CASE REPORT

Liver Biopsy Discloses a New Apolipoprotein A-I Hereditary Amyloidosis in Several Unrelated Italian Families

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Background & Aims: Hereditary systemic amyloidoses are autosomal dominant, late-onset disorders caused by mutations in the genes for a group of plasma proteins including transthyretin, lysozyme, fibrinogen Aα chain, gelsolin, apolipoprotein A-I, and apolipoprotein A-II. We investigated both phenotypic and genotypic aspects of apolipoprotein A-I amyloidosis unexpectedly disclosed by liver biopsy in 13 unrelated individuals with asymptomatic, persistent elevation of alkaline phosphatase and γ-glutamyltransferase levels. Methods: Immunoelectron microscopy was used for in situ characterization of amyloid deposits on liver biopsy specimens. Mutation analysis was performed by sequencing of the apolipoprotein A-I gene in all patients. Wild-type/variant apolipoprotein A-I ratio in plasma high-density lipoproteins was assessed by a peptide mass fingerprinting approach after purification of total apolipoprotein A-I of 2 patients. Results: Family history was informative in 5 cases. Renal failure developed in 9 cases. Hypogonadism due to testicular involvement was observed. Amyloid fibrils specifically stained with anti–apolipoprotein A-I antibody. A novel (Leu75Pro) heterozygous mutation in the apolipoprotein A-I gene was present in affected individuals but not in controls. Variant apolipoprotein A-I was about 10% of the total protein in high-density lipoproteins. Conclusions: The high number of individuals with apparently sporadic disease might reflect widespread occurrence of this mutation in the population and a milder phenotype of this variant compared with other apolipoprotein A-I amyloidogenic mutants. These findings suggest that specific staining for amyloid should be performed on liver biopsy of individuals with asymptomatic chronic elevation of alkaline phosphatase and γ-glutamyltransferase levels.

Amyloidosis is a heterogeneous group of diseases characterized by the pathologic aggregation of proteins into insoluble fibrils that accumulate predominantly extracellularly in tissues and organs, causing damage and eventually death. Systemic forms of amyloidosis include hereditary amyloidoses, a group of autosomal dominant, late-onset disorders caused by variant transthyretin, apolipoprotein (apo) A-I, apoA-II, lysozyme, fibrinogen Aα chain, cystatin C, and gelsolin.[1]

ApoA-I hereditary amyloidosis (AApoAI, MIM 107680) is a rare disease characterized by progressive deposition of amyloid fibrils mainly constituted by N-terminal polypeptide fragments of this protein. Ten amyloidogenic apoA-I variants have been identified thus far,[2,3] and most of these are private. Amyloid deposits predominantly affect the kidneys, heart, and liver, causing either progressive nephropathy or cardiomyopathy and, rarely, hepatopathy. Although an extensive visceral amyloid load is frequently observed as a postmortem finding or by means of serum amyloid P component scintigraphy in patients with progressive kidney or heart disease,[4,5] deterioration of liver function is seldom observed. In a large Spanish kindred with an apoA-I deletion/insertion mutant, however, a severe hepatopathy progressing to fatal liver failure was the unique clinical presentation of apoA-I amyloidosis.[6] In this family, the disease course was characterized by a long-lasting asymptomatic phase with only biochemical evidence of liver damage, followed by progressive deterioration of organ function leading to frank cholestasis, portal hypertension, hepatic encephalopathy, and death.[6,7]

Here we describe a novel apoA-I variant, Leu75Pro, associated with systemic amyloidosis predominantly involving the liver and kidneys. The disease was unexpect-

Abbreviation used in this paper: apo, apolipoprotein.

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Table 1. Characteristics of Patients at Presentation

Patient	Sex/age (*yr*)	Age (*yr*) at biopsy	Alkaline phosphatase (*U/L*)	γ-glutamyltransferase (*U/L*)	Alanine aminotransferase (*U/L*)	Creatinine (*mg/dL*)	ApoA-I (*mg/dL*)	High-density lipoprotein (*mg/dL*)
1	M/55	55	476	319	60	1.2	96	26
2	M/63	53	458	606	78	3.4	44	23
3	M/69	67	352	181	38	2.3	61	25
4	M/68	65	580	764	39	3.4	74	19
5	F/64	46	360	375	34	2.7	92	27
6	M/53	52	313	158	49	1.42	100	38
7	M/50	48	470	315	40	1.2	100	44
8	F/58	56	187	313	64	2.9	113	37
9	F/59	57	525	271	60	1.7	68	24
10	F/61	60	111	19	35	1.08	87	36
11	M/68	67	422	1514	58	1.6	86	18
12	M/54	53	130	120	110	1.2	102	32
13	F/70	62	360	204	37	1.3	62	22

