Vol. 209, No. 1, 1995 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
April 6, 1995 Pages 372-378

DETECTION OF POLYMORPHISMS IN THE 5'-FLANKING REGION OF THE GENE FOR APOLIPOPROTEIN(a)

Akitada Ichinose* and Masaru Kuriyama#

Department of Molecular Pathological Biochemistry, Yamagata University School of Medicine, Yamagata 990-23, Japan

#Department of Internal Medicine III, Kagoshima University School of Medicine, Kagoshima 890, Japan

Received March 4, 1995

Summary. Concentration of lipoprotein(a) in plasma is inherited in an autosomal co-dominant manner and its high concentration leads to atherothrombotic disease. Nucleotide sequence analysis of the apolipoprotein(a) gene revealed the presence of polymorphisms in its 5'-flanking region, which can be analyzed by *in vitro* amplification employing gene-specific primers. Genomic DNAs from normal individuals have been subclassified into four alleles according to the patterns of restriction digestion. The ratios of these subtypes differed between Caucasians and Japanese and in patients with myocardial infarction. It is very likely that the 5'-alleles in part control the plasma lipoprotein(a) level.

Apolipoprotein(a) [apo(a)] is a glycoprotein of 300-700 kD in lipoprotein(a) [Lp(a)] (1-4). Concentrations of Lp(a) in plasma vary among individuals from <1.0 to >150 mg/dl over a range of 1,000-fold. A population with a higher plasma Lp(a) level than 25-30 mg/dl has a higher incidence of atherothrombotic disease, such as acute myocardial infarction and cerebral infarction (5,6). Although the plasma Lp(a) concentration roughly correlates with the size and number of kringle 4 repeats of apo(a) (7,8), a significant variation in the Lp(a) level exists among cases having the same isoform (9,10). Several lines of evidence indicate that the plasma concentration of Lp(a) is determined by the rate of its synthesis (11-13) and is related to amounts of apo(a) mRNA in liver (14,15).

*Address correspondence to Akitada Ichinose, M.D., Ph.D., at Yamagata University. Fax: 81-236-24-4534.

0006-291X/95 $5.00

Differences in the nucleotide sequence of the 5'-flanking region of the apo(a) gene were characterized among individuals in the present study to explore the control mechanism of its expression.

MATERIALS AND METHODS

Venous blood was drawn after informed consent had been obtained from normal Japanese individuals and patients with myocardial or cerebral infarction. Genomic DNA samples were prepared from the leukocytes by a standard technique. PCRable genomic DNAs of normal American individuals were also purchased from BIOS Lab. (New Haven, CT).

0.1-1.0 μg of a genomic DNA was amplified in a 50 μl reaction mixture as described (16) employing 2.5 units of *Thermus aquaticus* DNA polymerase (Taq polymerase, StrataGene). After 30 cycles of amplification, nine μl of each reaction mixture was applied to a 0.8% agarose (International Biotechnologies Inc.) or 2% NuSieve and 1% Seakem (FMC BioProducts) gel.

Oligonucleotides were prepared with an Applied Biosystems' synthesizer. Genomic DNAs were amplified by employing primers designed from the genomic sequences for apo(a) (17,18); for amplification and sequencing to detect polymorphisms in the 5'-flanking region, 5' side-CTTGAATTCCCAAAGTGCTGGGATTACAGAG (A2-53; underlined nucleotides are identical to those of the apo(a) gene) & 3' side-TAAGGATCCGGCATATGTATTTTTACTACATTGTGGGAG (A5FL-3); for amplification and digestion of the 1.2kb fragment with *Taq*I and *Mae*II, 5' side-CTTGAATTCCCAAAGTGCTGGGATTACAGAG (A2-53) & 3' side-CCATTTTGGGACTGGCCAGCAG*C*G (A2-331, the C in italic replaces the authentic T in order to introduce a *Hha*I site); for amplification and digestion of the 228 bp fragment with *Mae*II and *Hha*I, 5' side-CCAGGATCCAGCATCTATTGACATTGCACT (A2-511) & 3' side-TTAGAATTCATTTTGGGACTGGCCAGCAG*C*G (A2-33). In order to classify the 5'-flanking region of the apo(a) gene, the amplified DNAs by A2-53 and A5FL-3 were digested first with *Taq*I at 65°C, then with *Mae*II at 50°C. The digested DNAs were re-amplified by A2-53 and A2-331, then by A2-511 and A2-33. The amplified DNAs were digested with *Mae*II at 50°C and/or *Hha*I at 37°C for 1.5 hrs.

