



Short communication

Lipoprotein(a) phenotyping using a computerized micro scale and phenotype frequencies in a healthy Japanese population

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1. Introduction

Lipoprotein(a) (Lp(a)) was first described in human plasma by Berg [1] and was believed to be inherited as an autosomal dominant trait. This lipoprotein is isolated in a density of 1.05–1.12 kg/l. The lipid composition of Lp(a) closely resembles that of low density lipoprotein (LDL), but the particle diameter is slightly larger than that of LDL [2]. Lp(a) contains a specific glycoprotein, apolipoprotein(a) (apo(a)), which is linked to apo B-100 by disulfide bridges [3,4]. The cDNA sequence of apo(a) has revealed a high degree of homology with plasminogen [5]. Apo(a) contains the signal domains, lacks the tail and kringles 1 through 3, but includes a variable number of kringle 4-like domains, a single copy of kringle 5-like domain and the serine protease domain of plasminogen. The differences in size of apo(a) isoforms are due to allelic differences in the number of kringle 4-like units. Utermann et al. [6] reported that the size polymorphism of apo(a) is controlled by at least six alleles (Lp(a)^B, Lp(a)^{S1}, Lp(a)^{S2}, Lp(a)^{S3}, Lp(a)^{S4} and Lp(a)⁰) at a single locus and is associated with plasma

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Lp(a) levels and with premature coronary heart disease [7]. Recently, 11 different isoforms of apo(a) have been identified [8] and a total of 19 and 26 different isoforms were detected by the techniques of pulsed-field gel electrophoresis and genomic blotting, respectively [9,22].

In the present study a new system has been employed to determine the apo(a) phenotypes using a computerized digital micro scale after sensitive immunoblotting of serum specimens. The Lp(a) phenotype frequencies were determined in healthy Japanese subjects using this system. The Lp(a) phenotype frequencies were set in relation to Lp(a) lipoprotein concentrations in serum.

2. Materials and methods

2.1. Samples

For the population study, fresh sera ($n = 500$) were obtained from blood donors ($n = 385$) and medical students ($n = 115$). They were healthy adult subjects — 275 males (mean \pm S.D. age 32 ± 12 years) and 225 females (29 ± 13 years). For the initial R_f values determinations with the micro scale 210 serum samples of the above subjects were taken for the S3 and S4 apo(a) isoforms, and for the determination of F, B, S1 and S2 isoforms, 149 subjects were collected with high levels of Lp(a). All sera were obtained under conditions minimizing proteolysis, and were used for Lp(a) analysis either within a week or were frozen immediately at -40°C until analysis.

2.2. Antibody against apo(a)

Lp(a) antigen and antiserum were prepared as described previously [10]. Antibody specificity was characterized by immunoblotting with human serum and tested for cross-reactivity against apo B-100 and plasminogen.

2.3. Electrophoretic procedures

SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and immunoblotting were performed exactly as previously described [14].

2.4. Classification of apo(a) phenotypes

According to Utermann et al. [11], apo(a) phenotypes were categorized into phenotypes F, B, S1, S2, S3 and S4 and into the respective double-band phenotypes compared with the mobility of apo B-100 as a marker. R_f values of apo(a) isoform bands were measured using a computerized digital micro scale (MAX-CAL, NSK Co., Japan) [12]. Briefly, the micro scale was connected to a microcomputer (PC-9801, NEC Co., Japan) using an RS 232C interface. After standardizing the system with selected samples the apo(a) phenotyping was done automatically.

2.5. Analytical methods

Lp(a) concentrations were determined by enzyme-linked immunosorbent assay (ELISA) as described previously using rabbit anti-apo(a) antiserum and Lp(a) reference standard (Immuno AG, Vienna, Austria) expressed in terms of total Lp(a) lipoprotein [10].

2.6. Statistical methods

Overall comparison of Lp(a) lipoprotein concentrations between phenotypes was done by the non-parametric Kruskal-Wallis test. Comparison of Lp(a) concentrations among phenotypes was performed by Mann-Whitney *U*-test. Comparison of the observed phenotype frequencies with expected values was done by χ^2 analysis. Apo(a) allele frequencies were calculated according to Wiener et al. [13].

3. Results

3.1. Apo(a) phenotyping using the computerized micro scale

Serum samples were analyzed by SDS-PAGE under reducing conditions and the apo(a) isoforms were visualized by immunoblotting. Two types of patterns were observed which were characterized by the presence of a single-band or double-bands of apo(a) isoforms.