NOTE. Reference ranges are as follows: alkaline phosphatase, 60–260 U/L; γ-glutamyltransferase, 11–53 U/L; alanine aminotransferase, <40 U/L; creatinine, <1.2 mg/dL; apoA-I, 110–205 mg/dL in men and 125–215 mg/dL in women; HDL, 55–140 mg/dL in men and 55–125 mg/dL in women.

edly diagnosed in 13 unrelated patients who underwent a liver biopsy for chronic liver test abnormalities of unknown origin.

Materials and Methods

Thirteen unrelated patients (8 men and 5 women; age range, 50–70 years; Table 1) were independently referred to our center for biopsy-proven liver amyloidosis. The biopsy was performed for asymptomatic chronic liver test abnormalities in the absence of a history of drug and alcohol use, specific biochemical markers, and abnormal imaging in 11 patients and for chronic hepatitis C in the remaining 2 patients. Common histologic features included conserved lobular architecture with anisocytosis of the hepatocytes and the presence of enlarged portal areas, filled with variable amounts of an amorphous, hyaline material that showed green birefringence under polarized light after Congo red staining. Amyloid deposits were localized both in the stromal and perivascular regions and occasionally resulted in bile duct compression. A mild, mononuclear inflammatory infiltration without evidence of piecemeal necrosis and a limited periportal fibrosis were also present. All patients underwent complete clinical and laboratory evaluation.

All subjects are of Italian ancestry and originate from the Lombardy region in northern Italy, where our hospital is located. Pedigree analysis showed a significant family history for possible amyloid-related liver or kidney disease in 5 of 13 patients (patients 1–3, 10, and 11). Patient 1 (Figure 1; family A, III:3) was the only known affected member of his family at presentation. Later, his first-degree cousin (III:1) was found to have amyloidosis on liver biopsy performed for chronic hepatitis C. Their fathers (II:1 and II:8), who were siblings, died in their 80s from apparently unrelated causes. The patient's mother (II:9) is alive and well and is aged 89 years. Patient 2 (Figure 1; family B, IV:4) had a similarly affected brother (IV:3). Later, their 76-year-old cousin (III:3) presented with renal failure and amyloid deposits on abdominal fat biopsy. The proband's father (III:1) and grandfather (II-1) died in their 80s from undefined causes. Patient 3 (Figure 1; family C, III:8) had a brother (III:4) who presented with hepatomegaly, abnormal liver function tests, and renal failure in his 60s. Amyloid deposits were shown on liver and adrenal gland biopsy specimens. He died at the age of 76 years from stroke. A younger brother (III:11) developed mild renal failure and hepatopathy at the age of 60 years. A second-degree cousin (IV:1) had amyloid deposits on a testicular biopsy performed at the age of 40 years for gynecomastia and testicular failure, but he was not available for study. The proband's mother (II:2) died at the age of 61 years from stroke.

Patient 10 (Figure 1; family D, III:8) was retrospectively found to have a first-degree cousin (III:2) with amyloid deposits documented on a liver biopsy performed at the age of 58 years during cholecystectomy. Mild renal failure had been documented since the age of 53 years. He died at the age of 70 years from apparently unrelated reasons. Later, a younger brother (III:4) and his 2 sons (IV:3 and IV:4) were similarly found to be affected by renal failure of unknown origin.

Revision of medical records showed that patient 11 (Figure 1; family E, II:1) had a sister (II:2) who underwent a liver biopsy for chronic liver test abnormalities and hepatomegaly at the age of 58 years. The biopsy specimen showed the presence of amyloid deposits that were not further characterized. Type 2 diabetes mellitus and mild renal failure were also documented on that occasion. She died at the age of 67 years from undefined reasons.

