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The Use of a Nonradioactive Digoxigenin Labeled Probe for Detection of Apo(a) Gene Size

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Abstract: Elevated lipoprotein(a) [Lp(a)] concentrations are associated with premature coronary heart disease and early myocardial infarction. In the general human population the sizes of apolipoprotein [apo(a)] isoforms are inversely associated with lipoprotein(a) levels. Large isoforms are associated with low Lp(a) and small isoforms with high Lp(a) in the plasma. The size of the polymorphism is directly correlated with the number of kringle-4 domains in apo(a). It has been shown by quantitative Southern blotting, and more recently by pulsed-field gel electrophoresis, that polymorphism is the result of differences in the number of tandem kringle-4 repeats in the apo(a) gene.

In our current study, we used a digoxigenin labeled MP-1 probe which is specific for the

apo(a) gene and does not detect other genomic sequences under the blotting conditions. We determined plasma Lp(a) levels by ELISA and compared them with the size of the apo(a) gene. The latter were determined by dot blotting of plasma samples from 50 coronary artery disease patients (angiographically documented) and 50 healthy controls. Our results showed an inverse relation between the Lp(a) levels and the apo(a) gene size. This is consistent with the literature. Our method has no radioactive steps. It is rapid and easy to perform.

Key Words: Lp(a), coronary artery disease, dot blot analysis, DIG-labeling, apo(a) gene.

Introduction

Lp(a) represents a class of lipoprotein particles defined by the presence of apo(a), a unique glycoprotein linked by a disulfide bond to apolipoprotein B-100 to form a single macromolecule (1). Apo(a) contains three different structural domains each having high amino acid sequence homology with plasminogen. One of the domains, called kringle-4, is present in multiple copies, the number of which varies and is genetically determined. This accounts for the size heterogeneity of apo(a) and thus of Lp(a) (2-5). The size variation is inversely related to the plasma concentration, thus the lower molecular weight isoforms are present in higher concentrations (6).

The Lp(a) particle is more atherogenic than LDL, probably because it disturbs the balance between thrombogenesis and fibrinolysis. High concentrations of Lp(a) may favor atherosclerotic plaque formation by inhibiting plasminogen activation (7, 8).

In the general human population, plasma concentrations of Lp(a) have been found to vary between 0 and more than 20 mg/dl. When Lp(a) is >30 mg/dl, the relative risk of coronary atherosclerosis is almost doubled (9). The concentration of Lp(a) is genetically determined and only slightly influenced by environmental factors (10, 11). Numerous studies demonstrated an association between elevated Lp(a) levels and the risk for coronary heart disease, stroke and vessel reocclusion after aorto-coronary bypass surgery (12, 13). Increased concentrations of apo(B) and Lp(a) may act synergistically to promote atherosclerosis. Subjects with familial hypercholesterolemia have a mean Lp(a) concentration two to three times higher than the mean of the healthy controls (14).

The apo (a) gene is localized on chromosome 6 (q26-27) in a linkage group with the plasminogen gene. Apo (a) contains three motifs that are also present in plasminogen: 1. a protease domain with 94% homology

to the plasminogen's protease domain, 2. a single plasminogen like kringle 5-domain, 3. a multiple plasminogen like kringle-4 domain. The apo(a) and plasminogen cDNAs have been cloned and the two genes are closely linked on chromosome 6 (q26-27). The two cDNA sequences are remarkably similar (15, 16).

The size variation of the apo(a) gene is inversely related to the plasma Lp(a) concentration because it is observed that small alleles tend to be dominant over large alleles in the establishment of the Lp(a) levels (17).

Thus when the gene size increases there is decreased possibility for the Lp(a) molecule to be assembled from the large size apo(a) protein which allows low levels of Lp(a) concentration in the plasma. Conversely, when the size of the gene is relatively small, then the small alleles are dominant and the Lp(a) concentration in the plasma increases. Thus it can be concluded that the Lp(a) level in the plasma is genetically determined (18). In our study, we used a probe MP-I, which was hybridized specifically to the kingle-4 encoding region of the apo(a) gene but not to the plasminogen or to any other of the apo(a) or plasminogen pseudogenes. We determined plasma Lp(a) levels by ELISA and compared them with size of the apo(a) gene which was determined by dot blotting in 50 angiographically documented plasma samples from coronary artery disease patients and 50 healthy controls. Our results showed inverse correlation between an Lp(a) levels and the apo (a) gene size.

Materials and Methods

Subjects

High molecular weight DNA was isolated from leucocytes of 50 coronary artery disease patients and 50 healthy controls following standard protocols (19). The purity and amount of DNA samples were determined spectrophotometrically (20).

