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Influence of exonic polymorphisms in the gene for LDL receptor-related protein (LRP) on risk of coronary artery disease

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Abstract

The low density lipoprotein (LDL) receptor-related protein (LRP) is a multifunctional receptor involved in numerous biological processes relevant to vascular biology including lipoprotein metabolism. Several polymorphisms in the LRP gene have been described and in this study we examined their influence on coronary artery disease (CAD). We compared the frequencies of the exon 3 (C766T), exon 6 (C663T), exon 22 (C200T), and four rarer and more recently described polymorphisms in approximately 600 Caucasian subjects aged <50 years with angiographic CAD and approximately 700 similarly aged subjects without symptomatic CAD randomly selected from the community. We found the distribution of exon 22 C200T genotypes to differ significantly between the CAD (CC: 52%, CT: 39%, TT: 9%) and control subjects (CC: 43%, CT: 46%, TT: 11%, $P=0.005$), with the CC genotype conferring an odds ratio (OR) for CAD of 1.5 (95% CI: 1.2–1.8, $P=0.001$) despite a lack of significant influence on plasma cholesterol or triglyceride. The other LRP polymorphisms were less common. Two showed an association with CAD; for the exon 3 C766T polymorphism the TT genotype was significantly lower (1.0 vs. 2.7%; OR: 0.36; $P=0.04$) and, for the exon 6 C663T polymorphism, the heterozygote frequency was higher (6.2 vs. 3.4%; OR: 1.9; $P=0.03$) in CAD subjects. In conclusion, LRP gene polymorphisms, particularly the relatively common exon 22 C200T polymorphism, are a significant risk factor for premature CAD in Caucasians.
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Keywords: LDL receptor-related protein; Coronary artery disease; Genetic polymorphism

1. Introduction

Low density lipoprotein (LDL) receptor-related protein (LRP) is a multi-ligand receptor. It is also known as the α2-macroglobulin receptor, and its structure has a very high degree of homology to the LDL-receptor and other members of the LDL-receptor family [1]. It can bind and endocytose over 30 unrelated ligands many of which are of potential importance to the pathogenesis of obstructive coronary artery disease (CAD), including apolipoprotein E rich lipoproteins [2], chylomicron remnants [3–5], very low density lipoprotein [6], lipoprotein and hepatic lipase [7,8] and other non-lipid related ligands such as plasminogen activator inhibitor I (PAI I) [9–11]. LRP has been found in smooth muscle cells and macrophages in both early and advanced atheromatous lesions in the human aorta, as well as in the normal aorta [12]. This is of interest because of the potential for LRP to promote unlimited cellular uptake of cholesterol-rich lipoprotein, a possibility related to the absence of any sterol regulatory element in its promoter region [13].

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The human gene coding for LRP is located on chromosome 12q13–q14 [14]. It covers ~90 kb of genomic DNA, contains 89 exons and codes for a 15 kb mRNA [15]. The gene is highly conserved with 97% amino acid homology between human and mouse LRP [16]. We have previously shown, by sequencing the 89 exons of human LRP, that the gene is polymorphic; most of the polymorphisms initially described, including the exon 3 C766T and exon 22 C200T polymorphisms, are not expressed [17]. The C766T polymorphism has been reported to be associated with Alzheimer's disease, albeit weakly [18–20]. LRP is a good candidate gene for CAD, however, there have been very few attempts to determine the influence of the aforementioned polymorphisms, or that of the single previously well described and expressed polymorphism, exon 6 C663T (A217V), on the risk of CAD. Four expressed polymorphisms, some of which involve non-conservative amino acid substitutions, were recently described: exon 14 G121C (A775P), exon 39 G52A (D2080N), exon 48 C92A (D2632E), and exon 85 G44A (G4379S); the influence of these on CAD is, likewise, also unknown.

To test the hypothesis that these LRP polymorphisms influence the risk of CAD, we carried out a case–control study focusing on relatively young subjects both because of the importance of premature CAD and because this strategy might be more likely to reveal a genetic influence. We examined LRP genotypes in a large number of patients aged <50 years, who have angiographic CAD with or without myocardial infarction (MI) and a similarly aged community group randomly selected from the electoral roll. Consecutive CAD subjects were all recruited prospectively at the one hospital for the purpose of risk factor and genetic studies.

