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ORIGINAL ARTICLE

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Polymorphisms in human apolipoprotein(a) kringle IV-10 and coronary artery disease: relationship to allele size, plasma lipoprotein(a) concentration, and lysine binding site activity

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Abstract Elevated plasma levels of lipoprotein(a) [Lp(a)] represent a major independent risk factor for the development of atherosclerosis. The kringle IV type 10 of apolipoprotein(a) [apo(a)] is the primary lysine binding site (LBS) of Lp(a) and is associated with lesion formation in transgenic mice. The purpose of this study was to search for mutations in the apo(a) kringle IV type 10 which could alter the LBS activity of Lp(a) from patients with coronary artery disease. We found the DNA region of kringle IV type 10 of apo(a) to be mutable but relatively well preserved in the Spanish population. We identified a novel mutation which probably leads to a truncated form of apo(a) in a patient heterozygous for the mutation and with low lysine binding activity and low plasma Lp(a) concentration. Two other mutations have been previously identified in humans, the substitutions W81R and M75T. The W81R was not found in our sample, but the M75T mutation was present in 43% of patients with coronary artery disease and 23% of age-matched controls. The genotype TT conferred a significant risk for myocardial infarction (odds ratio 2.53). This association was not due to linkage disequilibrium with kringle IV repeats. The M75T polymorphism was not associated with the LBS function of apo(a), but it influenced plasma Lp(a) concentration.

Keywords Apoprotein(a) · Kringle IV 10 · Lysine binding site activity · Lipoprotein(a) · Mutation · Polymorphism

Abbreviations *apo(a):* Apolipoprotein(a) · *CAD:* Coronary artery disease · *KIV:* Kringle IV · *LBS:* Lysine binding site · *Lp(a):* Lipoprotein(a) · *SSCP:* Single-stranded conformational polymorphism

Introduction

Elevated plasma lipoprotein(a) [Lp(a)] concentrations are associated with an increased risk of coronary artery disease (CAD) [1]. Lp(a) is a lipoprotein particle that has as a protein moiety apo B-100, linked by a single disulfide bridge to a multikringle structure, apolipoprotein(a) [apo(a)], which has a high degree of homology to plasminogen [2]. The cDNAs of apo(a) and plasminogen have been cloned, and the two genes are found to be closely linked on chromosome 6 (q26–27) [3]. Apo(a) contains multiple copies of the plasminogenlike kringle IV (KIV) domain which are similar but not identical to each other, and ten distinct classes have been designated (KIV types 1–10). The tandem repeats of KIV type 2 constitute the molecular basis of Lp(a) isoform size heterogeneity (see Fig. 1A). Plasma Lp(a) concentration segregates as an autosomal quantitative dominant trait under the control of a single locus [4, 5]. In most populations there exists an inverse association between the number of KIV type 2 repeats and plasma Lp(a) concentrations [6].

The kringle domains of plasminogen contain lysine binding site(s) (LBS) which interact with the carboxyl terminal lysines of proteins, mediating binding to cells and substrates [7]. Lp(a) also exhibits LBS properties, and the KIV type 10 is the primary LBS of Lp(a) which can mediate the interaction of Lp(a) with cells [8, 9], extracellular matrices [10], and fibrin [11]. The activity of LBS plays a key role in the pathogenic activity of apo(a) [12, 13]. Two mutations of KIV type 10 have been described which reduce LBS activity, one is the substitution W72R found in rhesus monkeys [14] which corresponds in humans [15] to the W81R, according to the HSALIPOA sequence (NCBI), and a second mutation, the substitution D57N, which has been reported in the chimpanzee [16]. The substitution in the chimpanzee is associated with poor fibrin binding, and a substitution of D55 and D57 to A in r-apo(a) causes reduced LBS activity but does not abolish activity [13]. Another mutation with a substitution M75T, located in the vicinity of the postulated LBS pocket, is not associated with a LBS defect [17, 18] and showed no effect on plasma Lp(a) concentration [18]. To date no mutations in KIV type 10 have been reported which increase LBS function in Lp(a), but these may be critical for identifying mechanisms of increased CAD risk. A second LBS (II) has been identified in the kringles KIV 5–8, which is important for the assembly of Lp(a) and is accessible only in isolated apo(a) [19]. The aim of this study was to search for mutations in the apo(a) KIV type 10 from patients with CAD which could relate apo(a) structure and function.

