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Variations in the Promoter Region of the Apolipoprotein A-1 Gene Influence Plasma Lipoprotein(a) Levels in Asian Indian Neonates from Singapore

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ABSTRACT

We studied the influence of two DNA polymorphisms (−75 bp G/A and +83 bp C/T) in the promoter region of the apolipoprotein A-1 (apoA1) gene on cord plasma level of lipoprotein(a) [Lp(a)] in 1076 newborns of both genders from the three major ethnic groups in Singapore—Chinese, Malays, and Asian Indians. The frequency of the *A* allele at −75 bp in the Indians was significantly lower than the Chinese and Malays. There was no linkage disequilibrium between the two sites studied. Both polymorphic sites were not significantly associated with any lipid factors except for Lp(a) levels in the Asian Indians. The *AA* and *CC* homozygotes were significantly associated with lower Lp(a) levels. These associations were specific only to the male Indian neonates. The genetic variations at the −75 and +83 bp explained 6.9% and 7.2%, respectively, of the total variability of plasma Lp(a) levels at birth in the Asian Indians. The Lp(a) levels were also significantly different between composite genotypes in the order *GG/TT* > *GA/CT* > *GG/CT* > *GA/CC* > *GG/CC* > *AA/CC*. The effects of the two polymorphisms seem to be additive as the composite genotypes were able to explain 14% of the Lp(a) variance, equivalent to the sum of the two constituent sites. Our results showed that there is significant ethnic- and gender-specific influence of the apoA1 gene on plasma Lp(a) levels at birth that is inherent and independent of known gene-environment interactions. **(*Pediatr Res* 49: 514–518, 2001)**

Abbreviations

apoA1, apolipoprotein A-1
Lp(a), lipoprotein(a)
CAD, coronary artery disease

ApoA1 is the predominant protein that is associated with the HDL cholesterol (HDLC) particle. Both HDLC and apoA1 are key components of the reverse cholesterol transport process and are inversely correlated with the risk of CAD.

Both genetic and environmental factors influence plasma HDLC and apoA1 levels (1, 2). There is evidence to link polymorphisms in the apoA1 gene to CAD prevalence and incidence (3, 4). Population and clinical studies suggest a major locus involved in the control of plasma HDLC and apoA1 (5–8). Recently, however, three groups reported that Tangier disease, a rare recessive disorder characterized by an extremely low HDLC and an apoA1 level of only 2–3% of normal range, is caused by mutations in the ATP-binding cassette transporter1 gene (9–11).

The gene for apoA1 is located on the long arm of chromosome 11 together with the apolipoprotein C-3 and apolipoprotein A-4 genes (12, 13). A common polymorphism described in the apoA1 promoter region consists of a *G* to *A* substitution at the 75 bp upstream of the transcription start site. Many studies have found consistent association of this polymorphism with HDLC levels in several populations (14–21) but not in others (22–24). ApoA1 production rate was shown to be significantly lower in *G/A* human subjects than *GG* homozygotes (22). Lower promoter activity (22) and expression rate (22, 25) were observed *in vitro* with the *A* allele. However, another study found that the *A* allele significantly decreased the binding affinity of a nuclear factor that enhanced the transcription efficiency of the promoter (26). In a meta-analysis (27) comprising over 3000 healthy subjects, the rare allele *A* was associated with mildly increased apoA1 levels of about 5 mg/dL.

Recently, another *Msp*I polymorphic site was identified in the first intron of apoA1 gene, creating a *C-T* and/or *G-A* transition at the +83 bp and/or +84 bp (28). These sequence variations were shown to be associated with increased HDLC levels (29, 30). Both the −75 bp and +83 bp sites were

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significantly predictive of CAD severity in terms of the number of diseased vessels in a case-control study (31). There was another report of higher *A* allele frequency in myocardial infarction patients relative to healthy control subjects (32) but not for alleles at the +83 bp.