The electrophoretic migration distance of the apo(a) isoforms was determined employing a computerized micro scale in 210 healthy subjects and in 149 individuals with high Lp(a) cores. The migration distance was expressed as R_f value ($R_f = 1.0$ means migration to the end of the gel). The histograms of these R_f values are shown in Fig. 1. Two peaks in healthy subjects and four peaks in high Lp(a) subjects were observed on these histograms. Apo(a) isoforms were classified using the R_f value of apo B-100 (Fig. 2) according to Utermann [11]. Table 1 shows the R_f values of five frequent apo(a) isoforms. Using these data the R_f limits of the different apo(a) isoforms were determined for phenotyping with the micro scale — S4: <0.057; S3: 0.058–0.087; S2: 0.088–0.119; S1: 0.120–0.148; B: 0.149–0.185; F: >0.186. The mean mobility ratios of six apo(a) isoforms to that of apo B-100 were 0.28, 0.47,

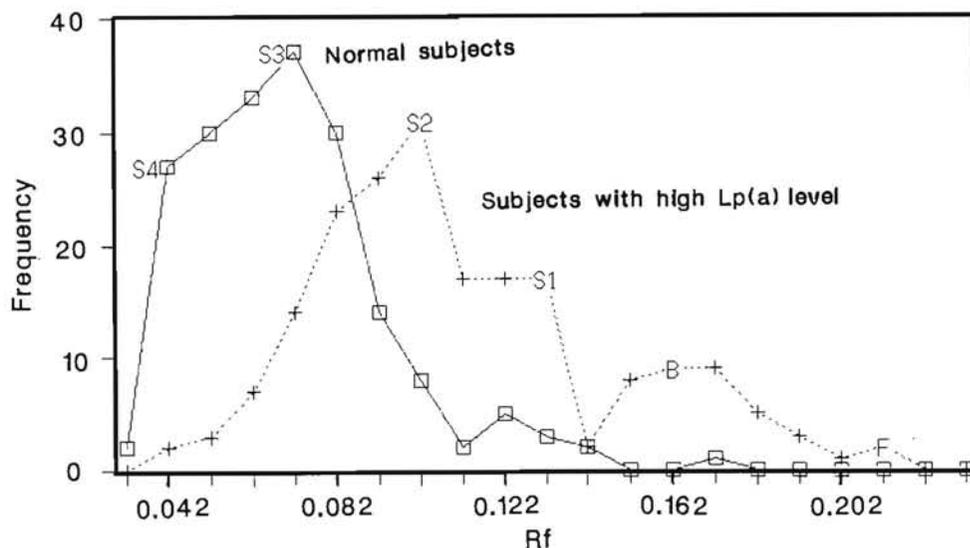


Fig. 1. R_f values of apo(a) isoforms from 210 normal Japanese subjects and from 149 subjects with high Lp(a) levels.