Histology and Immunohistochemistry

Sections of a formalin-fixed, paraffin-embedded liver biopsy specimen from patient 1 were stained with Congo red and examined under polarized light. Immunoelectron micros-

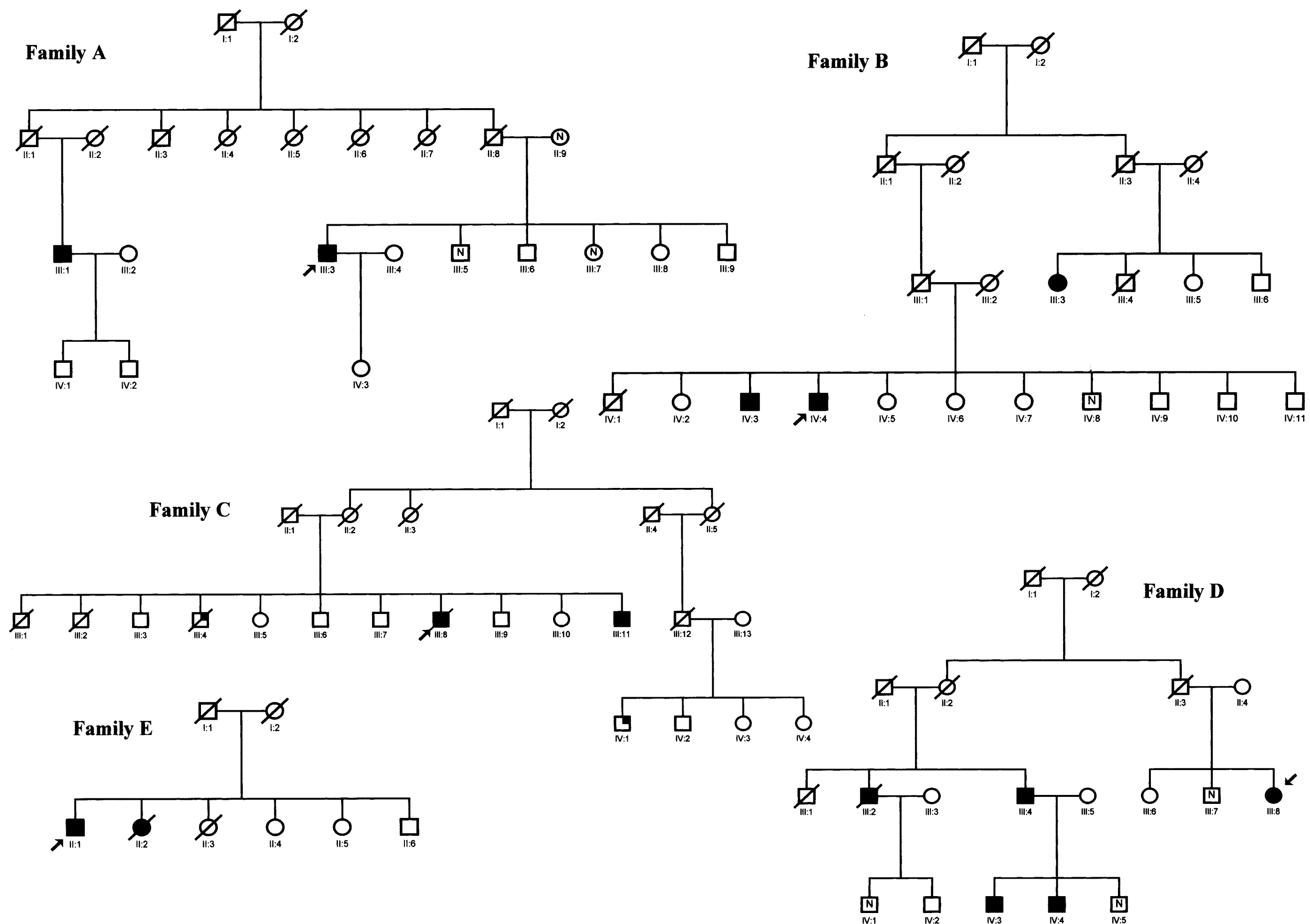

Figure 1. Family trees of 5 kindreds with apoA-I amyloidosis (families A–E). Probands are indicated by *arrows*. Clinically affected individuals and known carriers of the mutation are indicated by *closed symbols*. Dead individuals are indicated by a *diagonal line* through the symbols. *Open symbols* indicate asymptomatic individuals not tested. *Partially filled symbols* indicate amyloid confirmed histologically but genetic test not performed. N, tested individuals but no mutation found. See text for detailed clinical information on single members in families.

copy typing of amyloid deposits was performed as described[8] using antibodies against κ and λ light chains, transthyretin, fibrinogen, lysozyme, serum amyloid P, serum amyloid A, and apoA-I.

