterolemia (FH), as would be expected, a priori, because the affinity of very low density lipoprotein (VLDL) remnants for the LDL receptor is not affected as clearance of these remnants is mediated by apoE (1). Three mutations of apoB have been reported that cause reductions in binding affinity for the LDL receptor. These are the R3500Q (2), R3531C (3, 4), and R3500W (5) mutations.

Because each LDL particle carries a single molecule of apoB-100, it would be expected, a priori, that such defects would lead to the selective accumulation in plasma of those particles bearing the defective apoB allotype. We have identified nine additional R3531C probands and have in a number of cases measured the mass ratios of the defective Cys[3531] LDL to normal Arg[3531] LDL particles using a monoclonal antibody MB19/dynamic laser light scattering technique. This approach determines specifically whether an apoB mutation results in a change in lipoprotein metabolism. It is based on the pioneering work of Young and his co-workers (6). They showed that the monoclonal antibody MB19 against human apoB could be used to detect a polymorphism of apoB with LDL designated $MB19_1/MB19_1$ binding with high affinity, and $MB19_2/MB19_2$ with low affinity. LDL from heterozygotes had an intermediate binding affinity. This polymorphism was shown to be an ApaLI restriction fragment length polymorphism (RFLP) in codon 71, a Thr→Ile substitution (7), and represents the Ag(c/g) locus on apoB (8), with Ile[71] being the high binding and Thr[71] the

Abbreviations: apoB, apolipoprotein B; CAD, coronary artery disease; DGGE, denaturing gradient gel electrophoresis; FH, familial hypercholesterolemia; HDL, high density lipoprotein; LDL, low density lipoprotein; RFLP, restriction fragment length polymorphism; TC, total plasma cholesterol; TG, total plasma triglyceride; VLDL, very low density lipoprotein; VNTR, variable number of terminal repeats.

[1]Both authors contributed equally to this study.

[2]To whom correspondence should be addressed.

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low binding allotype. The polymorphism itself has no influence on plasma levels of apoB or lipoproteins (9). A solid-phase MB19 radioimmunoassay assay (6) was used with patients in kindreds with familial hypobetalipoproteinemia to show different mass ratios of the two apoB allotypes in plasma (10, 11). It has also been used with R3500Q subjects, who are also Thr^{71}/Ile^{71} heterozygotes, to determine the percentage of defective LDL in plasma (12, 13). A refinement of the method using dynamic scattering of laser light from LDL-MB19 complexes (14) has been used in the present study to generate more precise estimates of allelic ratios. A maximum of two LDL particles can be bound by the bivalent antibody. MB19 will promote substantial dimer formation with Ile^{71} apoB LDL, but little with Thr^{71} apoB LDL. When compared to a calibration curve, constructed from mixtures of Ile^{71}/Ile^{71} and Thr^{71}/Thr^{71} apoB LDL, the amount of dimer formed with LDL isolated from heterozygous Thr^{71}/ Ile^{71} individuals yields an estimate of the ratio of the two apoB allotypes.

The ability of LDL prepared from R3531C subjects to promote growth of a cell line with a growth requirement for LDL was compared with that of normal LDL. In individual cases, the effect of the mutation on the binding affinity for the LDL receptor was measured using both conventional displacement and dual-label fibroblast assays. We have also assessed the inter-individual variation in the expression of this mutation in terms of its effects on the levels of blood lipids and lipoproteins in seven kindreds.

The serendipitous discovery of three novel apoB gene mutations as a result of these studies is reported.

METHODS

Preparation of genomic DNA

Genomic DNA was routinely prepared from whole blood obtained from patients in the lipid clinics of University of California, San Francisco (UCSF) and the Glasgow Royal Infirmary as previously described (4, 5). DNA was also prepared from volunteers who took part in the Determinants of Coronary Disease in Women study at UCSF.

Identification of R3531C probands

To detect the apoB R3531C mutation, two approaches were used. First, DNA prepared from 1372 patients and unselected volunteers attending the Lipid Clinic of the University of California, San Francisco, was screened using a previously described method (4). The PCR product spanning nucleotides 9561 to 11142 was digested with NsiI prior to agarose electrophoresis. The R3531C mutation is detected as the gain of an NsiI site due to the change at nucleotide 10800.

Second, DNA prepared from 1171 patients attending the Lipid Clinic of the Glasgow Royal Infirmary was subjected to amplification by polymerase chain reaction (PCR) followed by analysis by denaturing gradient gel electrophoresis (DGGE). Primers were chosen for PCR using the Melt 87 programs (written by Dr. Leonard Lerman, 1988, copyright with Massachusetts Institute of Technology, Cambridge, MA). A region of apoB spanning the 3531 codon was amplified using buffer, primer, and enzyme concentrations as recommended by the manufacturer (MBI Fermentas, Tyne and Wear, UK). The primers used (**Table 1**) flank 207 base pairs of sequence from codon 3512 to codon 3581 (Table 1, fragment I). Thirty five cycles of PCR were performed on a Hybaid Touchdown thermal cycler (Hybaid, Teddington, Middlesex, UK), consisting of denaturation (95°C for 5 min initially and 1 min thereafter), annealing (66°C for 1 min), and extension (72°C for 1 min). After the final extension step the products were heated to 98°C for 12 min and cooled slowly. The products of the PCR amplification were subjected to DGGE on an 8% polyacrylamide gel at 60°C for 16 h at 55 volts. The gradient was 35% to 60% denaturant as defined by Myers, Maniatis, and Lerman (15). Other conditions were as previously described (5). To confirm the pattern observed on DGGE with each R3531C patient (J.K., H.M., J.C., and J.R.) the PCR product was synthesized as for DGGE, but with rapid cooling to encourage homoduplexes. After digestion with NsiI the samples were run on a nondenaturing 8% polyacrylamide gel. The absence of an NsiI site resulted in an uncut band of 286 bp, (normal allele and normal controls) while