Material and methods

Study population

The procedures used complied with the ethical standards of the Hospital Universitari de Sant Joan. Blood samples were obtained from 94 male patients who participated in a previous study [20] and had had one episode of acute myocardial infarction, as defined by the criteria of the World Health Organization [21], before the age of 50 years (range 29–49). They had no history of familial hypercholesterolemia, renal failure, liver disease, hypertension, obesity, or diabetes mellitus. The patients were compared with 94 ostensibly healthy (that is to say, free of any disease, and recruited at their place of work during a routine medical examination) male controls living in the same geographic area and with a similar age (±4 years), body mass index (±1.1), and ethnic background (Mediterranean whites).

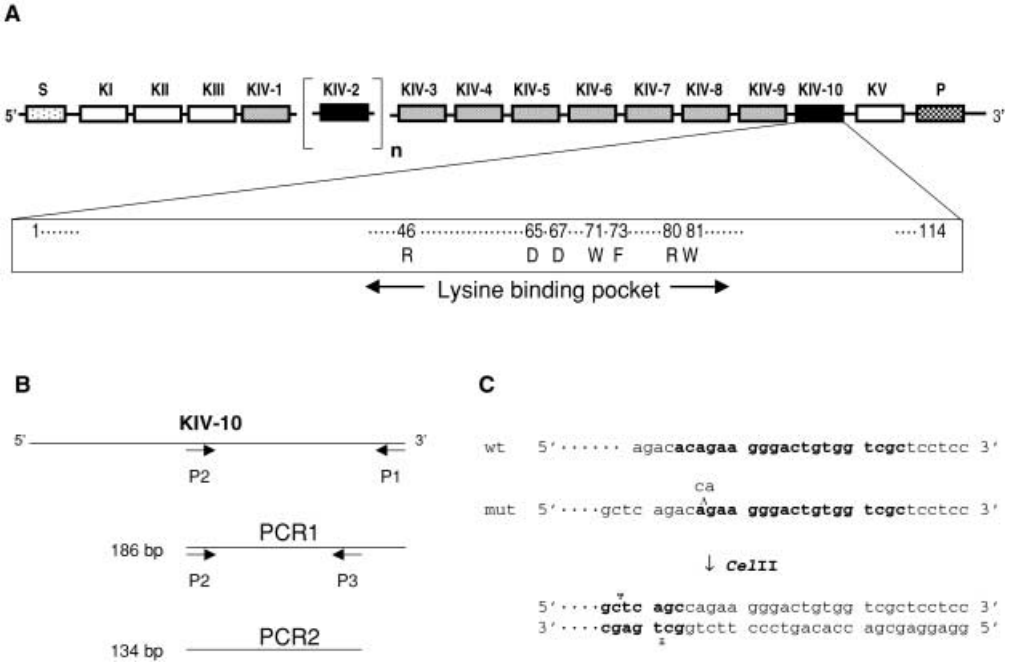


Fig. 1A–C Schematic representation of the human apolipoprotein(a) gene. **A** Protein domains of apo(a); *S* signal peptide; *K* kringles; *P* protease domain. **B** Schematic drawing of the KIV type 10 DNA sequence and localization of the primers used. **C** Localization of the CA deletion (12656–12657del, *white arrowhead*) and creation of the *Cel*II restriction cutting sites (*black arrowheads*). *Bold type* P3 primer sequence; *Mu* mutant; *wt* wild type