Paraffin-embedded liver biopsy specimens from 6 patients were also studied by immunoelectron microscopy. Paraffin-embedded specimens were rehydrated through a series of graded ethanol solutions, fixed in osmium tetraoxide, and re-embedded in Epon araldite resin. Immunohistochemical analysis was then performed as previously described.

Mutation Search in the apoA-I Gene

DNA was obtained using standard procedures from peripheral blood mononuclear cells of 13 patients, 8 affected relatives (family A: III:1; family B: III:3 and IV:3; family C: III:11; family D: III:2, III:4, IV:3, and IV:4), 9 healthy family members, and 100 controls. For affected individual II:2 in family E, genomic DNA was extracted from a paraffin-embedded liver biopsy specimen as previously described.[9]

Mutation detection was performed in all patients on exons and exon-intron boundaries of the apoA-I gene. Amplification conditions for exons 3 and 4 were as described.[9] A single 514–base pair amplicon containing the first 2 exons and the 5′ region of the gene was also obtained using the following pair of primers: 5′-AGGGACAGAGCTGATCCTTG-3′ and 5′-GTGAGAAACCTGCTGCCTCTG-3′. Amplification conditions consisted of an initial denaturation step (at 95°C for 10 minutes) followed by 30 cycles of denaturation (95°C for 2 minutes), annealing (63°C for 1 minute), and elongation (72°C for 1.5 minutes), with a final step of elongation (72°C for 5 minutes).

Polymerase chain reaction products were sequenced on both strands using the same forward and reverse primers as used in the amplification reactions. Sequence reactions were performed with the Big Dye Terminator Cycle Sequencing kit (Applied Biosystems, Foster City, CA), and the products were analyzed on an ABI PRISM 377 DNA sequencer (Applied Biosystems).

Restriction Analysis of the Leu75Pro Mutation

Restriction fragment length polymorphism analysis with the enzyme *Hpa*II, for which the mutation introduces a

new restriction site, was performed in patients, their relatives, and 100 controls by digestion of a 392–base pair DNA fragment, corresponding to the 5′ of exon 4, previously amplified for sequencing analysis.

To avoid possible problems expected from DNA fragmentation, genomic DNA extracted from paraffin was tested for the presence of the mutation after polymerase chain reaction amplification of a 134–base pair DNA fragment using the same conditions and the following primers: 5′-CAGCGTGACCTCCACCTTC-3′ and 5′-TGGCCTTCACCTGGTGGAG-3′. The same restriction enzyme *Hpa*II was used on this product to detect the mutation.

Characterization of Wild-Type and Variant apoA-I in Plasma High-Density Lipoprotein

Lipid-free apoA-I was purified from plasma high-density lipoproteins and digested by *Staphylococcus* V8 protease; separation of peptides and identification by mass spectra analysis was performed by reversed-phase chromatography coupled with an LCQ ion trap spectrometer as previously reported.[10]

Results

Clinical Features

Patients were symptom-free at presentation. Their clinical characteristics are shown in Table 1. On physical examination, hepatomegaly without spleen enlargement was present in 10 patients. Liver function tests showed increased alkaline phosphatase and/or γ-glutamyltransferase levels, more than twice the upper reference limit, in all but one patient. Aminotransferase levels were within reference range in 6 individuals and showed values less than twice the upper reference limit in 6 patients. In one patient with chronic hepatitis C (patient 12), the alanine aminotransferase level was markedly altered (110 U/L; upper reference limit, <40 U/L). Serum bilirubin level, albumin level, and prothrombin time were within reference range in all cases. Serum creatinine level was increased in 9 patients, with concentrations ≥2 mg/dL in 5 cases. In contrast to what is usually observed in amyloid kidney involvement, only 2 patients (patients 2 and 4) had a significant proteinuria (1 g/24 h and 2.43 g/24 h, respectively). No patient had cardiac symptoms, and echocardiography at presentation showed no evidence of heart involvement in all cases. Two patients (patients 6 and 7) had evidence of testicular failure, with low testosterone levels and elevated plasma gonadotropin levels. Patient 6 underwent surgical correction of gynecomastia at the age of 46 years and was then treated with testosterone for 4 years.