TABLE 1. PCR amplified fragments of apoB gene exon 26 subjected to DGGE analysis

Fragment	Oligonucleotide Primers	Nucleotides (cDNA)	Codons Screened[a]	Size[b]	Restriction Enzyme Site	Size after digestion[b]		Number of Patients Screened
						5′	3′	
				bp				
I	5′-CTG,CAG,GGC,ACT,TCC,AAA,ATT,G-3′ 5′-cgc,ccg,ccg,cgc,ccc,gcg,ccc,gtc,ccg,ccg,ccc,ccg--CAG,GGA,AAT,CAT,GGA,AGG,AAC-3′	10722–10971	3512–3581	250 (286)	–	–	–	1171
II	5′-cgc,ccg,ccg,cgc,ccc,gcg,ccc,gtc,ccg,ccg,ccc,ccg--CCA,CTG,AAA,GAT,TTC,TCT,CTA,TGG,G-3′ 5′-cgc,ccg,ccg,cgc,ccc,gcg,ccc,gtc,ccg,ccg,ccc,gcg--,cCT,AGG,GAC,ATG,AAG,GAC,TGG,CAG,CTC-3′	9564–10060	3127–3275	497 (570)	RsaI 9824	262 (298)	235 (272)	27
III	5′-cgc,ccg,ccg,cgc,ccc,gcg,ccc,gtc,ccg,ccg,cgc,ccc--GCC,TTC,ATA,CAC,ATT,AAT,CCT,GCC-3′ 5′-cgc,ccg,ccg,cgc,ccc,gcg,ccc,gtc,ccg,ccg,ccc,ccg--cGA,AAT,CAT,ACT,TAA,ATT,CCA,TGG,AG-3′	10004–10520	3273–3429	517 (590)	PstI 10257	256 (292)	261 (298)	27
IV	5′-cgc,ccg,ccg,cgc,ccc,gcg,ccc,gtc,ccg,ccg,ccc,ccg--CCA,AGT,CAA,AAC,CTA,CTG,TCT,CTT,CC-3′ 5′-cgc,ccg,ccg,cgc,ccc,gcg,ccc,gtc,ccg,ccg,ccc,gcG--CCA,CTT,CCT,GGC,CAA,GGT,CAG,GGA,AAT,C-3′	10471–10990	3430–3584	520 (591)	EcoRI 10691	223 (259)	297 (332)	27

Lower case lettering in oligonucleotide sequences indicates GC-rich clamps.
[a] Excludes codons in primers.
[b] Size in parentheses incudes GC-rich clamps.

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its presence (R3531C mutation) resulted in the two additional bands on the gel of 206 and 80 bp.

Other DGGE analysis

As a result of routine screening of LDL prepared from patients attending the UCSF lipid clinic, a number of patients were identified whose LDL had a binding affinity for the LDL receptor that was below the normal range. A fibroblast dual-label equilibrium binding assay was used for this purpose as described previously (4, 16). DNA prepared from 27 of these patients and from individuals with known apoB variants (Q3405E, R3480P, R3500Q, R3500W, and R3531C) was subjected to three separate PCR amplifications yielding overlapping fragments II, III, and IV (Table 1). These fragments were digested with the appropriate restriction enzyme (Table 1) and subjected to DGGE analysis on 7.5% acrylamide (2.7% BIS) gels containing either a 20–50% (fragments II and III) or a 30–50% (fragment IV) denaturing gradient (15). The gels were stained with SYBR Green DNA stain (17) and photographed using 254 nm epi-illumination.

Cloning and sequencing strategies

Fragment I PCR products from subjects that exhibited novel DGGE banding patterns were cloned using the T vector system (Northumbria Biologicals Ltd., Cramlington, UK) and sequenced using the Sequenase Version 2.0 kit (Amersham Life Science, Buckinghamshire, UK) as described previously (5). By using sequencing primers in both orientations from the plasmid vector, and using the sense PCR primer (Table 1) the entire amplified segment was sequenced. In the case of fragment IV, a novel banding pattern was investigated by sequencing using asymmetric PCR as previously described (18) with the primers listed in Table 1 and a Thermo Sequenase cycle sequencing kit (Amersham Life Science Ltd., Arlington Heights, IL). DNA from the four patients identified by PCR and NsiI digestion as having the R3531C mutation (A.M., S.L., L.T., A.P., and J.A.) was sequenced as previously described (4) to confirm the presence of this mutation.