2. Methods

2.1. Study subjects

The cases comprised approximately 600 Caucasian subjects aged less than 50 years (44 ± 0.2, mean ± S.E.M.), who presented to Royal Perth Hospital over a 6-year-period (1994–1999 inclusive) with clinical ischaemic heart disease, with $>50\%$ diameter obstruction in at least one coronary artery assessed by coronary angiography, either with or without acute MI as assessed by historical, electrocardiographic and enzymatic parameters as previously described [21]. The characterisation as MI required all three of the following: typical chest pain >20 min duration, serum creatine kinase levels at least twice normal, ST segment elevation in at least two leads of a 12 lead electrocardiogram. All of those with MI also had confirmatory coronary angiography and left ventriculography. The exact numbers of DNA samples available for genotyping each polymorphism are noted subsequently.

The control subjects comprised approximately 700 Caucasians, aged 41 ± 0.2 years, selected randomly from the electoral roll, who took part in a National Heart Foundation community survey of CAD risk factors in the Perth metropolitan area in 1994 [21]. Each subject was documented with respect to lifestyle and medical demographics which included blood pressure, height and weight. Only those without a history suggestive of CAD were included in this study. There were no Australian Aboriginals in this community group and, although there were Aboriginals who presented with CAD, they and those of Asian descent were not included in this study because the frequency of polymorphisms in the gene for LRP might be racially dependent [22].

2.2. Laboratory investigations

Fasting blood samples were collected for measurement of plasma total cholesterol (TC), HDL-cholesterol (HDL-C), triglyceride (TG), and extraction of DNA. Samples from community subjects were taken at presentation to the survey centre and those from CAD subjects immediately preceding coronary angiography. Lipids were measured using enzymatic methods. The Friedewald formula was used to calculate LDL-cholesterol (LDL-C) in those with TG levels <4.5 mmol/l. DNA was extracted from 5 ml of K_2EDTA anti-coagulated blood using a Triton X-100 method [23]. The nature of the polymorphisms studied is listed in Table 1. Genotyping for LRP polymorphisms was carried out by restriction enzyme analysis of PCR amplified DNA spanning the polymorphic sites. Genotypes were determined by electrophoresis on 12% polyacrylamide gels (25 V/cm for 1.5 h) and ethidium bromide staining after restriction enzyme digestion. The primer sequences, PCR amplicon size, PCR annealing temperatures, restriction enzymes and product sizes are shown in Table 2. Informed consent was obtained from all subjects and the study protocol was approved by the Royal Perth Hospital Ethics Committee.

2.3. Statistical analyses

To test for association between a polymorphism and CAD, we compared the genotype and allele frequencies between CAD and control subjects using chi-square (χ^2) tests. Comparisons of plasma lipids including TC, LDL-C, HDL-C and TG levels between subjects of different genotypes were carried out using analysis of variance (ANOVA). The lipid values were analysed separately in CAD and community groups, and separately for males and females. Variables are expressed as mean ± S.E.M. except for TG results which are expressed as geometric means and 95% confidence intervals. Significance was

Table 1
Genomic details of the LRP gene polymorphisms studied

Polymorphism	Location[a]	Polymorphic site[b]	Amino acid change	Accession number
Exon 3 C766T	Exon 3, 516	atgga**c**ggct	None	AF058399
Exon 6 C663T (A217V)	Exon 6, 663	ggggc**c**cagg	Alanine to valine	AF058401
Exon 14 G121C (A775P)	Exon 14, 343	gaggc**g**cacc	Alanine to proline	AF058407
Exon 22 C200T	Exon 22, 1209	ctctg**c**ggtg	None	AF058411
Exon 39 G52A (D2080N)	Exon 39, 239	ggatc**g**acct	Aspartic acid to asparagine	AF058416
Exon 48 C92A (D2632E)	Exon 48, 1569	gagga**c**gcct	Aspartic acid to glutamic acid	AF058419
Exon 85 G44A (G4379S)	Exon 85, 2181	gcgtc**g**gcca	Glycine to serine	AF058427

[a] Exon numbers and nucleotide positions in the indicated Gene Bank accession numbers are shown.
[b] Polymorphic sites are indicated in bold.

considered to be at $P < 0.05$; however, P values are also shown for statistical comparisons of borderline significance (P: 0.05–0.1). Statistical analyses were performed using SPSS for Windows version 9.0.1.