A long follow-up since identification of amyloid deposits was available for patient 5. Liver biopsy was performed at the age of 46 years because of mild hepatomegaly and elevated alkaline phosphatase and γ-glutamyltransferase levels. Eighteen years later, we observed severe hepatomegaly (10 cm below the costal margin), elevation of alkaline phosphatase (360 U/L) and γ-glutamyltransferase (375 U/L) levels, and renal failure (serum creatinine level, 2.7 mg/dL).

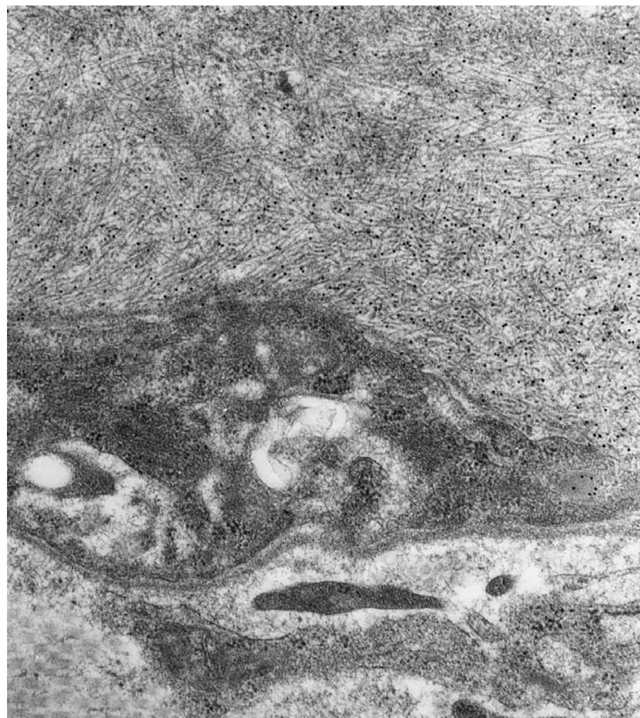

Figure 2. Electron micrograph showing 10-nm immunogold labeling of liver amyloid fibrils with anti–apoA-I antibody.

A mild to moderate reduction of apoA-I and high-density lipoprotein levels, with normal total cholesterol and triglyceride levels, was observed in all patients (Table 1) and their affected relatives.

Unfortunately, patient 3 suddenly died from stroke at the age of 70 years, 3 years after biopsy and 1 year after diagnosis.

Liver Histology

Histologic examination of the liver biopsy specimen from patient 1 showed conserved lobular architecture and the presence of amorphous material in portal areas, presenting typical green birefringence under polarized light, after Congo red staining. Amyloid deposits corresponded to about 20% of the sample area and were mainly localized at the perivascular level and as nodular aggregates. A mild, mononuclear inflammatory infiltration was also present in portal areas. On electron microscopy, the liver appeared heavily infiltrated by amyloid fibrils that strongly reacted with anti–apoA-I (Figure 2) and anti–serum amyloid P antibodies but not with the other antibodies tested.

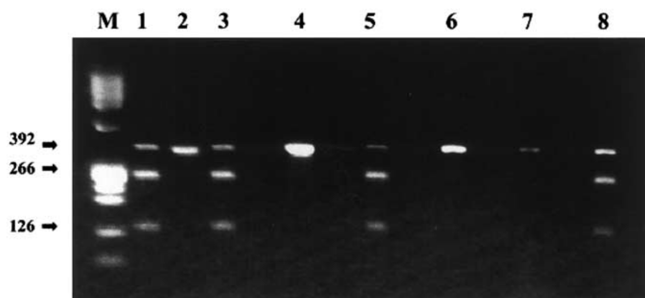

Figure 3. Restriction fragment length polymorphism analysis with the enzyme *Hpa*II, for which the mutation creates a new restriction site, shows the presence of 2 additional fragments in patients (*lanes 1, 3, 5,* and *8*) compared with healthy relatives (*lanes 2, 4, 6,* and *7*). M, marker ϕX174 *Hae*III.