Percentage mass ratio of defective to normal LDL

LDL (1.021–1.055 g/ml) were prepared from 6 individuals heterozygous for the R3531C mutation and 5 control subjects who had all been determined as heterozygous for the Thr^{71}/Ile^{71} polymorphism, detected as an ApaLI RFLP as previously described (4, 7). In all six cases the mutant Cys^{3531} allele is associated with the Thr^{71} allele (presence of an ApaLI site at nucleotide 416) as determined by haplotype analysis (see below). The mass ratio of the two apoB allelic products was measured by dynamic laser light scattering (4, 14). Briefly, the diameters of LDL and LDL–antibody complexes were measured using a NICOMP Model 270 particle sizer (Particle Sizing Systems, Santa Barbara, CA) equipped with an argon ion laser (514.5 nm wavelength). Monoclonal antibodies MB19 and MB47 have been described previously (6, 19, 20). MB47 (the binding of which is not influenced by the Ile^{71}/Thr^{71} polymorphism) was used as a control to take account of other factors causing variations in binding (4, 14). LDLs from apoB (Ile^{71}/Ile^{71}) and apoB (Thr^{71}/Thr^{71}) homozygotes were mixed at known ratios to construct a calibration curve. With these LDL mixtures, at concentrations of 15 nm, the mean diameters were measured in the absence of antibody, with an equivalent amount of MB19 and with MB47. The relative percentage diameter increase was plotted against the percentage of strongly binding apoB (Ile^{71}) and fitted using a second-order polynomial. For the LDL from apoB (Ile^{71}/Thr^{71}) heterozygotes, the percentage Ile^{71} apoB LDL was calculated as the mean of at least five separate measurements performed on each sample.

Fibroblast LDL receptor binding assay

Competitive dual-label ($^{125}I/^{131}I$) equilibrium binding assays were carried out on radiolabeled LDL, isolated in the density range 1.021–1.055 g/ml, as previously described (4, 16). By way of comparison to R3531C patients and normal subjects, binding affinities were measured on LDL prepared from three patients with the R3500Q mutation. These were identified by differential oligonucleotide hybridization (4). A competitive displacement binding assay was also performed using ^{125}I-labeled LDL as described by Goldstein, Basu, and Brown (21).

LDL-induced U937 cell growth assay

The binding properties of LDL from R3531C subjects were tested using the LDL-induced growth assay as previously described (5). For this purpose LDL (1.021–1.055 g/ml) were isolated from 17 subjects heterozygous for the R3531C mutation and from 11 normal individuals.

ApoB gene haplotype analysis

The genotypes of eight apoB gene markers were determined as previously described (4, 5). The 3′ VNTR was assessed, in terms of numbers of repeats, according to Ludwig and co-workers (22).

RESULTS

NsiI and DGGE screening

Using PCR plus digestion with NsiI followed by agarose gel electrophoresis or PCR with DGGE (**Fig. 1**) we screened a total of 2570 individuals for the presence of the apoB R3531C mutation. All mutations were confirmed, either by sequencing or by restriction digestion with NsiI. The result of these studies was the identification of nine new R3531C heterozygous probands. Five were patients at lipid clinics and four were unselected volunteers. One of the volunteers (A.P. II-1) had a family history of heart disease.

Three silent mutations were discovered by DGGE analysis. These were a T for G transition at nucleotide 10829 (T3540T), a T for C transition at nucleotide 10835 (N3542N), and a T for C transition at nucleotide 10865 (T3552T).

Laser light scattering

In the six heterozygous R3531C individuals who were also heterozygous for the apoB MB19 polymorphism (Ile^{71}/Thr^{71}) 42.6% of LDL was bound strongly to the antibody, i.e., carried the Ile^{71} site (**Table 2**). The mean percentage mass ratio of defective Cys^{3531} (Thr^{71} apoB) to normal LDL (Ile^{71} apoB) particles was 57:43; a result significantly different ($P = 0.001$) from the ratio of 50:50 observed for the LDL from five normal MB19 heterozygotes. This showed that the defective binding affinity for the LDL receptor of the mutant apoB Cys^{3531} LDL particles leads to their accumulation in the plasma and that the R3531C mutation has an effect on lipoprotein metabolism in affected individuals. These results are similar to those obtained previously on three other individuals with this genotype, when the mean ratio was found to be 59:41 (4).