The characteristics of the two study groups in terms of conventional risk factors are summarised in Tables 3 and 4. The CAD subjects had a history of hypertension or diabetes more frequently, were more often smokers or ex-smokers and BMI was higher in both males and females compared with their respective control groups. The CAD patients were predominantly male (88%) while approximately equal numbers of males (51%) and females (49%) were recruited in the community group. There were significant differences in lipids with CAD subjects of both sexes having a lower HDL-C and higher TG than their counterparts in the control group (Table 4); there was also a significant difference in TC between female, but not male, CAD and control subjects.

3. Results and discussion

The genotyping results are shown in Table 5. Genotype frequencies conformed to Hardy–Weinberg equilibrium in both the CAD and control groups, and in males and females separately. Most of our CAD subjects were male while there were similar number of males and females in the control group. However, there was no difference in the frequency of LRP genotypes between males and females in the control or CAD subjects and, on this basis, we consider pooling of genders for comparison of genotype frequencies to be valid. The four recently described expressed polymorphisms in exons 14, 39, 48 and 85 were extremely uncommon, consistent with the findings of the original report on several smaller cohorts [24], and with no homozygotes for the rarer alleles found (Table 5). Four heterozygotes for exon 14 G121C (A775P) were found in the CAD group and none in the control group ($P = 0.03$). How-

Table 2
Conditions for PCR and restriction enzyme analysis of LRP gene polymorphisms

Polymorphism	Primers	Amplicon size (bp)	PCR annealing temperature (°C)	Restriction enzyme	Restriction fragment sizes[a] by allele (bp)
Exon 3 C766T	5′-ggggtccaggactgcatgta 5′-aagtccgtacctcggcagtg	59	54	*Rsa* I	C: 32, 19, 8 T: 51, 8
Exon 6 C663T (A217V)	5′-aacatcttggccacgtacct 5′-cgtgctcgtaggtgtgatg	60	58	*Hae* III	C: 32, 18, 10 T: 50, 10
Exon 14 G121C (A775P)	5′-gcaactacctcttctggactg 5′-cattgttcacccggcattt	519	58	*Hae* III	G: 372, 104, 43 C: 372, 67, 43, 37
Exon 22 C200T	5′-gctcagatgagggcgagct 5′-gtccttggcccagactccta	86	59	*Mwo* I	C: 67, 19 T: 86
Exon 39 G52A (D2080N)	5′-aagctgtactggtgcgatg 5′-tcacctgtcactccagtagat	150	56	*Taq* I	G: 106, 44 A: 150
Exon 48 C92A (D2632E)	5′-agcctgtggtgtgggcgagt 5′-gctcgcagggctggaagagc	518	64	*Bsa* HI	C: 400, 87, 31 A: 400, 118
Exon 85 G44A (G4379S)	5′-gcccccagctgtctgacct 5′-tgctcaaatctggggaagg	144	65	*Hae* III	G: 67, 51,26 A: 93, 51

[a] Shown are restriction fragment sizes of the alleles for the various polymorphisms.

Table 3
Characteristics of CAD and control subjects[a]

	CAD group ($n = 603$)	Control group ($n = 694$)
Age (years)	44±0.2	41±0.2
Males	529 (88%)	352 (51%)
MI previous	173 (29%)	
current	200 (33%)	
previous and current	19 (3%)	
Diabetes	83 (14%)	8 (1%)
Smoking current	276 (46%)	168 (24%)
ex	208 (34%)	191 (28%)
History of hypertension	201 (33%)	113 (16%)
BMI (kg/m^2)	28.4±0.2	25.8±0.6
On lipid lowering drugs	116 (19%)	10 (1%)

CAD, coronary artery disease; n, number of subjects; BMI, body mass index.
[a] Continuous variables are expressed as mean±S.E.M. For dichotomous variables, percentages are given in parentheses. χ^2 analysis was used to assess differences between variables. Each of the variables was significantly different between the two groups ($P < 0.001$).

Table 5
LRP genotype frequencies in CAD and control subjects

LRP gene polymorphism	Genotype	CAD group n (%)	Control group n (%)	
Expressed polymorphisms				
Exon 6 C663T (A217V)	CC	556 (94)	597 (96)	a,c
	CT	37 (6)	21 (3)[b]	
	TT	1 (0.2)	1 (0.2)	
Exon 14 G121C (A775P)	GG	583 (99)	689 (100)	d
	GC	4 (0.7)	–[e]	
	CC	0	0	
Exon 39 G52A (D2080N)	GG	567 (95)	660 (96)	
	GA	29 (4.9)	30 (4.3)	
	AA	0	0	
Exon 48 C92A (D2632E)	CC	587 (99.8)	691 (99.9)	
	CA	1 (0.2)	1 (0.1)	
	AA	0	0	
Exon 85 G44A (G4379S)	GG	567 (99.8)	678 (99.9)	
	GA	1 (0.2)	1 (0.1)	
	AA	0	0	
Non-expressed polymorphisms				
Exon 3 C766T	CC	464 (78)	462 (74)	f
	CT	128 (21)	143 (23)	
	TT	6 (1)	17 (2.7)[g]	
Exon 22 C200T	CC	308 (52)	294 (43)[j]	h,i
	CT	232 (39)	321 (47)	
	TT	55 (9)	75 (11)	

