Characterization of a new human apolipoprotein A-I Yame by direct sequencing of polymerase chain reaction-amplified DNA

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Abstract A new genetic variant apolipoprotein (apo), A-I Yame, was discovered during screening for apoA-I genetic variants with isoelectric focusing gel electrophoresis. To investigate the structural abnormality of apoA-I Yame, we amplified the apoA-I gene isolated from the proband's peripheral blood leukocytes with the polymerase chain reaction (PCR) method and directly sequenced these PCR fragments. ApoA-I Yame was found to have aspartic acid (GAC) substituted by tyrosine (TAC) at residue 13. We also identified this substitution by an automated DNA sequencer. This substitution was confirmed with amino acid sequencing of the isolated apoA-I Yame by Immobiline gel electrophoresis. This combined method, direct PCR from genomic DNA-derived individual peripheral blood leukocytes and subsequent direct sequencing, can be used to identify the entire sequence of apoA-I in a short period of time. Furthermore, with this method, it is possible to identify both alleles in heterozygous individuals. —**Takada, Y., J. Sasaki, M. Seki, S. Ogata, Y. Teranishi, and K. Arakawa.** Characterization of a new human apolipoprotein A-I Yame by direct sequencing of polymerase chain reaction-amplified DNA. *J. Lipid Res.* 1991. **32:** 1275–1280.

Supplementary key words apoA-I • HDL • apoA-I gene

Apolipoprotein (apo) A-I is the major apolipoprotein among high density lipoproteins (HDL) and is a single polypeptide chain composed of 243 amino acid residues (M_r 28,000) (1, 2). ApoA-I plays an important role in nascent HDL to promote cholesterol efflux from tissues. Subsequently, the mature HDL is catabolized through HDL receptors (3). ApoA-I is also a cofactor of lecithin:cholesterol acyltransferase, which is responsible for esterification of almost all plasma cholesterol (4). Recently, there have been reports on apoA-I mutants, with and without altered apoA-I levels, and their metabolism (5–9).

To further understand the apoA-I structure and function relationship, we screened apoA-I variants by isoelectric focusing gel electrophoresis. Here, we report a new human apolipoprotein variant apoA-I Yame, characterized by combined polymerase chain reaction (PCR) and direct DNA sequencing.

MATERIALS AND METHODS

Subject

The proband of apoA-I Yame was a 54-year-old female. She was found to have apoA-I Yame during the screening of samples at an annual health check-up. Neither her three children nor one sister had the apoA-I Yame. Her total serum cholesterol level was 272 mg/dl, triglyceride 249 mg/dl, HDL cholesterol 44.3 mg/dl, and apoA-I 92 mg/dl. There were no signs of atherosclerotic disease.

Materials

Acrylamide, TEMED, and N,N'-methylenebis acrylamide were purchased from Bio-Rad. Sodium decylsulfate was obtained from Eastman Kodak, and ampholytes were from LKB (Bromma, Sweden). Oligodeoxynucleotides were synthesized on an Applied Biosystems Instrument, model 380B DNA synthesizer, and purified by high pressure liquid chromatography. Deoxyribonucleotides, dideoxyribonucleotides, and Sequenase Ver.2.0 were purchased from United States Biochemical Corporation. [α-^{32}P]dATP was purchased from Amersham Corp. T4 polynucleotide kinase was from TOYOBO Co. Taq DNA polymerase and other PCR reagents were purchased from Perkin-Elmer Cetus Co.

Abbreviations: apo, apolipoprotein; HDL, high density lipoprotein; TBS, Tris-buffered saline; PCR, polymerase chain reaction; TE buffer, 10 mM Tris-HCl, 1 mM EDTA, pH 8.0.

Electrophoretic analysis

Screening isoelectric focusing gel electrophoresis was performed by either the one-step screening method or in combination with ultracentrifugation as described previously (9). Two-dimensional gel electrophoresis of serum, consisting of isoelectric focusing followed by sodium dodecylsulfate electrophoresis, was performed as described previously (9).

Immunoblots

Two-dimensional electrophoretic spots of apoA-I by immunological methods were identified by immunoblots (*Instruction for the Bio-Rad Immuno-Blot* (GAR-HRP) Assay Kit). Proteins were transferred to nitrocellulose paper at 40 V for 1 h. Nitrocellulose paper was then incubated for 16 h in 1% gelatin in TBS (20 mM Tris, 0.5 M NaCl, pH 7.5) containing a monospecific rabbit antibody to apoA-I. After incubation, the apolipoprotein was visualized by indirect immunoperoxidase assay on nitrocellulose paper according to the manufacturer's instructions (Bio-Rad).