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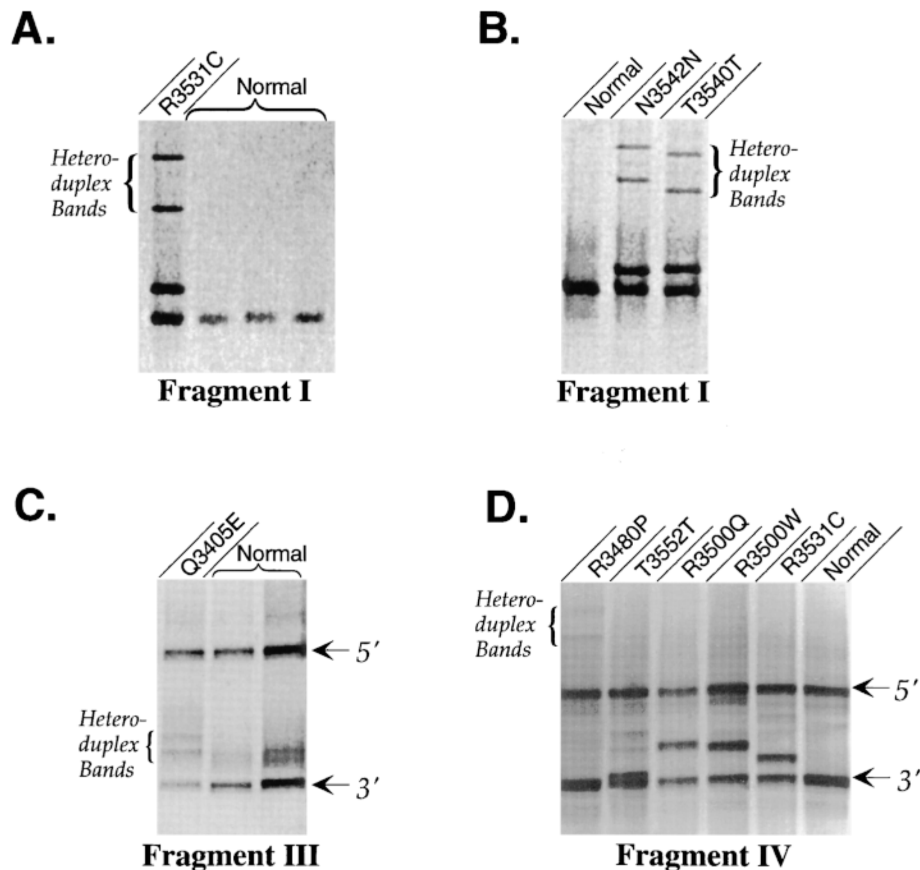

Fig. 1. DGGE analysis of part of exon 26 of the apoB gene coding for sequences that affect binding to the LDL receptor. The four fragments examined (I to IV) are as described in Table 1. A: Detection of the R3531C mutation by analysis of codons 3512 to 3581 (fragment I). B: Detection of novel conserved mutations, N3542N and T3540T in fragment I. C: Analysis of fragment III during screening of patients with LDL of low binding affinity revealed only the presence of the Q3405E polymorphism. D: Analysis of fragment IV could detect several mutations including the novel, conserved, T3552T.

Receptor-binding affinity of LDL

As measured using a dual-label assay, the affinities of LDL prepared from four heterozygous R3531C subjects was in each case considerably lower than the reference LDL (**Table 3**). The mean value of 49.3% is somewhat lower than we observed in our previous study where LDL was prepared from eight individuals and the mean affinity was 60.3% (4). The mean affinity of LDL from the three R3500Q individuals was 37.2%, similar to the value of 34.3% that we found previously (4) and also to that reported by others (13, 23). In a competitive binding assay (**Fig. 2**) LDL from two individuals heterozygous for the apoB R3531C mutation and from two control subjects were compared. In agreement with the dual-label assay results, LDL from the Cys[3531] subjects (IC_{50} 4.3 and 3.2 μg/ml)

TABLE 2. ApoB allelic ratios measured by dynamic laser light scattering

R3531C Kindred, Subject	%ApoB (Ile71) LDL	Normal Subject	%ApoB (Ile71) LDL
A.P., II-1	42.3	#1	50.4
A.M., III-10	37.4	#2	50.5
L.T., II-1	42.7	#3	49.9
J.C., II-1	47.8	#4	49.4
J.C., I-2	41.0	#5	49.0
J.C., II-3	44.5		
Mean ± SE	42.6 ± 1.4		49.8 ± 0.3

P = 0.001.

TABLE 3. Skin fibroblast dual-label LDL receptor binding assay

Subjects	LDL Binding Affinity As a % of Reference LDL	Subjects	LDL Binding Affinity As a % of Reference LDL
R3531C		Normal	
A.P -II-1	43.3	#1	105.9
J.C.-II-1	28.6	#2	98.8
J.C.-II-3	56.6	#3	95.7
L.T.-II-1	68.8	#4	110.1
Mean ± SE	49.3 ± 8.7[a]	#5	120.1
		#6	117.5
R3500Q		#7	84.7
#1	42.5	Mean ± SE	104.7 ± 4.7
#2	24.4		
#3	44.8		
Mean ± SE	37.2 ± 6.5[b]		

[a] *P* = 0.00017.
[b] *P* < 0.0001, compared to normal subjects.

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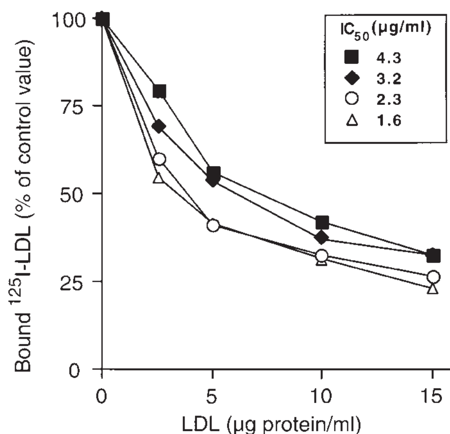

Fig. 2. Comparison of the ability of LDL from two individuals heterozygous for the apoB R3531C mutation (LT II-1■ and J.C. II-3◆) and two control subjects △ and ○) to compete with ^{125}I-labeled normal LDL for high-affinity binding to human skin fibroblasts at 4°C.

was a less effective competitor than normal LDL (IC_{50} 2.3 and 1.6 μg/ml).