Comparison of genotype distributions between CAD and control groups (χ^2): [a]$p = 0.07$; [d]$p = 0.03$; [f]$p = 0.06$; [h]$p = 0.005$. Comparison of individual genotypes and/or alleles: [b]$p = 0.03$ (CT); [c]$p = 0.04$ (T and C alleles); [e]$p = 0.03$ (GC); [g]$p = 0.04$ (TT); [i]$p = 0.004$ (T and C alleles); [j]$p = 0.001$(CC).

ever, because of the rarity of this polymorphism and the number of comparisons we have carried out, we consider this result to be tentative rather than convincing evidence for a role in CAD. The other expressed polymorphism studied, the exon 6 C663T (A217V), has been described [17] but only previously examined in relation to Alzheimer's disease [25]. The T allele was associated with CAD, with CT heterozygosity conferring an OR of 1.9 for CAD (95% CI: 1.1–3.3; $P = 0.03$).

Of the two exonic non-expressed polymorphisms studied, the exon 22 C200T polymorphism was common and significantly related to CAD, C homozygosity being associated with a higher risk when comparing the total CAD and control groups (OR: 1.5; 95% CI: 1.2–1.8; $P = 0.001$), and in males (OR: 1.4; 95% CI: 1.0–1.8; $P = 0.03$) and females (OR: 1.9; 95% CI: 1.1–3.2; $P = 0.02$) separately. The C allele frequency was also significantly higher in the whole CAD group compared with the control group ($P = 0.004$), in males ($P = 0.04$) and females ($P = 0.02$) separately and, reciprocally, the T allele was significantly lower in the CAD group. This result is contrary to that reported, in smaller cohorts (153 CAD cases, 166 controls) in abstract form [26].

Table 4
Lipid values in CAD and control subjects[d]

	CAD group		Control group	
	Male ($n = 529$)	Female ($n = 74$)	Male ($n = 352$)	Female ($n = 340$)
TC	5.52±0.05	5.28±0.14[a]	5.42±0.06	5.01±0.05[a]
HDL-C	0.98±0.01[b]	1.06±0.03[b]	1.21±0.02[b]	1.50±0.02[b]
LDL-C	3.35±0.07[c]	3.40±0.17	3.53±0.05[c]	3.07±0.04
TG	1.92 (1.83, 2.02)[b]	1.73 (1.49, 2.01)[b]	1.22 (1.15, 1.31)[b]	0.83 (0.79, 0.88)[b]

TC, total cholesterol; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol; TG, triglyceride. The P values shown are for comparisons between CAD and control subjects of the same gender.
[a] $P = 0.024$.
[b] $P < 0.001$.
[c] $P = 0.039$.
[d] The values (mmol/l) are expressed as mean±S.E.M., except for TG where geometric means and 95% confidence intervals are quoted.

Table 6
Lipid values in CAD and control subjects for LRP Exon 22 C200T polymorphism[a]

	Male			Female		
	CC	CT	TT	CC	CT	TT
CAD group	(266)	(205)	(51)	(42)	(27)	(4)
TC	5.44 ± 0.07	5.58 ± 0.09	5.81 ± 0.23	5.20 ± 0.21	5.39 ± 0.20	5.83 ± 0.73
HDL-C	1.00 ± 0.02	0.99 ± 0.02	0.91 ± 0.03	1.05 ± 0.05	1.09 ± 0.04	1.00 ± 0.16
LDL-C	3.38 ± 0.10	3.35 ± 0.12	3.17 ± 0.22	3.19 ± 0.30	3.50 ± 0.20	4.4
TG	1.86 (1.74, 1.98)	1.99 (1.83, 2.16)	2.12 (1.75, 2.56)	1.71 (1.39, 2.12)	1.85 (1.48, 2.33)	1.44 (0.39, 5.33)
Control group	(153)	(158)	(41)	(139)	(163)	(34)
TC	5.46 ± 0.08	5.36 ± 0.09	5.53 ± 0.20	5.04 ± 0.07	5.01 ± 0.07	4.92 ± 0.17
HDL-C	1.20 ± 0.02	1.23 ± 0.03	1.19 ± 0.04	1.48 ± 0.03	1.50 ± 0.03	1.59 ± 0.05
LDL-C	3.59 ± 0.07	3.50 ± 0.08	3.50 ± 0.18	3.10 ± 0.06	3.07 ± 0.06	2.95 ± 0.15
TG	1.22 (1.10, 1.34)	1.17 (1.06, 1.30)	1.41 (1.14, 1.73)	0.86 (0.79, 0.94)	0.82 (0.75, 0.89)	0.75 (0.64, 0.89)

