
GENETIC DISORDERS – DEVELOPMENT

Renal amyloidosis caused by a novel stop-codon mutation in the apolipoprotein A-II gene

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Background. Although apolipoprotein A-II (apoA-II) associated amyloidosis has been described in the senescent accelerated mouse (SAM) model of aging, so far there has been no report of human apoA-II amyloidosis except for a recent report of renal amyloidosis resulting from a stop-codon to glycine mutation of apoA-II. The mechanisms of amyloid formation in human apoA-II amyloidosis are not clear.

Methods. A 46-year-old Caucasian male with proteinuria noted at 42 years of age was studied. Renal biopsy revealed amyloid deposition in glomeruli. DNA analysis of genes known to be associated with hereditary renal amyloidosis revealed no abnormalities. To elucidate the type of his amyloidosis, apoA-II gene and plasma apoA-II were examined.

Results. DNA analysis revealed heterozygosity for a G to C transversion at the second position of the stop-codon of apoA-II gene, suggesting a stop to serine substitution at codon 78. Western blot analysis and amino acid sequence analysis of the patient’s plasma apoA-II showed both normal apoA-II and variant apoA-II with a 21-amino acid residue extension at the C-terminus.

Conclusions. These results indicate that the patient’s amyloid fibrils were derived from apoA-II and the amyloidogenesis is likely to be closely linked to the peptide extension at the C-terminus of variant apoA-II. The pathogenesis of human apoA-II amyloidosis is different from that of SAM.

Hereditary systemic amyloidosis may be caused by mutations in a number of plasma proteins including transthyretin (TTR), cystatin C, apolipoprotein A-I (apoA-I), gelsolin, fibrinogen Aα-chain, and lysozyme [1–6]. Each type of amyloidosis is inherited as an autosomal-dominant disease and is associated with a structurally altered protein that aggregates to form amyloid fibrils. These proteins may be the result of single nucleotide change, deletion or insertion in the gene coding for the amyloid precursor protein. To date, hereditary amyloidosis has been associated with over 70 mutations in TTR, 9 mutations in apoA-I, 4 in fibrinogen Aα-chain, 2 in lysozyme, 2 in gelsolin, and 1 in cystatin C [7]. Apolipoprotein A-II (apoA-II) is one of the major components of plasma high-density lipoprotein (HDL) and is mainly produced in the liver and small intestine with N-terminus pre- and pro-peptides. Mature apoA-II is composed of 77 amino acid residues and, in plasma, exists mainly as a disulfide-linked dimer with a molecular weight of approximately 17 kD [8]. While apoA-II was well known to form amyloid fibrils in the senescence accelerated mouse (SAM) model of aging [9], apoA-II amyloidosis had never been reported in humans. Moreover, no mutant human apoA-II proteins have been described. Recently, Benson et al identified a novel type of amyloid composed of a variant apoA-II isolated from kidney of an affected patient in a kindred originally reported in 1973 [10, 11]. Although the causative relationship between the mutant apoA-II and amyloid fibril formation has been recognized, there have been no other reports of human apoA-II amyloidosis. The mechanisms of amyloid fibril formation from apoA-II and similarities or differences between human and SAM have not been addressed.

This study reports a patient with renal amyloidosis who has a novel variant of apoA-II, and discusses possible mechanisms of amyloid formation from variant apoA-II.

Case report

The proband, a 46-year-old Caucasian male, was first seen at age 42 to evaluate proteinuria and a serum creatinine of 2.4 mg/dL. Renal biopsy showed global sclerosis of approximately 40% of glomeruli and amyloid deposition in glomeruli (Fig. 1A). Amyloid deposits also were
Fig. 1. Histopathology of the patient’s biopsied kidney. Congo red-stained section revealed amyloid deposition in the glomeruli (A) and vascular walls (B, arrow), which by polarization microscope showed typical green birefringence of amyloid. No tissue remained for immunohistochemistry.

present in the walls of medium-sized blood vessels (Fig. 1B); Significant past history was a serum creatinine determination of 1.3 mg/dL at age 35. An abdominal fat pad aspirate was positive for amyloid deposition, and amyloid was noted in blood vessel walls of a bone marrow biopsy. There were no signs of neuropathy, cardiomegaly, or hepatosplenomegaly on physical examination. Family history revealed that the patient’s mother died at age 67 of cancer of the breast, and the father died at age 75 of cancer of the urinary bladder. One sister and three half siblings were reported to be in good health. The patient’s two children, ages four years and eight months, were well. Evaluation for systemic amyloidosis revealed normal electrocardiogram and echocardiogram. There was no evidence of monoclonal gammopathy on immunofixation of serum or urine. There was only modest proteinuria (595 mg/24 h), and creatinine clearance was 47 mL/min.