T3552T was the only mutation found on DGGE screening of the 3127–3584 region in a group of patients whose LDL had defective binding affinities as measured using the dual-label fibroblast assay. The mean binding affinity of the LDL prepared from these 27 individuals was 62.8 ± 1.2% (mean ± SE) of that of the reference LDL. Thus, mutations in this region of apoB do not seem to account for the defective binding seen in these assays. It is possible that some mutations will not have been detected using the DGGE approach although this method has been acknowledged to detect nearly all mutations (24).

R3531C family studies

The pedigrees of seven of the R3531C probands are shown in **Fig. 3**. **Table 4** displays the plasma lipid profiles of the probands together with those of family members. **Table 5** shows the clinical histories of the probands and, in addition, age- and sex-adjusted lipid values. No one had tendon xanthomas. Plasma levels of total cholesterol and LDL cholesterol in affected individuals were 32 mg/dl and 21 mg/dl higher, respectively, than the levels observed in their unaffected relatives (23 mg/dl and 16 mg/dl, when age and sex adjusted), consistent with our earlier findings (4). However, in the present study these differences in lipids were not statistically significant.

The plasma lipid profiles of the individuals with the three novel silent mutations found by DGGE analysis are presented in **Table 6**.

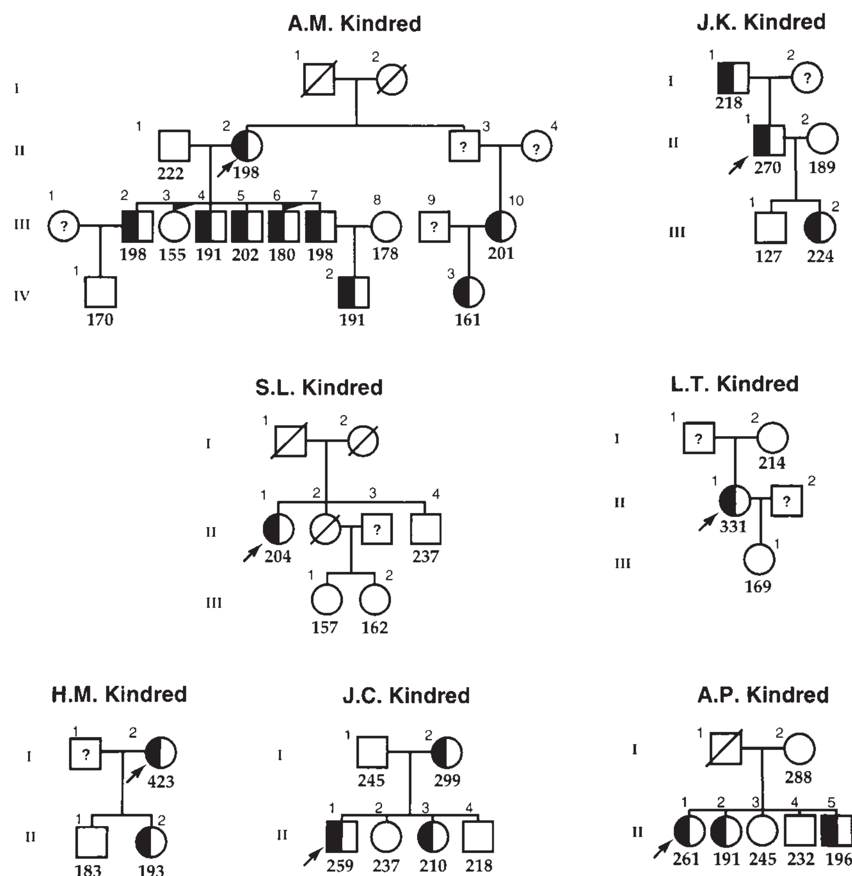

Fig. 3. Pedigrees of seven of the probands studied; ◧ and ◐ indicate males and females, respectively, who are heterozygous for the presence of the apoB R3531C mutation. Beneath the symbols are the plasma levels of total cholesterol (in mg/dl).

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TABLE 4. Plasma lipid profiles of R3531 families