Preparative Immobiline isoelectric focusing

For analytical purposes, the pure apoA-I isoforms were isolated by preparative Immobiline isoelectric focusing gel electrophoresis (9). One ml of apoHDL solution (10 mg/ml) was applied and electrophoresed overnight at 10°C at a constant current of 1 mA. The focused apolipoproteins were visualized by soaking the gel in water. Individual visualized bands were excised and eluted with 0.1 M Tris-HCl, pH 7.4, containing 4 M guanidine and 1 mM EDTA.

Determination of amino acid sequences

Purified samples of apoA-I (30 μg) were analyzed for amino acid sequences with an Applied Biosystems Model 477A Gas-Phase Sequencer with an on-line Model 120 A phenylthiohydantoin derivative analyzer according to instructions provided by the manufacturer.

DNA amplification by PCR

PCR was carried out essentially according to Saiki et al. (10). Genomic DNA was isolated from 100 μl of peripheral blood. The proband's peripheral blood (100 μl) was added to 400 μl of cell lysis buffer (0.32 M sucrose, 10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 1% Triton X-100), 5 μl of proteinase K (10 mg/ml), and 25 μl of 10% SDS. This solution was incubated for 60 min at 60°C and extracted with an equal volume of phenol–chloroform 1:1 saturated with TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and with chloroform. Twenty μl of this supernatant was used as the PCR template. PCR reaction was performed using a modified protocol from Perkin-Elmer Cetus Instruments. Twenty pmol of each primer was mixed in a 100-μl reaction mixture consisting of 10 μl of the 10 × reaction buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, 15 mM $MgCl_2$, 0.1% (w/v) gelatin), and 16 μl of 1.25 mM dNTP (dATP, dCTP, dTTP, dGTP). Then, the mixture was incubated at 95°C for 10 min and centrifuged briefly, and 2.5 units of Taq DNA polymerase (Cetus) was added. The mixture was sealed in 100 μl of mineral oil. The region of primer pairs 1 and 2 was PCR-amplified as follows: denaturation for 1 min at 94°C, annealing for 2 min at 60°C, extension for 1 min 30 sec at 72°C, and this heating cycle was repeated 35 times, using a Perkin-Elmer Cetus Thermocycler (model PCR 1000). To amplify the region of primer pairs 3 and 4, it was denatured for 30 sec at 96°C, annealed for 30 sec at 65°C, and extended for 1 min at 72°C in 35 cycles.

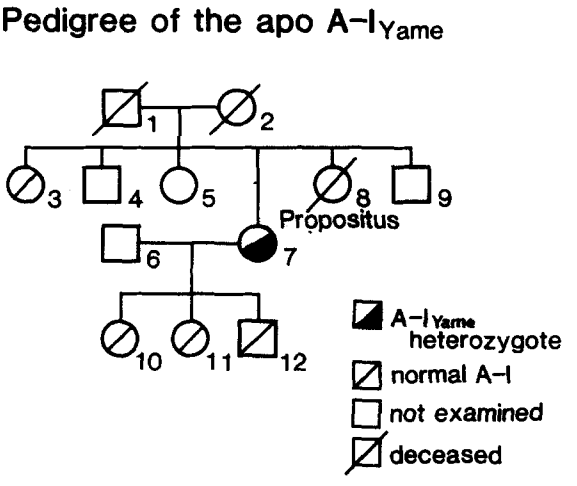


Fig. 1. Pedigree of family with apoA-I Yame.

Manual direct DNA sequencing

The manual direct sequencing of the PCR fragments was performed using a modified protocol as described by Higuchi et al. (11). The amplified DNA was purified by electrophoresis on a 5% polyacrylamide gel. The band was cut from the gel and the DNA was eluted electropho-

TABLE 1. Serum and lipoprotein lipid levels of the apoA-I Yame family

No.[a]	Subject	Age	Sex	Cholesterol Total	Cholesterol LDL	Cholesterol HDL	Triglyceride
				mg/dl			*mg/dl*
Carrier							
7	TN	54	F	272	186	44.3	249
Non-carrier							
10	HA	28	F	265	127	79.5	76
11	NE	25	F	192	89	69.0	48
12	AN	23	M	191	95	49.1	343

[a]See Fig. 1.