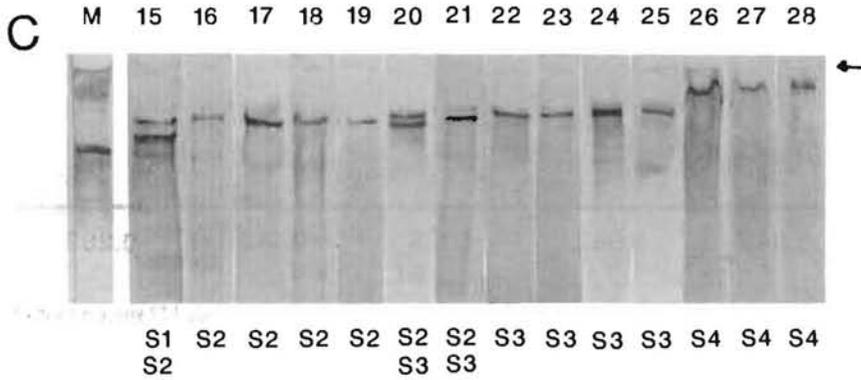
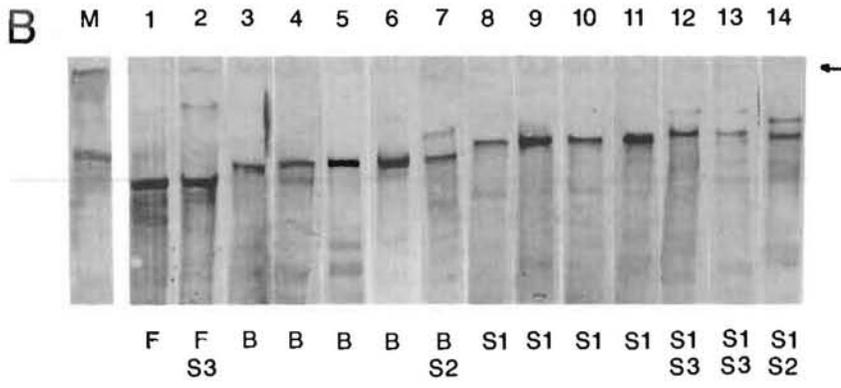
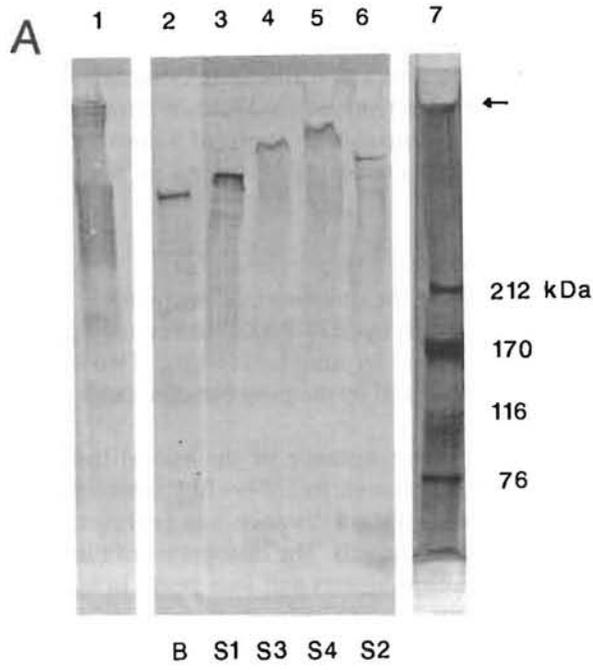


Table 1
 R_f values of apo(a) isoform on SDS-PAGE

	R_f of apo(a) isoform				
	S4	S3	S2	S1	B
<i>n</i>	81	109	48	13	2
Mean*	0.042	0.074	0.102	0.137	0.160
S.D.	0.036	0.011	0.019	0.019	0.016
Mean $\frac{R_f \text{ apo(a)}}{R_f \text{ apo B}}$	0.28	0.47	0.65	0.88	1.03

*Significant between S4 and S3, S3 and S2 or S2 and S1: $P < 0.001$.

0.65, 0.88, 1.03 and 1.32, respectively. Inter-assay reproducibility (CV) of ($n = 70$ blot sheets) of apo B-100 mobility was 5.5%. Only R_f values of apo B-100 sampling from 0.145 to 0.165 were used for the identification of apo(a) isoforms; 89% of the blots fell within this range, i.e. 11% of the blots had to be carried out once again.

Positive correlation was found between Lp(a) concentration and R_f values in the single-band phenotypes (Fig. 3) ($n = 111$, $r = 0.675$, $P < 0.001$). These results

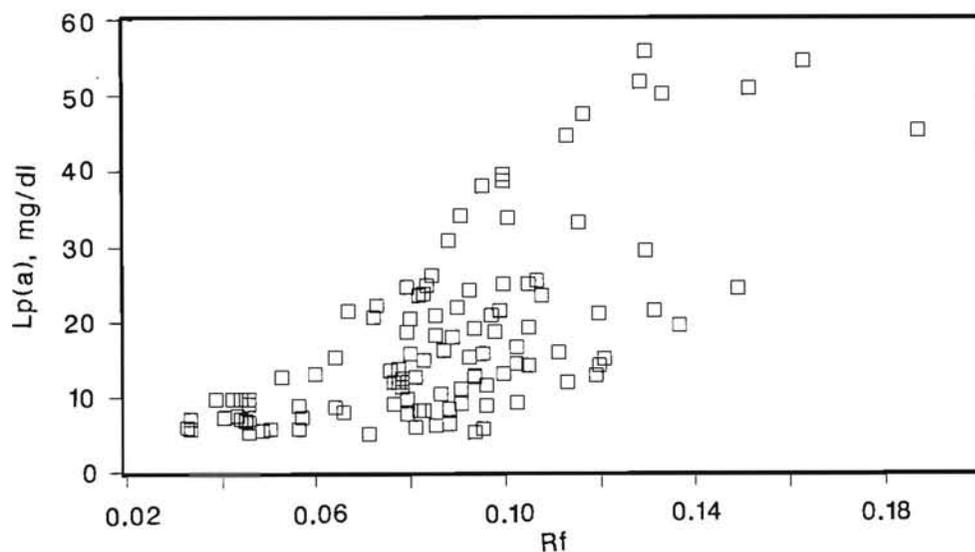


Fig. 3. Correlation between Lp(a) concentration and R_f values of single-band phenotypes. The relation was as follows: $y = 253x - 7.0$ ($n = 111$, $r = 0.675$, $P < 0.001$).