DNA studies searching for mutations in genes known to be associated with systemic amyloidosis including TTR, apoA-I, lysozyme and fibrinogen Aα-chain failed to reveal any abnormalities. Subsequently, the patient has continued to work without medical problems except for hypertension that has been controlled medically.

METHODS
DNA isolation
Genomic DNA was isolated from peripheral blood leukocytes using a standard phenol/chloroform method.

Single-strand conformation polymorphism analysis
Exons 3 and 4 of the apoA-II gene were examined by single-strand conformation polymorphism (SSCP) analysis using oligonucleotide primers as follows: exon
3, (A2E3F) TGC TGT GGA CCC AGC TGA and (A2E3R) GAA CCC CTT GCC CTG AGA yielding a 217 bp product; exon 4, (A2E4F) CTA ATC CCC TCA CCT A and (A2E4R) GGA AGA CAA TGG TCT G yielding a 161 bp product. Polymerase chain reaction (PCR) was done in a total volume of 50 μL, containing 5 μL of 10 × PCR buffer (100 mmol/L Tris-HCl, pH 8.3, 500 mmol/L KCl, 12 mmol/L MgCl₂), 8 μL of 1.25 mmol/L dNTP, 15 pmol of each primer, 2.5 units of *Taq* polymerase (Sigma, St. Louis, MO, USA) and 100 ng of genomic DNA. Amplification was performed using a Perkin-Elmer Thermal Cycler (Norwalk, CT, USA) for 40 cycles consisting of denaturing at 95°C for 30 seconds, annealing at 63°C (exon 3) or 60°C (exon 4) for one minute, and extension at 72°C for 30 seconds. Six microliters of polymerase chain reaction (PCR) product was diluted in 20 μL of SSCP buffer containing 98% formamide, 0.05% xylene cyanol FF, 0.05% bromophenol blue, 10 mmol/L NaOH and 10 mmol/L ethylenediaminetetraacetic acid (EDTA), and heated at 95°C for five minutes. Six microliters of diluted samples was electrophoresed on a 15% polyacrylamide gel including 10% glycerol in the Tris-glycine buffer, pH 8.3. Single-stranded PCR products were visualized by silver staining [12].

**Direct DNA sequence analysis**

DNA was analyzed by the direct sequencing of exons 3 and 4 of the *apoA-II* gene. PCR was done as indicated in the last section of this article. The PCR product was purified by QIAquick PCR purification kit (Qiagen, Valencia, CA, USA) according to the manufacturer’s protocol. Sequencing reaction was performed by using 1.0 μL of purified PCR-product as the template and the Thermo Sequence [α-33P]dNTP radiolabeled terminator cycle sequencing kit (USB, Cleveland, OH, USA). Samples were electrophoresed on a 6% polyacrylamide gel at 45 W for three hours using a glycerol tolerant gel buffer, dried and exposed to Kodak X-Omat film (Eastman Kodak, Rochester, NY, USA).

**Restriction fragment length polymorphism (RFLP) analysis**

In the absence of a spontaneous creation of an endonuclease restriction site by the TGA to TCA mutation in the stop codon of the *apoA-II* gene, a PCR-induced mutation restriction analysis (IMRA) was performed [13]. A mutation primer (A2S78) corresponding to nucleotides 2151 to 2129 of the *apoA-II* nucleotide sequence [14] with an A instead of T at the second position from the 3′ end (5′-GGA AGA CAA TGG TCT GGA CAC AT-3′) was used to create a restriction site for *Nla*III (CATG) only in the mutant gene PCR products. PCR was performed with A2E4F and A2S78 primers under the same conditions as in the DNA sequencing of exon 4 (yielding a 161 bp product). After purification of PCR products by QIAquick PCR purification kit (Qiagen), 12.5 μL of amplified DNA was added to 1 μL of *Nla*III (New England Biolabs, Beverly, MA, USA), 1.6 μL of its specific buffer, 0.16 μL of 100 × bovine serum albumin (BSA) and 0.74 μL of distilled water, and incubated for three hours at 37°C. The samples were electrophoresed through 3% (wt/vol) Nusieve GTG agarose gel (FMC BioProducts, Rockland, ME, USA), stained with ethidium bromide and photographed under ultraviolet light.