TABLE 2. Apolipoprotein levels of the apoA-I Yame family

No.	Subject	Age	Sex	Apolipoproteins A-I	A-II	B	C-II	C-III	E
				mg/dl					
Carrier									
7	TN	54	F	91.9	30.3	149	7.5	12.4	4.9
Non-carrier									
10	HA	28	F	146.3	39.6	93	3.2	6.1	2.9
11	NE	25	F	133.1	31.9	62	3.5	7.6	3.7
12	AN	23	M	147.4	35.8	91	8.1	16.4	4.6

retically and extracted with an equal volume of phenol–chloroform 1:1 and with chloroform. In 12 μl of TE, 0.25 pmol PCR product was mixed with 2.5 pmol of primer 5. This sequence primer had been labeled with ^{32}P using T_4 kinase (11, 12). The primer-template mixture was heated to 95°C for 5 min, and immediately placed on ice. Reactions were initiated by adding 2.8 μl of this mixture to 3.25 μl of dideoxy A, G, C, and T reaction mixtures composed of reagents provided in the sequence kit (USB) as follows: 2.5 μl of A, G, C, and T termination mixes, 0.15 μl of Sequenase, 0.38 μl of 5 × buffer, and 0.22 μl 0.1 M DTT. After incubation at 37°C for 10 min, the reaction was stopped by adding 4 μl of stop solution. This mixture was then heated to 95°C for 2 min and analyzed by standard electrophoresis and autoradiography.

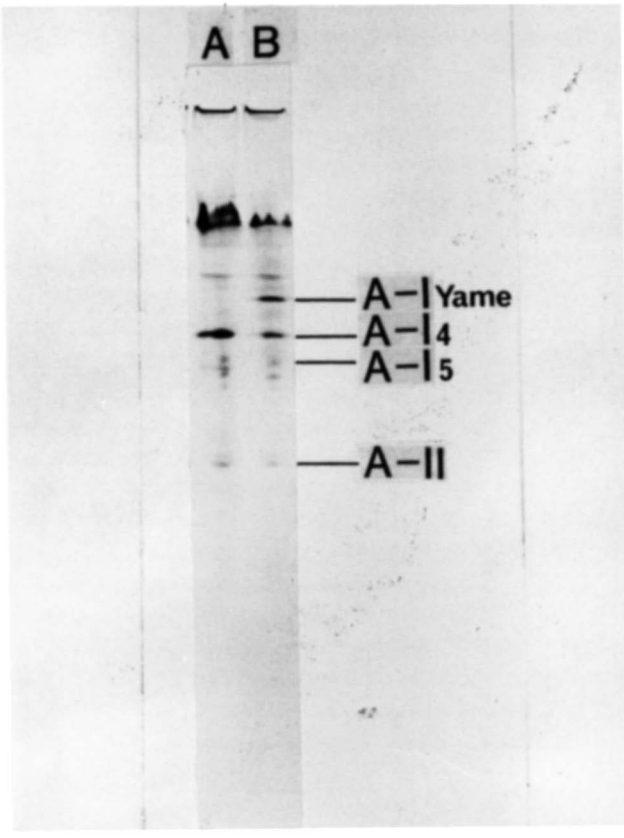


Fig. 2. Isoelectric focusing gel electrophoresis of 1 μl of the proband's serum in a pH gradient from 4 to 6. Lane A is a normal control and lane B is the apoA-I Yame variant. ApoA-I Yame has a relative charge of +1 compared to normal apoA-I_4 isoform. Isoelectric focusing gel electrophoresis was performed as described by Menzel et al. (6).

Automated direct sequence of PCR fragments

Automated direct sequence of PCR fragments was performed using a DNA sequencer GENESIS 2000 (DuPont). The amplified DNA (0.8 pmol), and 8 pmol of sequencing primer (primer 1 or 2) were suspended in 15 μl of TE. This mixture was heated to 95°C for 5 min, immediately placed on ice, and 6 μl of 5 × Sequenase™ buffer was added. The annealing reaction was conducted on ice for 30 min. Reactions were initiated by adding this mixture to 7.5 μl of reaction mixture composed of reagents provided in the Reaction Sequenase kit as follows; 2.5 μl DTT, 3.0 μl dNTP mix, 1.0 μl ddNTP fluorescent mix, and 1.0 μl T7 Sequenase. After incubation at 37°C for 5 min, unincorporated ddNTP fluorescent mix was removed with the Sephadex G-50 spin column. The column eluant was dried in a vacuum centrifuge, and the pellet was washed with 500 μl of 70% ethanol. The sample was dried in a vacuum centrifuge and redissolved in 3 μl of G-505 17 mer loading solution and denatured at 95°C for 2 min. This denatured sample was electrophoresed at a constant 20 W on an 8% polyacrylamide gel using a DNA sequencer.