TC, total cholesterol; HDL-C, HDL-cholesterol; LDL-C, LDL-cholesterol; TG, triglyceride.

[a] The values (mmol/l) are expressed as mean ± S.E.M., except for TG where geometric means and 95% confidence intervals are quoted. The number of subjects in each genotype category are shown in parentheses. Lipid values were not available for two female control subjects, both with the CC genotype.

The other exonic non-expressed polymorphism studied, the exon 3 C766T, has been considered, in several publications, to be associated with Alzheimer's disease although there has been recent doubt that any polymorphism in the LRP gene is so related [24]. There have been few studies in relation to CAD. In a Japanese association study on 445 cases and 464 controls, Yamada et al. [27] found no evidence of a role in MI ($P \geq 0.1$). While there is concern regarding the appropriateness of the control group used in this Japanese study, as discussed by Peters and Boekholdt [28], the findings are consistent with those of Benes et al. [29]; in a Czech study of Caucasian CAD and control subjects older than ours, these investigators found no difference in C766T genotypes although there was a relationship of the T allele with CAD in those with a certain PAI 1 genotype, namely 5G homozygosity. By contrast, in our study, T homozygotes, which are relatively uncommon, were under-represented in those with CAD (OR: 0.36 for CAD; 95% CI: 0.1–0.9; $P = 0.04$). The difference between the study of Yamada et al. [27] and ours may be due to ethnic differences or gene-environment interactions such as those involving lifestyle and diet. However, given that T homozygosity is not common, and in view of the number of statistical comparisons we have carried out, we do not consider our results on the exon 3 C766T polymorphism to be a strong refutation of the negative findings of Yamada et al. [27] and Benes et al. [29]. If the exon 22 C200T and exon 3 C766T polymorphisms, neither of which are expressed polymorphisms, genuinely influence the risk of CAD, as clearly seems true for the C200T, it must clearly be through linkage to some other polymorphism.

As already mentioned in Section 2, the CAD and control groups had significant differences in plasma lipids. However, there was no significant influence on TC, HDL-C, TG, or indeed LDL-C, in males or females, in either the CAD or control group for any of the polymorphisms studied. The results for the most relevant polymorphism to CAD, the exon 22 C200T, are shown in Table 6. A significant proportion (19%) of the CAD subjects were on lipid lowering drugs. This and other aspects of their management may have confounded the results; however, this is not a consideration for the control group where only 1% were so affected. Of the few previous studies examining the influence of LRP polymorphisms on plasma lipids, none have shown a substantial effect; in a similarly sized study to ours, Benes et al. [29] found the C766T polymorphism to have no influence on plasma lipids and Harris et al. [22] and Hegele et al. [30] found a very weak association between the tetranucleotide repeat polymorphism in the LRP promoter, which was not studied here, and plasma lipids. In spite of our findings, we cannot completely exclude an influence on lipid metabolism; the subjects in our study were fasting and, given the role of LRP in chylomicron metabolism [31,32], studies on post-prandial chylomicron remnant clearance may be more relevant than the fasting lipids studied here. On the other hand, given the many potential roles of LRP in vascular biology, it is possible that the influence of LRP polymorphisms on CAD is not mediated through an influence on plasma lipids per se.

In summary, we have studied the influence of several LRP gene polymorphisms on the risk of premature CAD. A number, including the exon 14 G121C (A775P) and exon 6 C663T (A217V) polymorphisms, showed a marginally statistically significant association with CAD and their low frequency renders them relatively unimportant in the broader aetiological context. However, the C200T polymorphism in exon 22 is common and homozygosity for the C allele is significantly related to CAD. The mechanism responsible for this is unclear but

demonstration of the association opens the way for further studies on the biological role of the LRP gene.

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