Quantification of plasma Lp(a) and Apo(a) genotype determination

The concentration of Lp(a) in plasma was determined by immunoturbidimetry using antibodies, calibrators, and standards supplied by Incstar Corporation (Stillwater, Minn., USA) [22]. The specificity of the apo(a) antibodies has been already described, and they recognize equally the different apo(a) size isoforms [23]. The assay is not affected by the presence of apoprotein B, plasminogen, or hyperlipidemia and shows a good correlation with a double monoclonal enzyme-linked immunosorbent assay used at the Northwest Lipid Research Laboratories [22]. Apo(a) alleles were determined as described elsewhere [4]. Intact DNA was digested with *Kpn*I and size-fractionated by pulsed-field gel electrophoresis, blotted to a nylon membrane and hybridized with an [^{32}P]dCTP radiolabeled human apo(a) KIV-specific single-stranded fragment (MP1; kindly provided by Dr. H. Hobbs, Southwestern Medical Center, Dallas, Tex., USA).

Measurement of LBS activity

The LBS function of Lp(a) was measured with a quantitative LBS-Lp(a) immunoassay as previously described [13]. The assay uses a monoclonal antibody to apo(a) to selectively capture Lp(a), an anti-K IV antibody that reacts only with unoccupied LBS, and a secondary disclosing antibody conjugated to alkaline phosphatase.

Amplification of human apo(a) kringle IV-10 from genomic DNA and detection of mutations and polymorphisms

The following procedures were modifications of previously published methods [24, 25]. Genomic DNA, extracted from the frozen cellular blood component by a salting-out method, was cut with *Bam*HI (5 U/µg DNA) at 37°C for 2 h to cleave all kringles other than KIV types 4, 9, and 10. The digested DNA was repurified and dissolved in Tris EDTA buffer to be used for PCR amplification with primers between residues 54 and 59 (Swiss-Prot: APOA HUMAN, P08519) (P2; 5′ AGTGGCCTGACAATGAACTA) and between residues 109 and 114 (P1; 5′ACCTTGTTCAGAAGGAGGCC) which give the PCR1 product (Fig. 1B).

Described polymorphisms at amino acid positions 75 and 81 were revealed by simultaneous digestion of the PCR1 product with *Nco*I and *Mnl*I, respectively, and the resulting fragments separated by electrophoresis in a 12% polyacrylamide gel and silver stained. We applied single-stranded conformational polymorphism (SSCP) analysis to the detection of single base changes at the final product. Although the effect of temperature and the addition of glycerol, formamide, sucrose, or urea were assayed, we found the best results in the patterns of separation using precast gels (12.5% acrylamide with 2% crosslinking), run at 4°C in a GenePhor (Pharmacia). To further confirm a mutation detected by an heteroduplex in the PCR, a distinct SSCP pattern and sequence analysis, we designed a nested PCR using the primers P2 and P3 (P3; 5′ GCGACCACAGTCCCTTCTGG) to introduce a restriction site for the enzyme *Cel*II in the mutated allele (see Fig. 1B, C). Primers P2 and P3 also recognize an apo(a)-like gene (locus HSU19517, NCBI) that would interfere with the 134-bp product from the apo(a) gene. To avoid this problem the second PCR was prepared with the PCR1 product as template. Additionally, the fragments were directly sequenced after asymmetric PCR in a Perkin Elmer ABI Prism 310 following the indications of the manufacturer.

Other laboratory measurements

Cholesterol and triglycerides were determined enzymatically with the CHOD-PAP and the lipase/GPO/PAP methods, respectively. High-density lipoprotein cholesterol was measured with a recently described homogeneous assay [26].