RESULTS

ApoA-I Yame was only observed in the propositus; one sister and three children so far examined did not have apoA-I Yame (**Fig. 1**). Lipid and apolipoprotein levels of

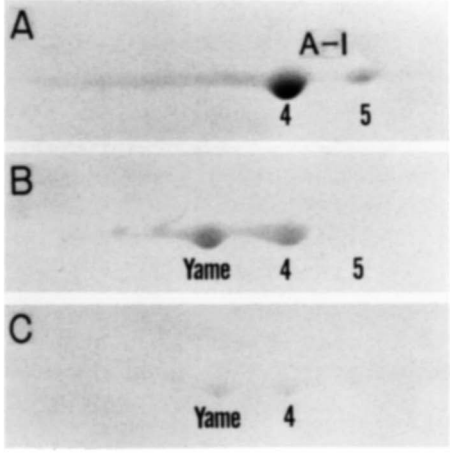
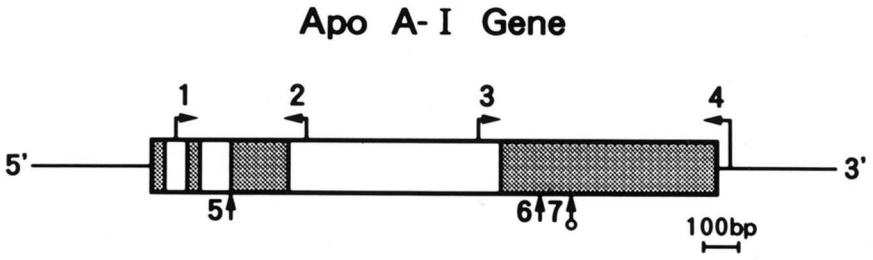


Fig. 3. Immunoblot of two-dimensional gel electrophoresis. The spot of apoA-I Yame was treated with a monospecific antibody of apoA-I. A, normal apoA-I; B, apoA-I Yame; C, immunoblot of apoA-I Yame. The basic side is on the left and acidic side is on the right.



1. (21-mer) 5'- AAG GCA CCC CAC TCA GCC AGG -3'
2. (24-mer) 5'- TTC AAC ATC ATC CCA CAG GCC TCT -3'
3. (23-mer) 5'- GTG TAC TGG AAA TGC TAG GCC AC -3'
4. (23-mer) 5'- CGT TTA TTC TGA GCA CCG GGA AG -3'
5. (17-mer) 5'- AGC CAG GCT CGG CAT TT -3'
6. (17-mer) 5'- GAG ATG AGC AAG GAT CT -3'
7. (17-mer) 5'- CAC CTT CTG GCG GTA GA -3'

Fig. 4. Oligonucleotide primers for PCR amplification and diagramatic representation of the apoA-I gene (16) with locations and directions of PCR primers indicated by arrows above bar. Arrows below the gene represent the location of primers used to sequence in the forward direction and arrows with open circles represent the location of primers used for reverse direction sequencing.

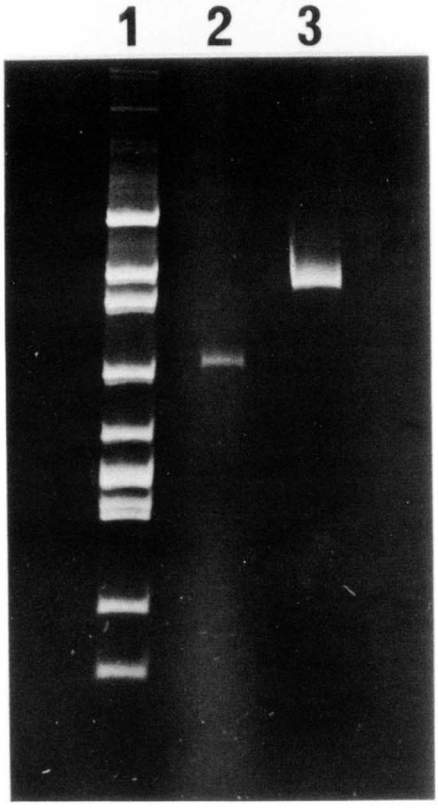


Fig. 5. Polyacrylamide gel electrophoresis of amplified apoA-I gene. Lane 1 is molecular marker *Hinc* II-digested ϕX 174. Lane 2 is the region of primer pairs 1 and 2 (508 bp) and lane 3 is the region of primer pairs 3 and 4 (762 bp).