Labeling of probe

The MP-I probe was enzymatically labeled at its 3' end with terminal transferase by incorporation of a single digoxigenin-labeled dideoxyuridin triphosphate (DIG-ddUTP). Tailing buffer (potassium cocadylate, 1mol/l; Tris-HCL, 0.125mol/l; bovine serum albumine, 1.25 mg/ml; Ph 6.6), CoCl₂ solution (25 mmol/l), 100 pmol MP-1 probe, 50 units terminal transferase, DIG-ddUTP (1 mmol/l) were mixed and brought to a final volume of 20 µl with ddH₂O. Incubation was at 37°C for 15 minutes. The labeled oligonucleotide was precipitated with LiCl₂ (4 mol/l). The solution was centrifuged. The

pellet was dried under vacuum and dissolved in sterile ddH₂O. The 3' End DIG labeling and detection kit was purchased from Boehringer Mannheim.

Hybridization and detection of the probes

DNA samples were genotyped by dot blotting using a DIG labeled MP-I probe which was specific to the kringle-4 region of the apo(a) gene. 1µg DNA was spotted into the positively charged nylon membrane. DNA was bound to the nitrocellulose by applying vacuum for 10 minutes and fixed by UV cross-linking with transillumination at 254 nm. The membrane was prehybridized at 68°C for 1 hour. The filter was incubated at 54°C for 6 hours to allow hybridization of the labeled probe. The membrane was washed and incubated with buffer containing antidigoxigenin-alkaline phosphatase conjugate. The filter was incubated with color substrate solution containing 5-bromo 4-chloro3-indolyl phosphate (X-phosphate) and nitroblue tetrazolium salt (NBT). The color precipitate started to form within a few minutes and the reaction was usually completed after 16 hours. After the desired spots or bands were detected, the reaction was stopped by washing the membrane. The plasma concentration of total cholesterol, triglycerid and HDL-cholesterol was determined using a Biotrol kit with a RA-1000 autoanalyser. LDL-cholesterol was calculated by the Friedewald formula and the plasma concentration of Lp(a) was determined with a Biotrol Tint ELISA kit.

Statistical methods

Statistical studies were performed with the Student's t-test. Significance at $p < 0.05$ was considered positive.

Results

To determine the molecular basis for the size heterogeneity found in the apo(a) protein, the structure of the apo(a) gene was explored with a genomic blot using an apo(a) gene specific probe (MP-I:5'-TTCATGATCAAGCCACTGGAAATTCCAAAACGATACA-3'), which does not detect other genomic sequences (Figure 1) Under the blotting conditions we concluded that the fragments which have a greater number of kringle-4 repeats tended to have more intense color development than the shorter fragments. Also, after dot blot analysis we observed dense purple spots on the MP-1 hybridized DNA samples of the control group, but only faint or no spots on sample from patients. Since the Lp(a) levels of the patient group were significantly higher than the levels of the control group ($p < 0.001$), we concluded that the level of plasma Lp(a) tends to be inversly related to the

size of the apo(a) gene. Triglycerides, total cholesterol, HDL-cholesterol and LDL-cholesterol levels of the patient and control groups were analyzed and compared within groups. Triglycerides, total cholesterol and LDL-cholesterol levels of the patient group were significantly higher than the levels of the control group ($p < 0.05$) (Table 1).

After dot-blot analysis we observed dense purple in the control group, and light, or no spots on the patients' MP-I hybridized DNA samples (Figure 2).

Discussion

Nucleic acid hybridization is a powerful technique for the detection of specific, complementary nucleic acid sequences and is being used extensively for detection of genetic disorders and molecular pathologies (21). With a

few exceptions, DNA probing techniques require the use of radioisotopes and toxic DNA extraction techniques which render the method expensive, potentially hazardous and time consuming. Most isotopic labeling techniques use the isotope ^{32}P (half-life 10-14 days) and require 3-10 days to visualize bands after hybridization (22). The nonradioactive DNA labeling and detection system is a safe, stable and sensitive system that is suitable for a wide variety of nucleic acid hybridization techniques (23, 24).

In this genomic blotting method we describe the use of a digoxigenin labeled apo(a) gene specific probe that has the advantage of using nonradioactive material. An alternative nonradioactive approach is based on the biotin-streptavidin system. This system suffers from two disadvantages; 1) the ubiquitous presence of biotin (vitamin H) in prokaryotic and eukaryotic cells, 2) a