Kindred	Subject	Age at Sampling	Sex	R3531C	ApoE Genotype	TC	TG	LDL-C	HDL-C
		yr					*mg/dl*		
A.M.	II-1	66	M	–	3/3	222	57	161	50
	II-2[a]	64	F	+	3/3	198	125	94	69
	III-2	39	M	+	3/3	198	76	127	56
	III-3	33	F	–	3/3	155	67	81	61
	III-4	38	M	+	3/3	191	39	120	63
	III-5	37	M	+	3/3	202	82	129	57
	III-6	33	M	+	3/3	180	114	103	54
	III-7	40	M	+	3/3	198	85	134	47
	III-8	42	F	–	2/3	178	58	102	64
	III-10	37	F	+	3/3	201	54	103	87
	IV-1	9	M	–	4/3	170	44	96	65
	IV-2	12	M	+	3/3	191	117	120	48
	IV-3	15	F	+	3/3	161	64	82	66
J.K.	I-1	80	M	+	3/3	218			50
	II-1[a]	49	M	+	3/3	270	150	201	41
	II-2	45	F	–	4/3	189			62
	III-1	15	M	–	4/3	127			50
	III-2	20	F	+	4/3	224			
S.L.	II-1[a]	64	F	+	3/3	204	108	106	76
	II-4	56	M	–	3/3	237	107	156	63
	III-1	33	F	–	4/3	157	52	104	49
	III-2	37	F	–	3/3	162	68	77	80
L.T.	I-2	66	F	–	3/3	214	165	149	39
	II-1[a]	46	F	+	4/3	331	214	254	39
	III-1	11	F	–	4/3	169	88	102	50
H.M.	I-2[a]	70	F	+	4/3	423	255	324	48
	II-1	46	M	–	3/3	183	123	124	41
	II-2	41	F	+	3/3	193	75	106	73
J.C.	I-1	53	M	–	4/4	245	405	112	41
	I-2	57	F	+	3/3	299	326	210	39
	II-1[a]	35	M	+	4/3	259	352	135	42
	II-2	22	F	–	4/3	237	251		
	II-3	32	F	+	4/3	210	132	147	31
	II-4	33	M	–	4/3	218	119	151	41
A.P.	I-2	77	F	–	3/3	288	99	205	70
	II-1[a]	49	F	+	3/3	261	154	191	50
	II-2	49	F	+	3/3	191	120	117	51
	II-3	51	F	–	3/3	245	88	176	58
	II-4	54	M	–	3/3	232	122	171	40
	II-5	52	M	+	3/3	196	136	125	51
J.A.[a]		66	M	+	4/3	240	195	176	25
J.R.[a]		36	M	+	3/3	369	378	245	31
Mean ± SE		44.1 ± 3.5		+		234 ± 13	152 ± 21	152 ± 13	52 ± 3
		41.6 ± 4.6		–		202 ± 10	120 ± 23	131 ± 10	54 ± 3

[a] Proband.

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ApoB haplotype analysis

Eight apoB gene markers (the signal peptide insertion/deletion polymorphism, the 3′ VNTR, and six RFLPs) were used to construct haplotypes of the Cys[3531] alleles. For six of the probands, it was possible to deduce the haplotype of the allele carrying the Cys[3531] codon. These are presented in **Table 7** together with the ancestries of these individuals. This table also includes, by way of comparison, the 3531C haplotypes of two families (L.S. and A.C.) that we reported previously (4). The 3531C haplotype present in the H. M. family is unique. The partial, but clearly distinct haplotype of another proband (J.A.) is also included in this table. The A.M., J.K., L.T., J.C., and A.P. families all had the same pattern as the L.S. kindred, which is of Celtic and Native American ancestry. Hence, we have now detected a total of four different haplotypes associated with Cys[3531] alleles.

U937 cell assays

LDL were prepared from 17 of the individuals who were heterozygous for the R3531C mutation, from 9 of their unaffected relatives and from 2 other normal subjects. This LDL was examined for its ability to promote growth of the cell line U937. These cells have an absolute requirement for extracellular LDL cholesterol for growth and have been used previously to identify LDLs with defective binding (5, 26, 27). The mean growth rate of the cells, relative to a standard reference LDL, was 74.1 ± 4.0% (mean ± SE) for LDL from the R3531C subjects and 96.6 ± 5.1% for the normal subjects ($P =$

TABLE 5. Clinical histories and adjusted lipoprotein lipid values

Subject	R3531C	Age- and Sex-Adjusted Lipids				Lipid Deposits					
		TC	TG	LDL-C	HDL-C	CA	XL	CAD	FAM	Ancestry	Notes
A.M. kindred											
II-1	–	184	45	121	44						
II-2[a]	+	152	106	68	49	no	no	no	no	Irish/Mexican	
III-2	+	170	56	104	57						
III-3	–	151	81	78	49						
III-4	+	165	29	99	64						
III-5	+	176	62	107	58						
III-6	+	162	93	89	54						
III-7	+	169	62	109	48						
III-8	–	161	61	92	50						
III-10	+	191	62	96	69						
IV-1	–	186	83	113	52						
IV-2	+	210	187	140	40						
IV-3	+	183	94	94	57						
J.K. kindred											
I-1	+	185			45						
II-1[a]	+	225	107	157	41	yes	yes	yes	no	Scottish/Irish	MI, defibrillator
II-2	–	165			48						
III-1	–	150			45						
III-2	+	235									
S.L. kindred											
II-1[a]	+	156	91	77	54	no	no	no	no	Scottish/English	
II-4	–	195	80	119	60						
III-1	–	153	63	100	39						
III-2	–	154	78	72	64						
L.T. kindred											
I-2	–	164	139	108	28						
II-1[a]	+	286	209	219	30	no	no	no	no	German	
III-1	–	185	131	117	43						
H.M. kindred											
I-2[a]	+	326	213	238	35	no	no	yes	sister	Scottish/Irish	angina/smoker >10/day
II-1	–	154	88	98	41						
II-2	+	176	80	96	58						
J.C. kindred											
I-1	–	203	297	86	40						angina/NIDD
I-2	+	233	284	159	28						angina/obese
II-1[a]	+	228	276	114	43	no	no	no	yes	Scottish/Irish	smoker >10/day
II-2	–	244	306								
II-3	+	206	161	142	25						
II-4	–	196	97	130	41						
A.P. kindred											
I-2	–	225	82	154	54						
II-1[a]	+	218	144	159	37	no	no	no	yes	Australia/ New Zealand	normal angiogram
II-2	+	159	112	97	38						
II-3	–	200	80	143	43						
II-4	–	192	90	131	39						
II-5	+	162	99	97	50						
J.A.[a]	+	199	155	132	22	no	no	yes	no	Austrian Jew	
J.R.[a]	+	323	291	206	31	yes	no	no	yes	Irish (probably)	smoker >80/day
Mean ± SE	+	204 ± 10	135 ± 17	127 ± 5	45 ± 3						
	–	181 ± 6	113 ± 19	111 ± 6	46 ± 2						