Fig. 2. Demonstration of typical apo(a) phenotypes. Sera were subjected to gel electrophoresis under reducing conditions and immunoblotted as described under Materials and methods. Arrow indicates the loading point of sample. (A) Lane 1: immunoblot developed with anti-apo B antibody (sample is the same with lane 2); lanes 2–6: apo(a) immunoblot, apo(a) phenotype B, S1, S3 and S2; lane 7: molecular weight marker. (B, C) Lanes 1–28: each lane indicates typical apo(a) phenotypes with single or double bands; lane M; immunoblot detected with anti-apoB.

Table 2
Frequencies of apo(a) alleles

Allele	Frequency		
	Male <i>n</i> = 275	Female <i>n</i> = 225	Total <i>n</i> = 500
Lp(a) ^B	0.005	0.020	0.012
Lp(a) ^{S1}	0.041	0.041	0.041
Lp(a) ^{S2}	0.171	0.165	0.168
Lp(a) ^{S3}	0.244	0.259	0.252
Lp(a) ^{S4}	0.182	0.171	0.177
Lp(a) ⁰	0.357	0.344	0.350

Frequency difference between male and female: $\chi^2 = 0.50$ ($P = 0.99$) not significant.

demonstrate again that the size of apo(a) is negatively correlated with Lp(a) levels in plasma.

3.2. Frequencies of Lp(a) phenotypes

Lp(a) phenotypes were determined using the computerized micro scale in 500 healthy unrelated subjects: 333 subjects exhibited a single band, 127 had double bands and in 40 samples (8.0%) no band was detected. Lp(a) levels of double-band phenotypes (mean = 12.7 mg/dl, S.D. = 11.3 mg/dl) were significantly higher than those of single band (mean = 24.9 mg/dl, S.D. = 19.2 mg/dl) ($P < 0.001$).

The allele frequencies were calculated according to Wiener et al. [13] and are shown in Table 2. When the observed phenotype frequencies in healthy subjects were

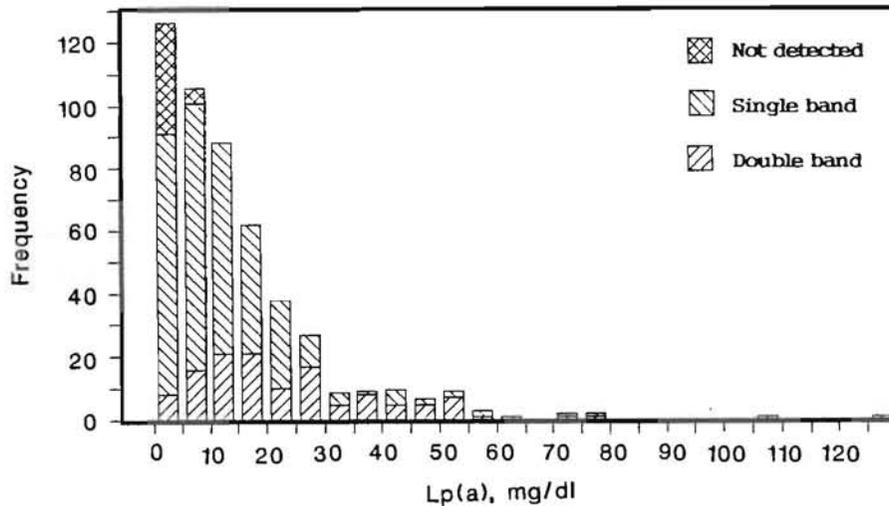


Fig. 4. Distribution of serum Lp(a) concentrations in 500 healthy Japanese.

compared with those expected assuming Hardy-Weinberg equilibrium, there was no significant deviation of observed from expected frequencies ($\chi^2 = 21.4$, $df = 15$, $P = 0.12$; full details of these results showing the similar isoform distributions are available from Ref. 14).

The distribution of serum Lp(a) concentrations in these healthy Japanese was highly skewed toward lower levels (Fig. 4) (concentration (mg/dl) for total: mean = 15.0, S.D. = 14.8, median = 11.0; for males: mean = 14.2, S.D. = 14.7, median = 10.0; for females: mean = 15.9, S.D. = 14.8, median = 12.1). Lp(a) concentrations did not differ significantly between males and females. The present method was sensitive enough to detect Lp(a) concentrations above 2.3 mg/dl (mean value of Lp(a) in null type; range, 0.3–7.2 mg/dl), with 92.0% of the subjects tested exhibiting single or double band(s).