RESULTS AND DISCUSSION

Since the plasma concentration of apo(a) is co-dominantly inherited (19), differences in its genomic sequence must exist among individuals. It is suggested that there are *cis*-acting sequences at the apo(a) locus, which would account for the variation in plasma Lp(a) levels (20), that is, apo(a) levels. Indeed, nucleotide polymorphisms have been discovered in the 5'-flanking region of the apo(a) gene (17). Nucleotide sequences of approximately 10 ssDNAs obtained from each of 10 individuals were found to be identical except for those at four positions: G or A, A or G, C or T, and

G or A at positions -914, -103, -49, and -21, respectively. The nucleotide substitution at position -914 has been also reported by Wade et al. (21).

The presence of these four nucleotide differences can be detected by cleavage of amplified DNA products with *Taq*I, *Mae*II, *Alu*I, and *Hha*I endonucleases, respectively (Figure 1). Digestion with *Alu*I endonuclease, however, was omitted from further analysis since the *Alu*I polymorphism turned out to be extremely rare (none in an additional 40 individuals).

Each of ssDNAs prepared by subcloning of the 10 individuals' amplified DNAs contained either G-C-G, A-C-G, A-C-A, or A-T-G as listed in Table 1. Thus, the amplified DNAs were digested by *Taq*I, *Mae*II, and *Hha*I, and classified into four 5'-alleles, which can be determined by a combination of the presence (+) or absence (-) of three restriction sites: type A, +++; type B, -++, type C, -+-; type D, --+ (+, cleaved; -, not cleaved) as described in the methods section. This procedure was successfully applied to genomic DNAs obtained from individuals. These three polymorphic sites in the 5'-flanking region were consistently co-inherited in two families of three generations (Figure 2, the other pedigree is not shown), confirming complete linkage of these polymorphic sites.

When 66 DNA samples (132 chromosomes) obtained from unrelated Caucasian individuals were analyzed by the present method, type A was found in more than half of the chromosomes tested (Figure 3A). Three other types, B, C, and D, shared one-third each of the remaining half. Analysis of 58 DNA samples (116 chromosomes) obtained from unrelated

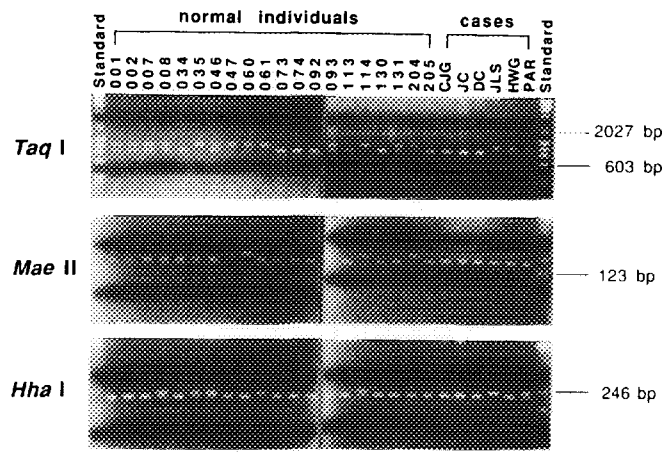

Figure 1. Restriction digestion with endonucleases of the 5'-flanking region. The amplified DNA fragments were digested with *Taq*I, *Mae*II, or *Hha*I and applied to a 0.8% agarose gel (Top) or a 2% NuSieve and 1% Seakem (Middle and Bottom) gel.

Table 1: Classification of 5'-flanking region

case	1st allele			2nd allele			Restriction sites			5'-allele	type
	-914	-49	-21;	-914	-49	-21	*TaqI*	*MaeII*	*HhaI*		
1	G	C	G;	G	C	G	+,+	+,+	+,+	+++,+++	A,A
2	G	C	G;	G	C	G	+,+	+,+	+,+	+++,+++	A,A
3	G	C	G	A	C	A;	+,-	+,+	+,-	+++,-+-	A,C
4	A	C	A;	A	T	G	-,-	+,-	+,-	--+,-+-	C,D
5	G	C	G;	G	C	G	+,+	+,+	+,+	+++,+++	A,A
6	G	C	G;	G	C	G	+,+	+,+	+,+	+++,+++	A,A
7	A	C	A;	A	C	A	-,-	+,+	-,-	-+-,-+-	C,C
8	G	C	G	A	C	G;	-,+	+,+	+,+	+++,-++	A,B
9	G	C	G;	A	C	A	+,-	+,+	+,-	+++,-+-	A,C
10	A	T	G;	A	T	G	-,-	-,-	+,+	--+,--+	D,D