Immunohistochemical analysis was also performed on 6 paraffin-embedded liver biopsy specimens on which amyloidosis was diagnosed by Congo red staining. Electron microscopic examination of these samples showed the presence of variable amounts of amyloid fibrils specifically immunostained by anti–apoA-I antibody.

DNA Analysis

ApoA-I gene sequencing showed in all patients a T to C transition at position 1772 of the nucleotide sequence, resulting in a proline for leucine substitution at codon 75 of the polypeptide chain. No other sequence variations, apart from at known polymorphic sites, were detected in the gene regions analyzed. All patients and their 9 affected relatives were heterozygous for this mutation, as confirmed by restriction fragment length polymorphism analysis with the enzyme *Hpa*II, for which the mutation introduces a specific cleavage site (Figure 3). No mutation was found among healthy relatives tested. Screening of 100 controls did not show any carrier.

Plasma apoA-I Studies

Total apoA-I, purified from 2 patients (patients 7 and 10), was digested by *Staphylococcus* V8 protease. Peptides were separated by reversed-phase chromatography. Wild-type and mutant peptides, encompassing codon 75, were eluted at different retention times (Figure 4*A*) and were recognized by their characteristic mass

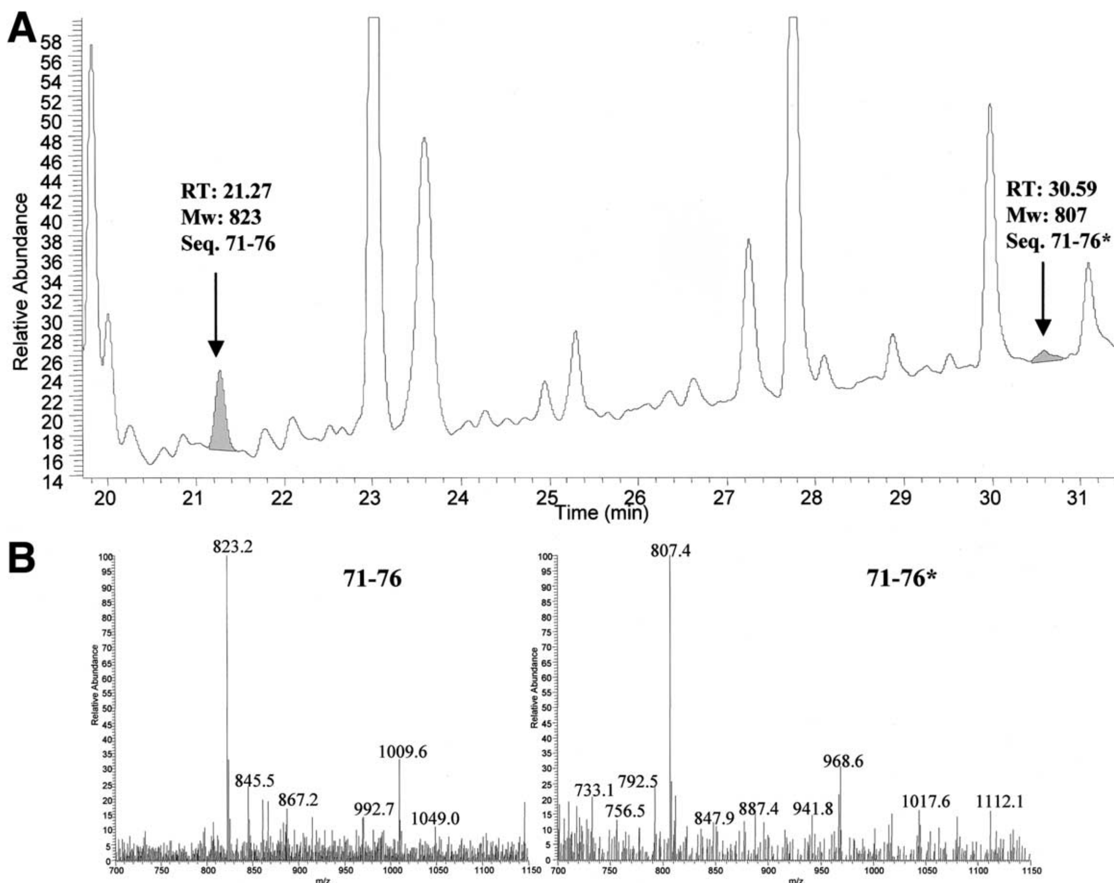

Figure 4. (*A*) Reversed-phase chromatography of V8 protease digestion of apoA-I purified from patient 7. The peptide indicated as 71–76* has Pro in position 75 and peptide 71–76 has Leu in position 75. The 2 integrated areas of the related peaks are shaded in *gray.* (*B*) Mass spectrum of peptide 71–76 (eluted at 21.27 minutes) and peptide 71–76* (eluted at 30.59 minutes) shown in *A.*