The non-parametric Kruskal-Wallis test showed a significant ($P < 0.01$) difference in Lp(a) concentrations between single-band phenotypes. Pairwise comparison by a Mann-Whitney *U*-test showed that the four frequent single-band phenotypes may be split up into two pairs, S1 and S2, and S3 and S4, with no significant difference between Lp(a) concentrations within a pair, but significant ($P < 0.01$) differences between the Lp(a) concentrations of phenotypes in different pairs, i.e. S1 vs. S3 and S1 vs. S4, S2 vs. S3 and S2 and S4. Phenotypes B, S1 and S2 were associated with high Lp(a) concentrations, whereas phenotypes S3 and S4 were associated with low Lp(a) levels in serum.

4. Discussion

Lp(a) lipoprotein contains two protein components, apo(a) and apo B-100, which are linked by a disulfide bridge in the intact particle. Apo(a) is released from Lp(a) after exposure to reducing agents, such as 2-mercaptoethanol. A sensitive, high resolution method for identifying apo(a) isoforms is described in the present paper. This method relies upon SDS-PAGE under reducing conditions and subsequent immunoblotting using a sensitive double antibody technique with gold-silver staining. After electrophoresis and blotting, R_f values of apo(a) isoform bands were measured using a computerized micro scale and apo(a) phenotypes were categorized automatically based on the R_f values. Apo(a) phenotype frequencies classified by this method were in agreement with those of our previous study [14] and of Caucasians by Utermann et al. [15]. These results suggest the accuracy of the present method with a microcomputer.

Recently, Farrer et al. [16] reported a simple and sensitive method for the identification and classification of apo(a) isoforms, based on R_f vs. apo B on SDS-PAGE. However, the present method was based on R_f values themselves, because good reproducibility for mobility of apo(a) isoforms and apo B-100 on SDS-PAGE was observed in the present system. As the sizes of apo(a) isoproteins are extremely large, their mobility will not be influenced greatly by duration of electrophoresis.

In the present study, we were unable to detect apo(a) isoforms in only 8% of the subjects tested. The frequency of null phenotype and allele in the present results are in agreement with those of Sandholzer et al. [17,18] and Haug et al. [19]. It is widely

believed that there are no true Lp(a)-negative sera. Therefore, the frequency of this allele depends on the sensitivity of the immunoblotting assay.

Gaubatz et al. [8] classified 66 different phenotypes based on 11 detectable bands in 686 subjects and observed that there were 412 plasma samples (59.5%) that contained a single band, 274 (39.6%) contained two bands and only 6 (0.9%) had no detectable apo(a) band. They concluded that phenotype expression did not fit a classical Mendelian inheritance pattern. Recent reports employing pulsed field gel electrophoresis of genomic DNA describe more than 20 different alleles on the basis of restriction fragments which included the variable number of kringle 4 repeats [9,22]. Also on a protein level more than 20 apo(a) size polymorphisms have been demonstrated by Kamboh et al. [20]. However, we used the apo(a) isoforms designated by Utermann et al. in the present study because of many comparable data and the known hereditary relationships. These data showed that, at least on the DNA level, the apo(a) alleles are in Hardy-Weinberg equilibrium. The present phenotyping with six polymorphic bands is considered to be convenient for clinical diagnostic testing.

Frequencies of Lp(a) phenotypes and apo(a) alleles in the Japanese population were in good agreement with the previous results for Caucasians [15], whereas they were significantly different from those for Chinese, Indians and Sudanese [18]. These results are ethnologically very interesting. An inverse association between apo(a) isoform sizes and Lp(a) concentrations was also observed in the present study, as has been observed previously [6,8,10,21,22]. According to Kraft et al. [22], 46% of the variability of Lp(a) levels can be explained by the size polymorphism, but the apo(a) gene locus controls over 90% of the variation. The remaining variability is still to be explained. Phenotypes B, S1 and S2 are associated with high and phenotypes S3 and S4 with low Lp(a) levels. There is, however, considerable overlap between the Lp(a) concentrations of the different phenotypes and it is impossible to predict Lp(a) concentrations from Lp(a) phenotypes, or vice versa. We could not find the F phenotype in the blood donors, and it is thought that this type is observed only in patients with hyperlipidemia or atherosclerosis.

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