carrier and non-carriers are shown in **Table 1** and **Table 2.** One of the non-carriers showed hypercholesterolemia and the other showed hypertriglyceridemia, indicating the presence of combined hyperlipidemia in this family. Decreased apoA-I and increased apoB levels were observed in the carrier of apoA-I Yame.

Fig. 2 shows this variant to have a relative charge of +1 compared to normal apoA-I_4, on isoelectric focusing in a pH gradient of 4 to 6. As determined by two-dimensional electrophoresis (**Fig. 3**), this band had a molecular weight identical to that of normal apoA-I and the abnormal spot was shown by immunoblot to react with a monospecific antibody to apoA-I (Fig. 2).

Fig. 4 shows the apoA-I gene with the locations and directions of PCR primers. PCR primer pairs 1 and 2 (508 bp) and 3 and 4 (762 bp) were used in amplification. Arrows below the gene (primer 5, 6, 7) represent the location of primers used to directly sequence the PCR fragments.

Fig. 5 shows the electrophoresis of the amplified products from the probands' DNA isolated from 100 μl of peripheral blood. Lane 1 shows molecular marker *Hinc* II which was digested ϕX 174. Lane 2 shows the region amplified using the primer pairs 1 and 2 (508 bp), and lane 3 shows the region amplified using the primer pairs 3 and 4 (762 bp).

Both PCR amplified nucleotides (508 bp, 762 bp) were sequenced. **Fig. 6** shows the manual direct sequence of the PCR fragment (508 bp) using primer 5. This primer was labeled with ^{32}P using T_4 kinase. The arrow shows the double band G and T. No other mutations were observed. This shows that apoA-I Yame proband is a heterozygote.

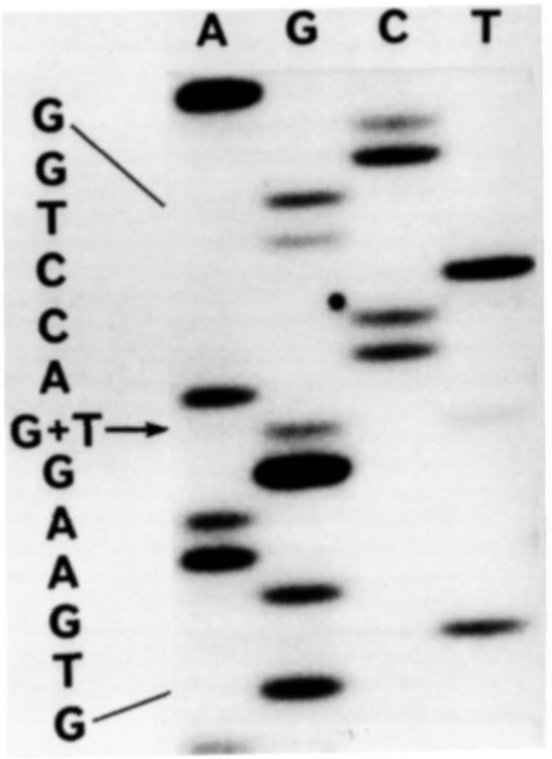


Fig. 6. Manual direct sequence of PCR fragments of apoA-I Yame heterozygote.

A one-point mutation, normal G to abnormal T, causes substitution of aspartic acid (GAC) with tyrosine (TAC) at residue 13.

The automated direct sequence of the PCR-amplified apoA-I Yame DNA was performed with a DNA sequencer (**Fig. 7**). The arrow shows the ratio to be higher than the T ratio but lower than the C ratio. Since the known nucleotide sequence at this position is G, this abnormal ratio represents the fusion wave form of G and T. We think this shear is caused by a difference in movement of the bases during electrophoresis.