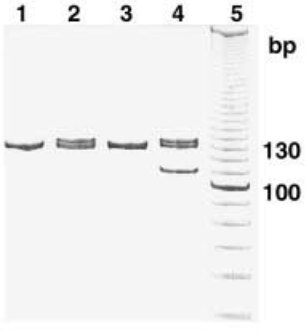
Statistical analysis

The χ^2 statistic was used to determine whether the genotype distribution was in Hardy-Weinberg equilibrium and to compare distributions of alleles and genotypes in the groups considered. The significance was set at $P<0.05$, and a Bonferroni correction to the P value was used where necessary. Variables were expressed as mean ±SD and geometric mean for Lp(a) concentration because tests were performed in the log-transformed variable. Data were initially analyzed by Snedecor's F test for the homogeneity of variances. The mean differences between groups were assessed using paired Student's paired t test or Mann-Whitney's U test following the indications of the F test, analysis of variance was used to compare variables according to the kringle number. The strength of the association of the selected polymorphisms with the occurrence of myocardial infarction was estimated by calculating the odds ratio with Epi-Info (Centers for Disease Control and Prevention, Atlanta, Ga., USA). Linkage disequilibrium between alleles at two different loci was assessed with the Arlequin program (University of Geneva; http://anthro.unige.ch/arlequin) [27].

Results

Identification of SSCP polymorphisms and description of a novel mutation

Among the 188 samples screened for mutations in or near the LBS pocket only one showed an abnormal pattern of migration. This particular sample already showed a double band in the electrophoresis gels of the PCR product compatible with the existence of a small deletion in heterozygosis (Fig. 2, lane 2). Therefore the PCR product was sequenced, and a 2-bp deletion (12656–12657del) was detected at amino acid 92 in one allele.



Expected bands (bp)

	wt/wt	wt/mut	mut/mut
PCR2	134	134,132 + heteroduplex	132
*Cel*II	134	134 + 108 + 24 + heteroduplex	108 + 24

Fig. 2 Detection and confirmation of the two nucleotide deletion (12656–12657del). Restriction analysis of the PCR2 product from control (*wt* wild type) and sample A53 (*mut* mutated) DNA. The product of the PCR was digested with the restriction enzyme *Cel*II following the indications of the manufacturer, separated by electrophoresis on 12% acrylamide gel and silver stained. *Lanes 1,3* wt; *lanes 2,4* mut; *lane 5* 10–bp ladder

To confirm such deletion a restriction analysis was performed as described in methods (Fig. 2, lane 4). This deletion is located in a dinucleotide repeat and produces a shift in the reading frame that creates a stop codon 63 bp downstream. These results suggest that a truncated protein would be produced from the translated allele. In the search for possibly undetected homozygous subjects we extended the restriction analysis to those individuals (14 patients and 17 controls) who had low plasma Lp(a) concentration (<40 mg/l), and we did not find the homozygous form of such mutation. The carrier of the described mutation had suffered an acute myocardial infarction at the age of 48 years, and clinical recovery was satisfactory.

Table 1 LBS activity and plasma Lp(a) concentration according to the kringle number of the smallest isoform in patients and controls. LBS activity is expressed as mean ±SD percentage of the reference Lp(a); Lp(a) concentration is expressed as geometric mean ±SD (mg/l)

	≤16	17–19	20–23	≥24	*P*
Patients	(*n*=35)	(*n*=22)	(*n*=20)	(*n*=17)	
LBS activity	66±22	59±19	41±26	36±20	0.0001
Lp(a)	289±34	280±25	101±42	87±32	0.001
Controls	(*n*=19)	(*n*=24)	(*n*=30)	(*n*=21)	
LBS activity	57±22	57±22	42±26	48±26	0.09
Lp(a)	166±29	124±32	75±30	101±32	0.105

Table 2 Genotype and allele frequencies for the M75T mutation at kringle IV-10 of the patients with myocardial infarction and controls (*MM* mutated, *MT* heterozygous the mutation, *TT* homozygous for the mutation)

	Myocardial infarction	Age-matched controls
Genotype		
MM	2 (2.1%)	6 (6.4%)
MT	49 (52.1%)	65 (69.1%)*
TT	43 (45.7%)	23 (24.5%)**
Allele		
M	0.282	0.406
T	0.718	0.594**