Association studies of apoA1 polymorphisms with lipid quantitative traits have thus far been carried out in adult populations. Such studies had to take into consideration the confounding nongenetic factors such as effects of smoking, diet, and medication on lipid profiles of the subjects. To be free from the confounding effects of the above environmental factors, we undertook this study with neonatal cord blood samples from the Chinese, Malay, and Asian Indian populations of Singapore. The Asian Indians will henceforth be referred to as Indians. As cord plasma lipid levels have yet to be influenced by any known environmental factors, any genotype-specific association of the apoA1 promoter gene polymorphisms with cord plasma lipid levels would reflect the extent of genetic control at birth.

MATERIALS AND METHODS

Study subjects. A sample of 1076 cord blood obtained from normal newborns of Chinese, Malay, and Indian heritage were studied. Participating babies had no history of mixed heritage in the preceding three generations. Verbal informed consent was obtained from parents of the newborns for inclusion of their child for this study. The details of sample collection and methods of lipid and lipoprotein level estimation were as reported in our earlier studies (33, 34). This study was approved by the National Medical Research Council of Singapore.

Isolation of DNA and genotyping of ApoA1 polymorphisms. Genomic DNA was extracted from leukocytes obtained from the cord blood samples by the sarcosine method (35). The ApoA1 polymorphisms were identified by PCR. A 433-bp fragment in the 5' region of the ApoA1 gene was amplified by using the following primers: sense, 5' AGG GAC AGA GCT GAT CCT TGA ACT CTT AAG 3' and antisense, 5' TTA GGG GAC ACC TAG CCC TCA GGA AGA GCA 3' (21). PCR was performed in a volume of 20 μL containing 1–10 μg of genomic DNA, 20 pmol of each primer, 0.5 mM of each deoxyribonucleoside triphosphate (dNTP), reaction buffer, 2% DMSO, and 1.0 U of *Taq* DNA polymerase. Amplified products were digested with 10 U of *Msp*I at 37°C overnight and the digested fragments were size-fractionated in 2% NuSieve (BioWhittaker, Rockland, ME, U.S.A.) agarose gel with incorporated ethidium bromide and photographed over a UV transilluminator.

The presence of the restriction site at −75 bp (*G* allele) and at +83 bp (*C* allele) resulted in four fragments of 209 bp, 113 bp, 66 bp, and 45 bp. The absence of the restriction site at −75 bp (*A* allele) resulted in three fragments of 209 bp, 179 bp, and 45 bp. The absence of the restriction site at +8 3bp (*T* allele) created a larger fragment of 254 bp instead of two fragments of 209 bp and 45 bp.

Statistical analysis. Statistical analysis was performed using SPSS for Windows (release 9, SPSS, Inc., Chicago, IL, U.S.A.). The significance of differences for the means of plasma total cholesterol (TC), triglycerides (TG), HDLC, LDL cholesterol (LDLC), apoA1, apolipoprotein B (apoB), and Lp(a) levels between races and gender were determined by the *t* test or ANOVA as appropriate. Allelic frequencies of the apoA1 gene polymorphisms were estimated by gene counting and 95% confidence intervals by the following formula: frequency $\pm$ 1.96 $[p(1 - p)/n]^{1/2}$ where p is the allele frequency and n is the total number of chromosomes. The χ^2 analysis was used to test for Hardy-Weinberg equilibrium and the z test for differences in allele frequencies between groups. Test of independence between the two apoA1 polymorphic sites was also estimated by the χ^2 statistic, and the strength of their associations (linkage disequilibrium) was determined by the correlation coefficient, Δ, according to Chakravarti *et al.* (36). ANOVA was performed to determine the effects of the apoA1 gene polymorphisms on the various lipid traits in the different ethnic groups after adjustment for significant covariates. The significance of the sample variance was tested by F and p values and percentage of explained variance ($R^2 \times 100$) was calculated from the sum of squares. Due to skewed distribution, the raw data for TG and Lp(a) were transformed by natural logarithm before comparison by *t* test and ANOVA. Because the raw values of triglycerides and Lp(a) in cord blood specimens were small, with most values below 1, and as the ln of <1 is negative, logarithmic transformation for these parameters were carried out following addition of 1 to the raw values. Statistical significance was taken at the 0.05 level.