The N-terminal amino acid sequence of apoA-I Yame was Asp Glu Pro Pro Gln Ser Pro Trp Asp Arg Val Lys Tyr Leu Ala Thr Val Tyr Val Asp, confirming the results of DNA analysis.

DISCUSSION

A new genetic variant of apoA-I, apoA-I Yame, was identified from the direct sequence of the PCR-amplified apoA-I Yame gene. DNA sequence analysis of apoA-I Yame revealed a G→T transversion at the codon for amino acid 13, resulting in a change from the normal aspartic acid codon GAC to tyrosine codon TAC. This mutation was confirmed by analysis of the amino acid sequence of the purified apoA-I Yame (purified by Immobiline gel).

A number of apoA-I variants have now been identified (13). Recently, Ladias et al. (14) reported the identification of a new apoA-I variant, apoA-I Baltimore (Arg10→Leu) using the polymerase chain reaction method. After restriction endonuclease analysis, they amplified 164 nucleotides around the mutant Taq I site, and subcloned the amplified product. Here, as shown in this report, we established the PCR conditions to enable amplification of the entire amino acid coded region of the apoA-I gene and directly sequenced this amplified product. Initially we tried asymmetric PCR using primer pairs 1 and 2, and pairs 3 and 4; however we could not amplify these regions,

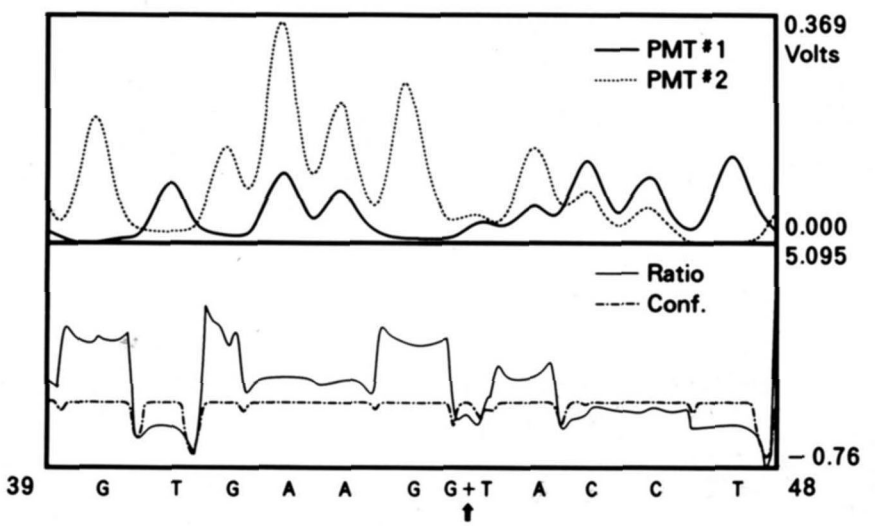


Fig. 7. Direct PCR fragment sequencing using an automated DNA sequencer (DuPont). Mutated site in apoA-I Yame at position 997 (16) is shown (arrow).

probably due to high GC content. We used a primer kinase method to sequence double-strand PCR products with a tenfold excess of primers over template and a snap-cooling method in the annealing step to prevent the fast renaturation of the template. Direct sequencing has the dual advantages of obviating the cloning of PCR products into DNA sequencing vector and eliminating the need to sequence multiple isolates in order to avoid DNA polymerase errors (15). Moreover, we performed automated direct sequencing on amplified products.

Von Eckardstein et al. (13) reported that the nonrandom distribution of amino acid substitution in human apoA-I reflects a sum of a hypervariable stretch of cytosines in the amino terminus (residues 1–10) and hypervariable CpG dinucleotides predominantly abundant in triplets coding for the protein's α-helical domain (residues 103–198). They also reported the relative distribution of charged amino acid substitutions within apoA-I subsequences. In 13 tandem apoA-I repeats, the first 3 repeats (amino acid residues 11–43) have the largest amount of potentially charged amino acid substitutions due to single base pair substitutions in the cDNA of apoA-I. However, no apoA-I variant that is a substituted charged amino acid has been reported in this region. The apoA-I Yame is the first case of substituted charged amino acid to be found in this region.

ApoA-I Yame was detected only in the proband; therefore, it is unclear whether slightly lower levels of HDL cholesterol and apoA-I with familial combined hyperlipidemia are associated with the mutation. Although the apoA-I Yame proband showed no advanced atherosclerosis, an extensive family study is needed to determine the potential contribution of this mutation to atherosclerosis.