Plasma lipid levels were adjusted as previously described (4) for age (to 25 years) and gender, by non-linear regression analysis using data in the Lipid Research Clinics Population Studies Data Book (25). CA, corneal arcus; XL, xanthelasma; CAD, coronary artery disease; FAM, family history of CAD; NIDD, non-insulin-dependent diabetes; MI, myocardial infarction.

[a] Proband.

0.0018). The relative growth rates using LDL from heterozygous R3500Q and R3500W subjects was found previously to be about 50% that of normal LDL (5, 28). Thus, our data presented here, which show that the LDL from individuals with the R3531C mutation is 74% as effective at promoting growth as normal LDL, are in line with the binding affinity and mass ratio data presented here and previously (4).

DISCUSSION

We report here the discovery of nine additional unrelated probands heterozygous for the apoB R3531C mutation as a result of screening 2570 individuals. Thus, together with our previous report, we have found 11 such cases out of 4130, a frequency of one in 375. The frequency in the general population is no doubt less, as most

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TABLE 6. Profiles of probands with novel apoB variants

Subject	Mutation	Age at Sampling	Sex	ApoE Genotype	TC	TG	LDL-C	HDL-C	Ancestry
						mg/dl			
K.A.	T3540T	32	M	4/3	151	57	95	44	Nigerian
A.M.G.	N3542N	53	M	4/3	398	154	344	37	Scottish
J.B.	T3552T	42	M	3/3	359	84	327	23	African American

subjects screened were pre-selected for lipid abnormalities. By comparison, the R3500Q mutation is present at 1 in 500 in populations of European descent (23). Other workers have recently reported the detection of additional individuals with the R3531C mutation. Four probands were found among a group of patients with coronary artery disease (CAD), with no family members available for cosegregation analysis (29); two families were identified by Wenham and co-workers (30) each with a history of atherosclerosis; and two probands, both with severe hypercholesterolemia, were reported in a French population (31).

LDL from a total of nine R3531C heterozygotes in this and a previous study (4) have been assayed using the MB19/dynamic laser light scattering method to measure the apoB allelic mass ratios. Ligand-defective Cys^{3531} LDL particles accumulated and comprised a mean of 58 ± 1% of the total particles present. Radioimmunoassays have previously been used to show that the defective apoB Gln^{3500} allotype accumulates in the plasma of patients with the apoB R3500Q mutation (12, 13). In these studies the mass ratio of Gln^{3500} to Arg^{3500} LDL was 73:27 (12). Comparable results are seen with laser light scattering, the percentage of Gln^{3500} LDL was found to be 87% and 74% in two R3500Q heterozygotes (14). The greater accumulation of mutant particles with R3500Q relative to R3531C is presumably due to the fact that Cys^{3531} LDL particles possess more residual affinity for the receptor. We have shown previously (4) and again in this study that LDL from R3531C heterozygotes is defective in binding to the LDL receptor. In the total of 12 heterozygous subjects who have now been tested, the overall binding affinity compared to reference LDL was 57 ± 4%. This value is for total LDL, being a weighted average of the normal and defective LDL present in the circulation. Defective apoB Cys^{3531} LDL itself has been calculated to have 27% of normal affinity (4) compared with less than 10% for Gln^{3500} particles (4, 12, 23). In line with these differences in the ratio of apoB allotypes and in binding activity, LDL prepared from R3531C heterozygotes exhibited an ability to promote U937 cell growth that was intermediate between that of R3500Q LDL and normal LDL.

Myant (32) has noted that, in all cases examined, LDL from R3500Q heterozygotes with normal levels of cholesterol had defective binding. In the present study one R3531C heterozygote, despite a normal level of cholesterol (J.C. II-3), had LDL with the expected defective binding. She, and another affected individual with a normal level of cholesterol (A.M. III-10), had an accumulation of the defective Cys^{3531} LDL particles. One R3531C subject reported by Wenham and co-workers (30) was normolipidemic, as was one of the eight affected individuals in the initial study by Pullinger et al. (4). LDL prepared from this latter subject had 48% of normal binding affinity. Thus, in evaluating any specific mutation of apoB, measurement of LDL binding affinity and, more significantly, determining whether there is an accumulation of the mutant allotype, is more revealing than the plasma level of cholesterol.