**Western blot analysis of plasma**

Plasma samples in sodium dodecyl sulfate (SDS) buffer were heated in a boiling water bath for 10 minutes with or without 5% 2-mercaptoethanol and electrophoresed on a 16.5% Tris-Tricine Ready gel (Bio-Rad, Hercules, CA, USA) for two hours at 100 V. Subsequently, proteins were transferred for one hour at 90 V onto nitrocellulose membrane (Trans-Blot Transfer Medium; Bio-Rad). After blocking the membrane with 5% nonfat dry milk in phosphate-buffered saline (PBS), pH 7.4, the apoA-II bands were detected with sheep anti-human apoA-II (1:1000 in 5% milk; Boehringer Mannheim, Mannheim, Germany). Alkaline phosphatase-conjugated donkey anti-sheep IgG (Sigma) was used as the second antibody (1:1000 in 5% milk). The immunoblots were visualized with a reaction to NBT/BCIP Alkaline phosphatase substrate (Sigma).

**Isolation of plasma apoA-II and amino acid sequence analysis**

Plasma apoA-II was isolated according to a method to isolate high density lipoprotein (HDL) described previously with some modifications [15]. Five milliliters of plasma was adjusted to a density of 1.21 g/mL with KBr and ultracentrifuged at 100,000 rpm for six hours (TL-100 ultracentrifuge; Beckman, Palo Alto, CA, USA). The top layer was collected, dialyzed against distilled water, and lyophilized. The sample was dissolved in 8 mol/L guanidine HCl containing dithiothreitol (DTT), alkylated with iodoacetic acid, and fractionated on a Sepharose CL6B column (Pharmacia, Uppsala, Sweden). Fractions were combined into three pools according to the absorbance at 280 nm, dialyzed against distilled water, and lyophilized. Protein samples were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot using anti-human apolipoprotein A-II antibody (Boehringer Mannheim) as indicated previously in this article. The Sepharose CL6B pool III was digested with trypsin and the resulting peptides were fractionated on a Beckman Ultrasphere-ODS high-pressure liquid chromatography (HPLC) column. Samples were subjected to Edman degradation on an ABI 473A protein sequencer using the manufacturer’s standard cycle programs.
RESULTS

DNA analysis

The SSCP analysis revealed an abnormally migrating band for apoA-II exon 4 PCR products of the patient in contrast to those of normal controls (data not shown). Direct DNA sequencing of the exon 4 PCR product showed a single base substitution in the stop codon for the apoA-II gene (TGA to TCA) indicating a stop to serine substitution at codon 78 followed by 60 bases before a new stop codon (Fig. 2) [14]. No other mutations were found in exons 3 and 4 of apoA-II gene, which encode the entire pro- and mature apoA-II protein, in this patient.

Restriction fragment length polymorphism analysis of the 161 bp PCR product from A2E4F and A2S78 primers revealed that the patient had the NlaIII recognition site associated with the guanine to cytosine mutation at the second position of the stop codon (Fig. 3). Electrophoresis of the digested PCR product in the patient gave a digestion band of 137 bp in addition to the normal 161 bp band indicating heterozygosity for this mutation. To exclude the possibility that the G to C mutation might be a polymorphism, we analyzed DNA from 50 unrelated individuals (100 alleles). None had the G to C mutation.

Characterization of plasma apoA-II

Plasma samples obtained from this patient, a patient with variant apoA-II (stop to glycine mutation of apoA-II) described previously [10], and a normal individual were studied by Western blot analysis using anti-human apoA-II (Fig. 4). In the patient in this study, positive bands migrating with molecular weight corresponding to dimeric normal apoA-II, heterodimeric apoA-II with normal and variant molecules, and dimeric variant apoA-II were identified in nonreducing conditions. In the presence of reducing conditions, Western analysis revealed two bands (~8 and 10 kD) corresponding to normal apoA-II monomer and the larger variant apoA-II mono-
these patients varied from adolescence to the fifth decade and they died in the fifth to sixth decades. While autopsy studies revealed that amyloid accumulation was present in most visceral organs, except for central nervous system and peripheral nerves, renal amyloid was the most prominent feature [11]. The severity of amyloid deposition in the liver, heart, and spleen was moderate and limited mainly to vascular walls. Benson et al isolated the amyloid fibrils from the kidney obtained from an autopsied patient in that family and identified that the amyloid fibrils were derived from variant apoA-II caused by a stop codon mutation (TGA; stop to GGA; glycine) [10]. This mutation results in a 21-amino acid residue extension at the C-terminus of normal apoA-II protein.