spectra (Figure 4*B*). The wild-type/mutant ratio, quantified on the basis of the integrated area of the correspondent chromatographic peak, indicates that the variant represents approximately 10% of the total apoA-I.

Discussion

The definition of the pathology underlying asymptomatic, persistent elevation of alkaline phosphatase and γ-glutamyltransferase levels is a thorny issue in gastroenterology practice. Here we report that, in several patients, this condition was sustained by liver amyloid deposits caused by a new hereditary apoA-I variant.

The presence of amyloid fibrils specifically immunoreacting with anti–apoA-I antibody in 7 patients and the demonstration that this mutation segregates with the disease strongly support its pathogenic role. Moreover, no other sequence variations in the apoA-I gene were observed.

This is the first apoA-I amyloidogenic variant identified in so many unrelated patients. The absence of a significant family history for the disease in most cases is an unexpected and previously unreported finding, probably related to the relatively milder disease associated with the Leu75Pro variant compared with all other known amyloidogenic apoA-I mutations.

With respect to derangement of liver function tests, the onset takes place in the fifth to sixth decade of life in all patients observed. The disease progresses through a long asymptomatic phase in which the only manifestations are a mild to moderate hepatomegaly and persistent elevation of alkaline phosphatase and γ-glutamyltransferase levels. This feature is common to all amyloidogenic apoA-I variants associated with massive liver deposition of amyloid fibrils[4–6] and reflects the slow infiltration of portal areas by amyloid deposits without hepatocyte damage, inflammatory infiltration, and fibrous distortion of the lobular architecture. Compared with the deletion/insertion mutation reported in a large Spanish kindred,[6,7] no evidence of progression to liver failure has been observed to date in individuals carrying the Leu75Pro variant, including obligate mutation carriers in family A (II:1 and II:8) and B (II:1 and III:1).

Two patients had gynecomastia and testicular failure a few years before evidence of liver dysfunction. Although bioptic proof is not available, it is likely that testicular failure is caused by amyloid deposits because localization of amyloid fibrils into the testis has been documented in another apoA-I variant.[9] In most patients, a slow progressive increase in serum creatinine levels without proteinuria appeared within a few years of liver biopsy. In one patient, in whom a positive liver biopsy specimen was obtained 18 years before diagnosis, renal function has deteriorated slowly over time, with the serum creatinine level increasing to 2.7 mg/dL. The lack of proteinuria is a characteristic feature of this type of amyloidosis because, in general, amyloid kidney involvement is associated with significant proteinuria, frequently in the nephrotic range. The structural basis of this peculiar renal dysfunction remains to be elucidated. Further data obtained from kidney biopsy specimens are warranted to assess the presence of amyloid fibrils and their pattern of deposition.

Presently, the lack of tissue samples for chemical characterization of the protein from natural fibrils of patients carrying the Leu75Pro variant does not allow us to characterize the precise size and sequence of the polypeptide that accumulates in the amyloid deposits.

Similar to other apoA-I amyloidogenic mutants,[5,6,9] all patients had a mild to moderate decrease in serum apoA-I concentration. Determination of wild-type and mutant species in total circulating apoA-I in 2 patients showed a 10:1 ratio or even less. This severe imbalance is in agreement with previously reported data for other amyloidogenic variants,[10] suggesting abnormal metabolism of the mutant compared with the wild-type protein.[10–12]

Although the role of liver biopsy in the evaluation of abnormal liver enzyme results in asymptomatic patients is still controversial,[13–15] in this case it was essential to establish a correct diagnosis, avoid further diagnostic procedures and unnecessary medications, and offer genetic counseling. Our findings indicate that a search for amyloid deposits using the Congo red method should be routinely performed in individuals with unexplained chronic elevation of cholestasis indices.

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