RESULTS

The genotype and allelic frequencies of both the apoA1 polymorphisms in the three ethnic groups are presented in Table 1. Genotype distributions were all consistent with a population at Hardy-Weinberg equilibrium except for the +83 bp site in the Indians. The marginal deviation was due to the presence of one rare homozygous *TT* subject. It was not likely that any of the assumptions for Hardy-Weinberg equilibrium were violated, and we attribute such departure from equilibrium to chance. The allele frequency of *A* at −75 bp in the Indians was significantly lower than both the Chinese and the Malays, whereas the *T* allele at +83 bp was significantly lower than the Chinese only. The two sites were not in linkage disequilibrium for all ethnic groups. Details of linkage disequilibrium tests are given in Table 2.

Cord plasma Lp(a) levels were not significantly different between genders in all the three ethnic groups. However, correlations of Lp(a) levels with other lipid traits were gender-specific. Spearman's correlation test showed Lp(a) levels to be weakly but significantly correlated with TC only in the females of all ethnic groups. Significant correlations with HDLC were also relatively stronger in the females compared with the males. In the Chinese only, Lp(a) levels were also significantly correlated with LDLC and TG in the females, and apoA1 in both genders. In the Indians, Lp(a) levels were only significantly correlated with gestational period in the females. The Spearman's correlation coefficients for each subgroup are presented in Table 3. Traits that were significantly correlated with

Table 1. *Genotype and allele frequencies of the two 5′ end polymorphisms at the −75 and +83 site in the neonates of three ethnic groups of Singapore*

−75 bp Promoter polymorphism				+83 bp Promoter polymorphism			
Genotypes	Chinese	Malays	Indians	Genotypes	Chinese	Malays	Indians
GG	223	137	200	*CC*	429	266	312
GA	209	114	113	*CT*	38	17	13
AA	35	32	13	*TT*	0	0	1
Total	467	283	326	Total	467	283	326
χ^2	2.17	1.22	0.36	χ^2	0.84	0.27	4.16
p	NS	NS	NS	*p*	NS	NS	<0.05
Allele *G*	0.70	0.69	0.79	Allele *C*	0.96	0.97	0.98
(95% CI)	(0.67–0.73)	(0.65–0.72)	(0.76–0.82)	(95% CI)	(0.95–0.97)	(0.96–0.98)	(0.97–0.99)
Allele *A*	0.30	0.31	0.21*†	Allele *T*	0.04	0.03	0.02‡
(95% CI)	(0.27–0.33)	(0.28–0.35)	(0.18–0.24)	(95% CI)	(0.03–0.05)	(0.02–0.04)	(0.01–0.03)

Significantly different from * Chinese ($p < 0.0001$), † Malays ($p < 0.0001$), and ‡ Chinese ($p < 0.05$) by *z* test.
CI, confidence interval.

Table 2. *Linkage disequilibrium between the −75 bp and +83 bp polymorphic sites*

	Chinese	Malays	Indians
χ^2	1.29	0.76	1.27
p	0.52	0.68	0.87
Δ	−0.01	0.02	0.01

Lp(a) levels were incorporated into the respective ANOVA as covariates to eliminate their confounding effects on Lp(a) levels when determining the influence of the polymorphic sites.

Among the lipid factors, only Lp(a) was found to be significantly associated with the two polymorphisms studied (Table 4). Nonsignificant results other than Lp(a) were not shown. The significant effect of both −75 bp and +83 bp sites was ethnicity specific for Indians only. Significantly lower plasma Lp(a) levels were found in *AA* and *CC* homozygotes. When analysis was carried out separately on the genders, we found that the effects of both −75 bp and +83 bp polymorphisms were confined to Indian male neonates only, whereas effects on the Chinese and Malays remained insignificant for both genders.