*Odds ratio 0.49 (95% CI 034–1.08), *odds ratio 2.53 (95% CI 1.31–4.88), ***P=0.03

Apo(a) size and differences in LBS activity and plasma Lp(a) concentration

Most individuals (96%) were heterozygous for number of KIV repeats and homozygous were distributed equally between groups. In analyzing the largest isoform of each subject, we found no difference in the distribution between patients and controls (for clarity, data are not shown). However, patients showed an statistically significant lower size for the smallest isoform than matched controls (18.6±18 vs. 20.4±20, P=0.008). The apo(a) size was divided into quartiles in order to explore its relationship with LBS activity and plasma Lp(a) concentration. There were only significant results for the smallest isoform (Table 1). The lower the kringle number, the higher the LBS activity and plasma Lp(a) concentration were, but this trend was statistically significant only in patients. This was further confirmed with Spearman's rank correlation values for the relationship between the sum of the apo(a) allele sizes and plasma Lp(a) concentration, which was significant in patients (–0.38, P<0.0005) but not in controls (–0.12, P=0.064).

Frequency of W81R and M75T substitutions

The W81R substitution was not found in our population sample, which suggests that this is infrequent in the Spanish population; all subjects studied were homozygous for W at position 81. The genotype and allele frequencies for the M75T polymorphism at KIV type 10 of the patients with myocardial infarction and controls are shown in Table 2. The distribution of the genotypes was in Hardy-Weinberg equilibrium but differed significantly between patients and controls. The frequency of the T allele was significantly higher in patients than in controls. Significantly fewer patients had the MT genotype than controls, and, conversely, the genotype TT was significantly more prevalent among the patients (Table 2). Homozygosity (TT) conferred a significant risk for myocardial infarction (odds ratio 2.53, 95% confidence interval, 1.31–4.88).

Influence of M75T substitution on relevant variables

The TT genotype conferred risk to myocardial infarction in our sample; therefore subjects were grouped accord-

Table 3 Relevant plasmatic variables in subjects homozygous for the T allele (M^-) and homozygous or heterozygous for the M allele (M^+) at position 75 according to their condition of patients with myocardial infarction or age matched controls. Results are expressed as mean ±SD (HDL high-density lipoprotein)

	Age matched controls		Myocardial infarction	
	M^+ (*n*=71)	M^- (*n*=23)	M^+ (*n*=51)	M^- (*n*=43)
Cholesterol (mmol/l)	5.51±0.92	5.36±0.92	5.79±0.69	5.72±0.74
Triglyceride (mmol/l)	1.58±0.94	1.63±0.89	2.11±1.57*	2.09±1.02
HDL cholesterol (mmol/l)	1.12±0.25	1.08±0.26	1.01±0.26*	1.01±0.21
Lp(a) (mg/l)	170±161	208±158	269±226**	395±340***
LBS activity (%)	48±25	57±22	55±25	53±25

*P=0.04, **P=0.003, M^+ vs. M^- between the different groups; ***P=0.03, M^+ vs. M^- in the same group

ing to the M75T genotype, and relevant variables were reassessed. Subjects with the MM or MT genotypes were considered M^+, and those with the TT genotype M^- (Table 3). There were no differences in age or body mass index between the groups considered. The percentage of current or former smokers were significantly higher in patients with myocardial infarction (81%) than in controls (31%), but it was virtually the same between M^+ and M^- individuals. Patients also had a higher enrichment of cholesterol and triglyceride in apoB containing lipoproteins and lower high-density lipoprotein cholesterol than controls, but the difference was significant only in M^+ subjects. There was no association between LBS activity and the M75T mutation. Patients had a significantly lower number of kringles (smallest isoform) than controls; The M^- subjects had also a significantly lower number of kringles than M^+ subjects, both in controls (18.2±3.9 vs. 21.2±4.9; $P<0.05$) and in patients (17.5±4.3 vs. 19.4±4.8; $P<0.05$). Correspondingly there was a trend toward higher plasma Lp(a) concentration in M^- individuals, but this was significant only in patients (Table 3). This result could be due to a possible linkage disequilibrium between the M75T mutation and the apo(a) size, as previously described [18], but this is not confirmed in our sample.