In the present study, DNA analysis revealed that the affected individual described here was heterozygous for a novel stop-codon mutation in the apoA-II gene (TGA to TCA) that causes substitution of serine for the stop codon at codon 78. Amino acid sequence analysis of the patient’s plasma apoA-II showed not only normal apoA-II, but also variant apoA-II with a 21-residue extension at the C-terminus. This 21-amino acid extension was completely identical to that in the patient with variant apoA-II described by Benson et al, except for serine at residue 78 [10].

The clinical picture of the patient described here resembles that in patients with variant apoA-II reported previously [10, 11]. During the clinical course, the most noticeable clinical manifestation was nephropathy caused by renal amyloidosis in both the patient described and the patients with stop to glycine mutation in the apoA-II gene. Moreover, the distinctive feature of amyloidosis in both families is that affected individuals do not have significant dysfunction of other visceral organs, including the heart, liver, and peripheral nerves due to amyloid deposition, although amyloid accumulation was seen systemically in affected patients of both families. While the mechanism of how amyloid proteins have organ specificity that leads to variation in clinical features is unknown, the non-neuropathic, nephropathic syndrome seems to be the prominent feature for apoA-II amyloidosis. The clinical features in both kindreds are similar to fibrinogen Aα-chain and lysozyme amyloidosis in terms of nephropathy without neuropathy [5, 6, 17]. Although there is no clear difference in the onset of amyloidosis among apoA-II, fibrinogen Aα-chain, and lysozyme amyloidosis, the latter forms (fibrinogen Aα-chain and lysozyme) usually show more extensive hepatic and splenic involvement.

Despite recent biochemical and molecular-genetic advances, possible mechanisms of amyloid fibril formation in apoA-II amyloidosis are not clear. In comparison with the previously reported family with variant apoA-II (stop to glycine mutation) [10, 11], the affected patients in both families have a novel 21-amino acid peptide in
Fig. 5. Chromatogram of top layer of patient’s plasma after ultracentrifugation, solubilized in 8 mol/L guanidine HCl on a Sepharose CL6B column equilibrated and eluted with 4 mol/L guanidine HCl, 0.05 mol/L Tris, pH 8.2. Fractions were pooled as indicated by the bars.

common, although the first residue of that 21-residue peptide (glycine or serine) is different. To date, there have not been reports of human apoA-II amyloidosis caused by other mutations except for the stop-codon site. It is likely, therefore, that the role of human apoA-II as a precursor of amyloid fibrils is closely related to the extension of the protein at the C-terminus. Similar cases of amyloid fibril formation caused by novel peptide sequences at the C-terminus have been identified with fibrinogen Aα-chain [18, 19], Abri in familial British dementia [20], and ADan in familial Danish dementia [21].

The new peptide sequence at the C-terminus in variant apoA-II with stop to glycine mutation is predicted to contain an α-helix that is different from the three class A amphipathic helices, important for lipid binding [22]. Benson et al emphasized that decreased binding capacity to HDL caused by variant apoA-II with extended sequence without lipid-binding capability could alter its metabolism and accelerate amyloid fibril formation [10]. The same thing may be hypothesized for amyloid fibril formation in variant apoA-II with stop to serine mutation in this study, although the precise conformational change with both types of variant apoA-II (stop to glycine and stop to serine mutation) has not been fully understood.