To examine the combined effects of both polymorphic sites, we used groupings based on composite genotypes. They were significantly associated with Lp(a) levels ($F = 6.4$, $p < 0.0005$) in the male Indian neonates. Consistent with the effect of individual polymorphisms, subjects bearing the composite genotype of *AA/CC* had the lowest Lp(a), whereas subjects who carried the *T* allele had higher Lp(a) level. Together, both polymorphisms could explain 14% of the total Lp(a) variation. The effects from the two sites appeared to be additive, as each could explain an equal proportion of 7%. The composite genotype and their corresponding means of transformed Lp(a) are also presented in Table 4. In a two-way ANOVA using both sites as independent variables, the interaction term was not significant. This indicates that their effects on Lp(a) levels were independent of each other. This is also supported by the lack of linkage disequilibrium between the two sites.

DISCUSSION

The allele frequencies of the two polymorphic sites in the Asian populations showed significant variations among the ethnic groups. In comparison with Caucasian frequencies, the Malays had the highest frequency, 0.3, for allele *A*, whereas the Indians had a frequency of 0.2. This was closer to the Caucasians, which range from 0.01 to 0.19 (14, 16, 21, 29, 37) and 0.1 in African blacks from Nigeria (38).

In accordance with previous reports (14–16, 21), the *A* allele in our study was associated with higher HDLC and/or apoA1 levels in the Chinese neonates, although the difference did not reach a statistically significant level (results not shown). An opposite trend was observed in the Malays and Indians, but it did not attain statistical significance.

Because the +83 bp polymorphism was described later than the −75 bp, relatively fewer reports on its effect are available. The rare *T* allele frequency in our study (0.02–0.04) was close to those that were reported for two Caucasian populations with 0.03 - 0.04 (29, 30) but much lower than the African population of 0.4 (38). Differing from the results reported Caucasians (29, 30), we did not find linkage disequilibrium between the two sites. There is also a lack of association of both polymorphic sites with HDLC and ApoA1 levels in our neonate population.

In this study, we found the effects of both the apoA1 polymorphisms on Lp(a) levels to be ethnicity specific to Indians and gender specific to male neonates only. Male-specific effect of the polymorphism was observed in other studies with European and Icelandic populations (14, 16) and in our earlier study on Singaporean Chinese adults (17). However, in Italian adults (15), French Canadians (39), and Europeans from 12 countries (20), the *A* allele was associated with higher HDLC/ApoA1 in women. It is interesting to note that gender-specific effects of the apoA1 genotypes could already be observed at birth. The possible reasons for such phenomenon has not been elucidated, but the effect of sex hormones would be the likely cause since they are known to affect Lp(a) levels in adults (40, 41), although not without controversies (42, 43). In our neonatal subjects, there was no significant difference between Lp(a) levels of male and female newborns.

Although there have been many reports on the roles of apoA1 containing lipoproteins and Lp(a) in atherosclerosis, only one had demonstrated the relationship of these two factors. Liu *et al.* (44) reported the protective effect of apoA1 on

Table 3. *Spearman's correlation coefficient (r) of Lp(a) levels with relevant parameters*

	Chinese				Malays				Indians			
	Males		Females		Males		Females		Males		Females	
	r	*p*	*r*	*P*	*r*	*P*	*r*	*p*	*r*	*P*	*r*	*P*
Gestational period	0.07	NS	0.03	NS	0.07	NS	0.06	NS	0.15	NS	0.25	0.003
Weight	0.02	NS	0.12	NS	−0.08	NS	0.14	NS	0.03	NS	0.08	NS
TC	0.14	NS	0.31	<0.0005	0.17	NS	0.30	0.001	0.14	NS	0.20	0.022
HDLC	0.24	0.001	0.28	<0.0005	0.19	0.04	0.32	0.001	0.16	0.046	0.23	0.008
LDLC	0.03	NS	0.18	0.01	0.11	NS	0.17	NS	0.07	NS	0.13	NS
TG	0.05	NS	0.22	0.03	0.17	NS	0.02	NS	0.09	NS	0.09	NS
ApoA1	0.21	0.006	0.24	0.002	0.14	NS	0.01	NS	0.12	NS	0.10	NS
ApoB	0.13	NS	0.15	NS	0.03	NS	0.06	NS	0.10	NS	0.07	NS