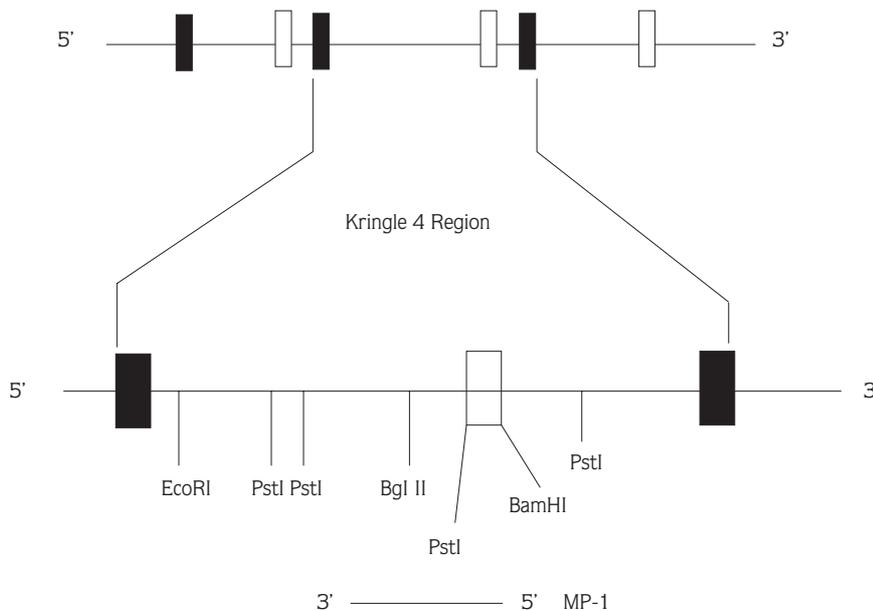


Figure 1. Kringle-4 encoding region of apo (a) gene. Location of apo(a) specific probe (MP-1) within the kringle 4-encoding sequence of the apo(a) gene is shown. Each kringle-4 encoding repeat is encoded by two exons, which are demarcated by boxes that are filled in (the first exon) or open (the second exon). The locations of Eco RI, Pst I, Bam HI restriction sites are given. A single kringle-4 encoding unit has been enlarged.

	PATIENT (n=50) (mg/dl) mean ± SD	CONTROL (n=50) (mg/dl) mean ± SD
Total Cholesterol	204.8 ± 47.8*	185.0 ± 42.0
Triglycerid	178.0 ± 75.2*	108.5 ± 53.9
HDL-Cholesterol	33.5 ± 11.3	48.3 ± 13.4
LDL-Cholesterol	135.8 ± 44.2*	115.3 ± 39.3
Lp(a)	38.3 ± 18.6**	25.6 ± 22.7

Table 1. The mean values ± standard deviations of patient and control groups.

* $p < 0.05$, ** $p < 0.001$

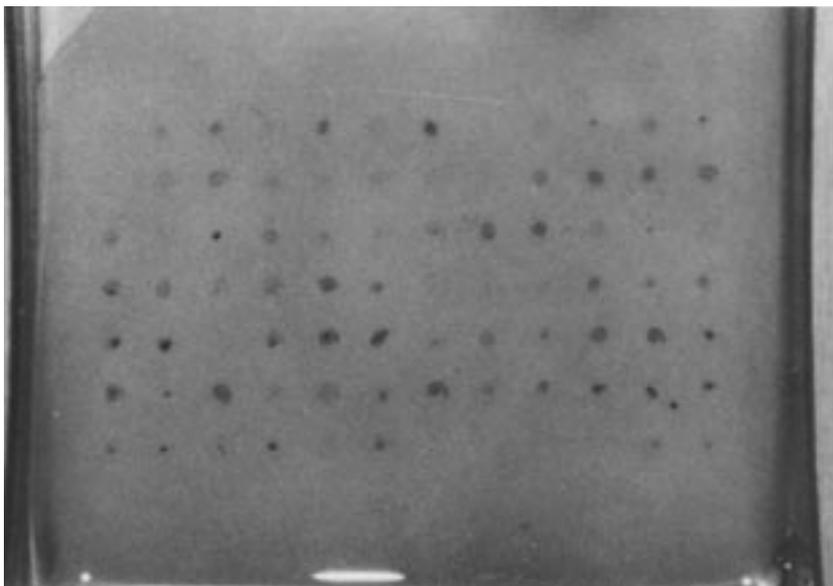


Figure 2. MP-I hybridized DNA samples. Dot blotting was performed using 1 µg genomic DNA. The blot was hybridized with 100 pmol/ml of the digoxigenin labeled apo(a) kringle-4 specific probe MP-I as described in methods.

tendency for streptavidin to bind unspecifically to immobilizing matrices such as nitrocellulose or nylon. A nonradioactive detection method based on digoxigeninated probes avoids these disadvantages (25).

The factors that influence the apo(a) gene size detection by dot blotting include the patient's Lp(a) concentration, the amount of DNA applied for blotting, the amount of digoxigenin labeled probe, and the incubation time for hybridization. The amount of DNA and incubation times for both prehybridization and hybridization were kept optimal and the MP-I probe was added in an optimized way to detect most, if not all, the kringle-4 domains in the apo(a) gene.

In 30% of the controls with Lp(a) levels, the darkest bands were observed. This was consistent with the inverse relation between plasma Lp(a) level and apo(a) gene size.

Lackner and co-workers used pulsed-field gel electrophoresis to identify a large Kpn-1 fragment from the apo (a) gene that contained most of the sequences

encoding the kringle-4 repeats (18). This site was identified by performing PCR using two oligonucleotides; one from the 3' end of kringle-5 and the other from the 5' end of the protease domain of the the apo (a) gene. Uterman and co-workers suggested an inverse relationship between the size of apo(a) gene and the amount of plasma Lp(a) (26). This observation was confirmed (27). Our findings are also consistent with these previous studies.

Although this method is not quantitative and not as sophisticated as pulsed-field gel electrophoresis, PCR and restriction procedures, our method is simple, rapid and does not involve any hazardous techniques. As a result it can be used for common screening of large numbers of samples.

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