Higuchi et al suggested importance of amino acid substitution in the N-terminal region of apoA-II for development of amyloid in the SAM, since the strain of mouse (SAMP) with the highest incidence of amyloidosis has a glutamine for proline substitution at the fifth residue from the N-terminus of apoA-II [23, 24]. However, human normal apoA-II with a proline in the fifth position is similar to apoA-II of SAM mice resistance to amyloidogenesis (SAMR), and affected patients in both families did not have a mutation at the fifth residue. Therefore, it is unlikely in the human that the N-terminal structure of the molecule plays an important role in its tendency to form amyloid fibrils [10]. Also, in human apoA-II amyloid patients, deposited amyloid fibrils are composed of only full-length variant apoA-II [10], whereas mature apoA-II and pro-apoA-II were detected in amyloid fibrils of SAM [25]. In addition, heavy amyloid deposition is seen in the liver, spleen, and heart as well as the kidney in SAM [26]. Hence, the pathogenesis of apoA-II related-amyloid formation in human is likely to differ from amyloid formation in the SAM model.

In the present study, the patient had no family history of amyloidosis. Since DNA for analysis was limited to only the patient, it could not be determined whether some asymptomatic carriers exist in this family or this stop to serine mutation was caused by de novo mutation. In the apoA-II amyloidosis family recently reported, the amyloid showed autosomal dominant inheritance [10], similar to all other types of hereditary amyloidosis [7].
The patient described here was heterozygous for the apoA-II mutation associated with amyloidosis. Accordingly, the chance that each of his children may have inherited this mutant allele is 50%. Careful observation will be required for family members, especially his children. Finally, this newly recognized apoA-II amyloidosis provides further knowledge of human amyloidogenesis and most likely will be of value in evaluating a number of kindreds worldwide with as yet unexplained autosomal dominant amyloidosis, especially with nephropathy but no neuropathy.

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REFERENCES

2. GHISO J, JENSSON O, FRANGIONE B: Amyloid fibrils in hereditary cerebral hemorhage with amyloidosis of Icelandic type is a variant of γ-trace basic protein (cystatin C). Proc Natl Acad Sci USA 83:2974–2978, 1986
9. HIGUCHI K, YONEZU T, KOGISHI K, et al.: Purification and character-

10. BENSON MD, LIEPNIERS J, YAZAKI M, et al.: A new human heredi-
tary amyloidosis: The result of a stop-codon mutation in the apolipo-
11. WEISS SW, PAGE DL: Amyloid nephropathy of Ostertag with special
12. WALLACE AJ, WILLIAMSON P, MOUNTFORD RC, RAMSDEN SC: Non-
13. ZELDENRUST SR, BENSON MD: A new test for detection of the His58
variant transthyretin allele in hereditary amyloidosis: Creation of
diagnostic restriction endonuclease recognition sites by PCR based
14. KNOTT TJ, WALLIS SC, ROBERTSON ME, et al.: The human apolipo-
protein AII gene: Structural organization and sites of expression.
16. OSTERTAG B: Familial Amyloid-erkrankung. Z Meeschl Verer-
17. UEMICHIT T, LIEPNIERS J, BENSON MD: Hereditary renal amy-
loidosis with a novel variant fibrinogen. J Clin Invest 93:731–736,
1994
18. UEMICHIT T, LIEPNIERS J, YAMADA T, et al.: A frame shift mutation in
the fibrinogen Aα-chain gene in a kindred with renal amyloidosis.
Blood 87:4197–4203, 1996
with a frame shift mutation in fibrinogen Aα-chain gene producing a
23. HIGUCHI K, YONEZU T, TSUMASAWA S, et al.: The single proline-
glutamine substitutition at position 5 enhances the potency of amy-
24. HIGUCHI K, KITAGAWA K, NAKI H, et al.: Polymorphism of apolipo-
protein A-II (apoA-II) among inbred strains of mice: Relationship
between the molecular type of apoA-II and mouse senile amy-
25. HIGUCHI K, KOUSHI K, WANG J, et al.: Accumulation of pro-apoallo-
659, 1997
26. HIGUCHI K, MATSUMURA A, HOMMA A, et al.: Systemic senile amy-
loidosis in senescence-accelerated mice: A unique fibril protein demon-
strated in tissues from various organs by the unlabeled immunoper-

Fig. 7. Amino acid sequence and cDNA sequence of human apoA-II from initiate codon to poly-A sequence. The translated amino acid sequence is given below the cDNA and the 21-amino acid residue extension resulting from mutation in the stop codon (resides 78 to 98) is indicated. Arrows show the sequence of plasma apoA-II identified by amino acid sequence analysis of tryptic peptides. The dotted line indicates the 21-amino acid residue extension in variant apoA-II. 

Yazaki et al: Novel stop-codon mutation in the A-II gene