Table 4. *Mean genotypic ln[Lp(a) + 1] levels in the three ethnic groups*

		Chinese		Malays		Indians				
		All		All		Males			Females	
		n	Mean ± SD	*n*	Mean ± SD	*n*	Mean ± SD		*n*	Mean ± SD
−75 bp	*GG*	184	0.77 ± 0.42	109	0.89 ± 0.44	89	0.90 ± 0.45	$F = 6.1$	88	0.94 ± 0.54
	GA	159	0.75 ± 0.42 NS	90	0.81 ± 0.35 NS	56	1.02 ± 0.59	$p = 0.003$	40	0.87 ± 0.39 NS
	AA	29	0.79 ± 0.41	26	0.75 ± 0.39	5	0.31 ± 0.52	$R^2 \times 100 = 6.9$	5	0.58 ± 0.44
+83 bp	*CC*	346	0.77 ± 0.42	214	0.84 ± 0.39	147	0.90 ± 0.49	$F = 12.7$	124	0.89 ± 0.46
	CT	26	0.67 ± 0.43 NS	11	0.89 ± 0.53 NS	3	2.06 ± 0.44	$p < 0.0005$	8	1.09 ± 0.93 NS
	TT	0	—	0	—	0	—	$R^2 \times 100 = 7.2$	1	1.57
−75/+83 bp	*GG/TT*		—	—		—	—		1	1.57
	GG/CT	16	0.73 ± 0.46	7	0.99 ± 0.62	2	1.81 ± 0.88		5	1.07 ± 1.19
	GG/CC	168	0.77 ± 0.42	102	0.89 ± 0.42	87	0.88 ± 0.44	$F = 6.4$	82	0.93 ± 0.49
	GA/CT	8	0.59 ± 0.40 NS	4	0.72 ± 0.34 NS	1	2.57	$p < 0.0005$	3	1.14 ± 0.44 NS
	GA/CC	151	0.76 ± 0.42	86	0.82 ± 0.36	55	0.99 ± 0.56	$R^2 \times 100 = 13.7$	37	0.85 ± 0.39
	AA/CT	2	0.45 ± 0.27	—	—	—	—		—	—
	AA/CC	27	0.82 ± 0.41	26	0.75 ± 0.39	5	0.31 ± 0.20		5	0.58 ± 0.44

Mean: ln[Lp(a) + 1]

atherosclerosis associated with apo(a) in transgenic mice. Their study showed that mice with the apo(a) transgene that were fed with atherogenic diet had the largest mean lesion area, whereas those with the apoA1 transgene were protected from this effect.

In our study, we found that the two polymorphisms in the apoA1 gene had effect neither on plasma HDLC nor apoA1 levels in all neonates of the three ethnic groups. However, we demonstrated significant associations of the two polymorphic sites with Lp(a) levels. This is the first study on neonatal population to report such a relationship between the big "A" and small "a." We had earlier reported Lp(a) levels at birth in the three ethnic groups in Singapore and showed correlation of their levels with the mortality rate of their respective ethnic groups (34). This indicates that an individual's level of Lp(a) was already determined at birth genetically. Our recent work on apolipoprotein(a) [apo(a)] gene polymorphisms, which included those at the kringle-4, pentanucleotide and the +93bp sites, had shown that these polymorphisms could explain 17% of the total variation in Lp(a) levels at birth (45). When we included the sum of kringle-4 repeats, pentanucleotide and the +93 bp sites as covariates in the ANOVA model, the effects of both apoA1 genotypes on Lp(a) remained significant (unpublished data), indicating that the associations were independent of apo(a) genotypes. We found in this study that the apoA1 gene could account for nearly as much (14%) variations in Lp(a) level.

As Scanu (46) pointed out earlier, it is of interest that the characteristics of the apoA1 and the apo(a) proteins, so diverse in nature, could have such influence on each other. At this point, our population study was only able to observe the association of the apoA1 polymorphic sites with Lp(a) levels, but explanation for the mechanism underlying this effect would have to come from future molecular studies of the interactions between these two genes and proteins.

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