As with the R3500Q mutation (32, 33), there would appear to be variable expression of the R3531C phenotype, which is modulated by environmental and other genetic factors. This variable penetrance makes it difficult to compare the clinical significance of this mutation with the R3500Q mutation and with FH. A recent study of 76 families identified 129 individuals with FH and 28 with the R3500Q mutation (34). The R3500Q mutation had the effect of raising total cholesterol by 93 mg/dl (42%) and FH 174 mg/dl (79%) above normal levels. Another study of 34 R3500Q subjects reported an increase of 67 mg/dl (35%) compared to unaffected relatives (29). In the present report, we found that the R3531C mutation was associated with an increase of 32 mg/dl or 16%, though this was not statistically significant. However, when the

TABLE 7. ApoB haplotypes of kindreds with the R3531C mutation

Kindred	5′-INS/Del	*ApaLI*	*AluI*	*XbaI*	*BfaI*	*MspI*	*EcoRI*	3′-VNTR	Ancestry
A.M.	Ins	+	−	+	−	+	+	34	Irish/Mexican
J.K.	Ins	+	−	+	−	+	+	34	Scottish/Irish
L.T.	Ins	+	−	+	−	+	+	34	German
J.C.	Ins	+	−	+	−	+	+	34	Scottish/Irish
A.P.	Ins	+	−	+	−	+	+	34	Australian/New Zealand
L.S. (ref. 4)	Ins	+	−	+	−	+	+	34	Celtic/Native American
H.M.	Ins	+	−	+	−	+	+	32	Scottish/Irish
J.A.	Ins	+	−	−	−	+	n.d.	34 or 46	Austrian Jew
A.C. (ref. 4)	Ins	+	−	−	+	+	+	34	Italian

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present data on our 24 individuals with the R3531C mutation was combined with that on the original 8 subjects (4) and with that on 12 other individuals (29–31), the mean cholesterol level was 253 mg/dl (n = 44) compared to 202 mg /dl (n = 29) for unaffected relatives, an increase of 51 mg/dl or 25% ($P < 0.0003$). LDL cholesterol levels were 171 mg/dl for 40 R3531C heterozygotes and 135 mg/dl for 25 unaffected relatives, an increase of 36 mg/dl or 27% ($P < 0.01$).

The original two families with the R3531C mutation had apoB haplotypes associated with the mutant allele that were different from one another (4). The first was of Celtic and Native American background. This haplotype is associated with the Cys^{3531} allele in five of the probands reported here, three of whom have Scottish or Irish ancestry. There is one new haplotype (H.M., Table 7) and one partial haplotype that is also clearly different from all the others (J.A., Table 7). In nine of the probands for which the analysis is meaningful there are four different haplotypes. This evidence, given that recombination events in this region of the apoB gene have not been detectable (35), points to multiple independent R3531C mutations. This is in contrast to the R3500Q mutation where one haplotype predominates in several populations in Europe and North America (36, 37), though two unique haplotypes, one of German (38) the other of Chinese ethnic origin (39), have been reported. The initial two probands reported with the apoB R3500W mutation had different haplotypes (5). The first was of Scottish and the second of Asian ancestry. Two other R3500W probands, a Chinese and a Malay, had this second haplotype (40).

We discovered three novel silent mutations of apoB. One (T3552T) was found by DGGE screening of 27 patients whose LDL had defective binding. However, no other mutations were detected among this group although we were able to detect all other known mutations in the region examined. Thus, mutations of apoB in the generally accepted receptor-binding domain do not seem to account for the defective binding affinity of the LDL here.

At the time of completion of this paper a report was published describing screening for the R3500Q, R3531C, and R3500W mutations of 9255 subjects from a general population in Denmark together with 948 patients with ischemic heart disease (41). Seven persons with the R3531C mutation were found in the general population (prevalence of 0.08%) and one among those with heart disease. The heterozygous R3531C carriers did not have a higher level of plasma cholesterol than the general population. The authors concluded that their results suggest that the R3531C mutation is not sufficient to cause hypercholesterolemia, is not associated with increased risk of ischemic heart disease, and that other additional factors are possibly required. It is difficult to project conclusions from only 7 patients compared to the 44 presented here and elsewhere (4, 29–31), particularly as the average age of the R3531C Danish people was 73 so they may represent a survivor population. The individual lipid values for the 7 subjects were not reported. The general population in this study had a mean total cholesterol of 235 mg/dl, and LDL cholesterol of 154 mg/dl, which is similar to our 24 R3531C carriers (234 mg/dl and 152 mg/dl, respectively, Table 4) and somewhat higher than the 18 unaffected relatives (202 mg/dl and 131 mg/dl, Table 4). This suggests that this Danish population is relatively hypercholesterolemic compared to the families we studied. The different conclusions of this study may reflect other determinants of cholesterol levels in the Danish population. Several key elements determine LDL levels in individuals including the production rate of VLDL and whatever determines the extraction rate of VLDL remnants from plasma. Receptor ligand defects may have a smaller impact when the subjects are relatively hypercholesterolemic and there is a partial diminution in LDL receptor activity.

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