Discussion

The KIV type 10 of apo(a) is the primary LBS of Lp(a). Transgenic mice with apo(a) mutations of KIV type 10 which have low LBS activity have reduced lesion [12]. The LBS activity is thought to mediate the pathogenic risk of Lp(a) by interfering with plasminogen binding or binding to cells and proteins by the LBS function which is unavailable to low-density lipoprotein. The purpose of this study was to search for mutations in the apo(a) KIV type 10 which could alter the LBS activity of Lp(a) from patients with coronary artery disease. We used SSCP and sequence analysis in the search for base changes at the DNA region coding for the tail end of KIV type 10 of human apo(a), and we found this region mutable but relatively well preserved. We have also found a novel mutation which consisted in a 2-bp deletion that presumably creates a truncated protein. The carrier of this mutation had undetectable levels of plasma Lp(a) and a low LBS activity. Although the mechanism underlying the pathogenetic role of Lp(a) in CAD is not fully understood, this combination is considered to be either beneficial or protective. This individual, however, was a survivor of a myocardial infarction with multiple angiographically confirmed lesions in two major coronary arteries. A considerable number of subjects showed phenotypically low LBS activity and low plasma Lp(a) concentration, and the distribution was virtually the same between patients and controls (14 and 17, respectively). Therefore the presence of either low plasma LBS activity or Lp(a) concentration does not indicate protection against the presence of the early-onset disease in our sample population. However, it is well known that Lp(a) is a risk factor primarily when interacting with other lipoprotein as well as non-lipoprotein-related risk factors [28].

The W81R substitution, which has been identified in 2% of a Chicago population, was not found in this sample or in 150 additional samples tested (data not shown), which suggests that this mutation, if present, is infrequent in the Spanish population. Furthermore, the two previously described subjects [15] were LBS defective and had a plasma Lp(a) concentration less than 10 mg/l and a single apo(a) allele of 120 kb, suggesting that this mutation can affect a major function of Lp(a). However, in our population sample we found seven subjects (four patients and three controls) with absent LBS activity and plasma Lp(a) levels less than 10 mg/l who do not bear such mutation. Therefore there is a considerable heterogeneity with respect to the LBS properties of Lp(a) which in some cases is extreme, and binding is not detected in vitro.

Another described mutation, the M75T substitution, was frequent in our population. Previous studies have determined the frequency of the M75T substitution in diverse populations [15, 18, 29, 30], and certain ethnic variation is evident. In our study the allele frequency differed significantly between patients and controls, and the genotype TT conferred a significant risk for myocardial infarction. This cannot be confirmed in whites [29], but it has been observed in Afro-Caribbeans [30]. All subjects included in the study population were unrelated whites and were born in an area that has the lowest incidence of myocardial infarction in the western world [31]. Although we recognize the limitations of association studies, these results may suggest the possibility of ethnic variation respect to the M75T substitution and that the presence of the mutation could be in linkage disequilibrium with other genetic risk factor [32] which in our sample is unrelated to the number of KIV repeats.

The putative mechanism is not related to the LBS activity because this study reveals comparable LBS activity in the M^+ and M^- subjects. However, we observed that M^- patients possess significantly higher values of plasma Lp(a). A linkage disequilibrium between the M75T mutation and a *Kpn*I allele corresponding to 18 kringles [18] has been described, but was not confirmed in our study. The difference in LBS activity is highly dependent on the number of kringles rather than on the presence or absence of the M75T substitution.

Survivors of myocardial infarction in the Spanish population are more frequently those subjects who are homozygotes for T, with high plasma Lp(a) levels, low number of kringles at the smallest isoform, and higher LBS activity. Further studies are needed to ascertain the possible relationships between these variables.

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