Japanese individuals revealed that the ratios of the four subtypes in the Japanese were quite different from those of the Caucasians (Figure 3B); type A was about half, types C and D were more than twice of the Caucasians, and type B was not found. The differences in the nucleotide sequence of the 5'-regulatory region may lead to differential transcriptional efficiency, which in turn results in a wide variety of plasma Lp(a) levels, not only among individuals but also between ethnic groups

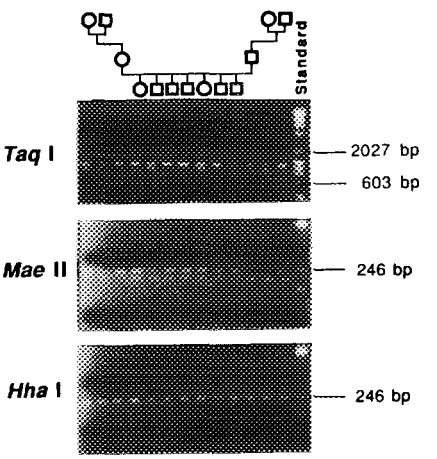

Figure 2. Linkage analysis of the 5'-alleles. One of the two 5'-alleles in children is transmitted from one parent and the remaining 5'-allele from the other parent.

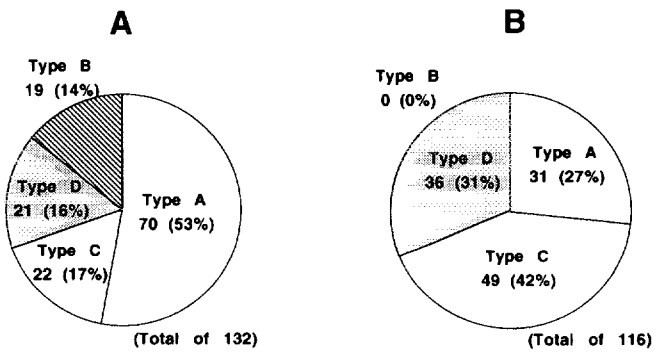

Figure 3. Ratio of each type of the 5'-flanking region among normal American (A) and Japanese (B) individuals.

(2,22). This hypothesis was confirmed by the CAT (chloramphenicol acethyltransferase) assay when each of the four types of 5'-alleles had been inserted into a promoterless CAT vector and its transcriptional activity had been compared directly (Suzuki and Ichinose, manuscript in preparation).

DNA samples of Japanese patients with myocardial and cerebral infarction were also examined by this genetic diagnostic method. The ratios of 5'-alleles in 68 patients with cerebral infarction were essentially the same as those of normal individuals (Figure 4A). In contrast, the ratios of 5'-alleles in 50 patients with myocardial infarction differed from those of normal individuals (Figure 4B): type A was about 1.5-fold and type D two thirds of normal, and two cases had type B. A new type, designated as type E (G-T-G and +-+), was also found in one case. The clinical significance of these nucleotide polymorphisms, however, remains to be elucidated.

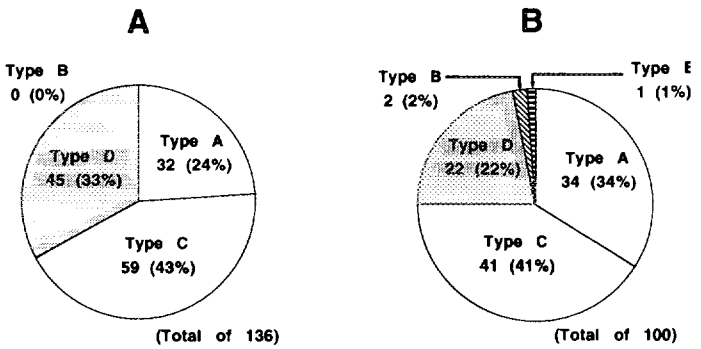

Figure 4. Ratio of each type of the 5'-flanking region among Japanese patients with cerebral (A) and myocardial (B) infarction.