Our overall conclusions are that the combined method of PCR amplification with subsequent direct DNA sequencing is faster and easier for characterizing the apoA-I variant. Furthermore, the present study shows the potential utility of automated direct DNA sequencing after PCR amplification.

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REFERENCES

1. Mahley, R. W., T. L. Innerarity, S. C. Rall, and K. H. Weisgraber. 1984. Plasma lipoproteins: apolipoprotein structure and function. *J. Lipid Res.* **25:** 1277–1294.
2. Brewer, H. B., T. Fairwell, A. LaRue, R. Ronan, A. Houser, and T. J. Bronzert. 1978. The amino acid sequence of human apoA-I, an apolipoprotein isolated from high density lipoproteins. *Biochem. Biophys. Res. Commun.* **80:** 623–630.
3. Stein, O., and Y. Stein. 1973. The removal of cholesterol from Landschutz ascites cells by high-density lipoprotein. *Biochim. Biophys. Acta.* **326:** 232–244.
4. Fielding, C. J., V. G. Shore, and P. E. Fielding. 1972. A protein cofactor of lecithin:cholesterol acyltransferase. *Biochem. Biophys. Res. Commun.* **46:** 1493–1498.
5. Franceschini, G., C. R. Sirtori, A. Capursc, K. H. Weisgraber, and R. W. Mahley. 1980. A-I Milano apoprotein: decreased high density lipoprotein cholesterol levels with significant lipoprotein modifications and without clinical atherosclerosis in an Italian family. *J. Clin. Invest.* **66:** 892–900.
6. Menzel, H. J., R. G. Kladetzky, and G. Assmann. 1982. One step screening method for the polymorphism of apolipoproteins A-I, A-II, and A-IV. *J. Lipid Res.* **23:** 915–922.
7. Utermann, G., G. Feussner, G. Franceschini, J. Hass, and A. Steinmetz. 1982. Genetic variants of group A apolipoproteins: rapid methods for screening and characterization without ultracentrifugation. *J. Biol. Chem.* **257:** 501–507.
8. Von Eckardstein, A., H. Funke, A. Henke, K. Altland, A. Benninghoven, and G. Assmann. 1989. Apolipoprotein A-I variants: naturally occurring substitutions of proline residues affect plasma concentration of apolipoprotein A-I. *J. Clin. Invest.* **84:** 1722–1730.
9. Takada, Y., J. Sasaki, S. Ogata, T. Nakanishi, Y. Ikehara, and K. Arakawa. 1990. Isolation and characterization of human apolipoprotein A-I Fukuoka (100 Glu→Lys). A novel apolipoprotein variant. *Biochim. Biophys. Acta.* **1043:** 169–176.
10. Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis, and H. A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science.* **239:** 487–494.
11. Higuchi, R., C. H. Von Beroldingen, G. F. Sensabaugh, and H. A. Erlich. 1988. DNA typing from single hairs. *Nature.* **332:** 543–546.
12. Scharf, S. J., G. T. Horn, and H. A. Erlich. 1986. Direct cloning and sequence analysis of enzymatically amplified genomic sequences. *Science.* **233:** 1076–1080.
13. Von Eckardstein, A., H. Funkel, M. Walter, K. Altland, A. Benninghoven, and G. Assmann. 1990. Structural analysis of human apolipoprotein A-I variants: amino acid substitutions are nonrandomly distributed throughout the apolipoprotein A-I primary structure. *J. Biol. Chem.* **265:** 8610–8617.
14. Ladias, J. A. A., P. O. Kwiterovich, Jr., H. H. Smith, S. K. Karathanasis, and S. E. Antonarakis. 1990. Apolipoprotein A-I Baltimore (Arg 10→Leu), a new apoA-I variant. *Hum. Genet.* **84:** 439–445.
15. Gibbs, R. A., P-N. Nguyen, L. J. McBride, S. M. Koepf, and C. T. Caskey. 1989. Identification of mutations leading to the Lesch-Nyhan syndrome by automated direct DNA sequencing of in vitro amplified cDNA. *Proc. Natl. Acad. Sci. USA.* **86:** 1919–1923.
16. Shoulders, C. C., A. R. Kornblihtt, B. S. Munro, and F. E. Baralle. 1983. Gene structure of human apolipoprotein A-I. *Nucleic Acids Res.* **11:** 2827–2837.