Determination of both 5'-alleles and the number of kringle 4 repeats [or isoform of apo(a)] will make it possible to predict the plasma apo(a) level of every individual in the near future.

ACKNOWLEDGMENTS

This paper was presented in part at the XIth ICF meeting in Copenhagen (1992), the XIIIth ICTH meeting in New York (1993), and the XIIth ICF meeting in Leuven (1994). The authors thank Prof. E. W. Davie for his support in the initial period of this study, Drs. T. Hashiguchi, S. Tsutsumi, K. Suzuki and N. Takabatake (cerebral infarction), and Dr. K. Takahashi and Prof. H. Tomoike (myocardial infarction) for their help in preparing genomic DNAs, and L. Boba for her help in the preparation of the manuscript. This work was supported by research grants from the NIH USA (HL 16919), Yamagata University, the Ministry of Education, Science and Culture, Japan (05454327), Yamagata Prefecture, Ono Medical Research Foundation, CIBA-GEIGY Foundation for the Promotion of Science, and T. Nanba Memorial Health Care Foundation.

REFERENCES

1. Berg, K. (1963) Acta Pathol. Microbiol. Scand. 59, 369-382.
2. Utermann, G. (1989) Science 246, 904-910.
3. Scanu, A. M., and Fless, G. M. (1990) J. Clin. Invest. 85, 1709-1715.
4. Miles, L. A., and Plow, E. F. (1990) Thromb. Haemostas. 63, 331-335.
5. Armstrong, V. W., Cremer, P., Eberle, E., Manke, A., Schulze, F., Wieland, H., Kreuzer, H., and Seidel, D. (1986) Atherosclerosis 62, 249-257.
6. Zenker, G., Koltringer, P., Bone, G., Niederkorn, G., Pfeiffer, K., and Jurgens, G. (1986) Stroke 17, 942-945.
7. Gavish, D., Azrolan, N., and Breslow, J. L. (1989) J. Clin. Invest. 84, 2021-2027.
8. Lackner, C., Boerwinkle, E., Leffert, C. C., Rahmig, T., and Hobbs, H. H. (1991) J. Clin. Invest. 87, 2153-2161.
9. Kraft, H. G., Kochl, S., Menzel, H. J., Sandholzer, C., and Utermann, G. (1992) Hum. Genet. 90, 220-230.
10. Perombelon, Y. F. N., Soutar, A. K., and Knight, B. L. (1994) J. Clin. Invest. 93, 1481-1492.
11. Krempeler, F., Kostner, G. M., and Bolzano, K. (1980) J. Clin. Invest. 65, 1483-1490.
12. Rader, D. J., Cain, W., Zech, L. A., Usher, D., and Brewer, H. B. (1993) J. Clin. Invest. 91, 443-447.
13. Rader, D. J., Cain, W., Ikewaki, K., Tally, G., Zech, L. A., Usher, D., and Brewer, H. B. (1994) J. Clin. Invest. 93, 2758-2763.
14. Koschinsky, M. L., Beisiegel, U., Henne-Bruns, D., Eaton, D. L., and Lawn, R. M. (1990) Biochemistry 29, 640-644.
15. Azrolan, N., Gavish, D., and Breslow, J. L. (1991) J. Biol. Chem. 266, 13866-13872.

16. Saiki, R. K., Gelfand, D. H., Stoffel, S., Scharf, S. J., Higuchi, R., Horn, G. T., Mullis, K. B., and Erlich, H. A. (1988) Science 239, 487-491.
17. Ichinose, A. (1992) Biochemistry 31, 3113-3118.
18. Ichinose, A. (1995) Biochem. Biophys. Res. Commun. 209, 365-371.
19. Utermann, G., Menzel, H. J., Kraft, H. G., Duba, H. C., Kemmler, H. G., and Seitz, C. (1987) J. Clin. Invest. 80, 458-465.
20. Boerwinkle, E., Leffert, C. C., Lin, J., Lackner, C., Chiesa, G., and Hobbs, H. H. (1992) J. Clin. Invest. 90, 52-60.
21. Wade, D. P., Clarke, J. G., Lindahl, G. E., Liu, A. C., Zysow, B. R., Meer, K., Schwartz, K., and Lawn, R. M. (1993) Proc. Natl. Acad. Sci. USA 90, 1369-1373.
22. Gaw, A., Boerwinkle, E., Cohen, J. C., and Hobbs, H. H. (1994) J. Clin. Invest. 93, 2526-2534.