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Short Communication

Meningocerebrovascular Amyloidosis Associated with a Novel Transthyretin Mis-Sense Mutation at Codon 18 (TTRD18G)

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We describe a novel transthyretin mutation at codon 18 where Asp is replaced by Gly (D18G) in a Hungarian kindred. This mutation is associated with meningocerebrovascular amyloidosis, producing dementia, ataxia, and spasticity. Fifty different transthyretin mutations are related to amyloid deposition, typically producing a peripheral neuropathy or cardiac dysfunction. These symptoms are absent in this family. Up to now, amyloid-β (Aβ), cystatin C, and prion proteins have been known to be deposited as amyloid in the brain, leading to stroke or dementia. With this report we establish that transthyretin amyloid deposition can also produce central nervous system dysfunction as the major clinical symptom. (Am J Pathol 1996, 148:361–366)

Transthyretin (TTR) is a carrier protein for thyroid hormone and retinol-binding protein in plasma and cerebrospinal fluid (reviewed in Ref. 1). Under pathological conditions, TTR can be deposited in the extracellular space as amyloid in several organs.[1,2] The peripheral nerves and the heart are particularly prone to TTR amyloid deposits with resultant neuropathy and cardiomyopathy as the major clinical manifestations. Fifty TTR mutations are associated with amyloid deposition,[1,2] although TTR amyloid deposition can also occur in the absence of any mutations.[3] The most common clinical syndrome associated with TTR amyloid is familial amyloid polyneuropathy, type 1 (FAP).[1] This autosomal dominantly inherited disorder is characterized by an initial presentation with sensory neuropathy in the lower limbs and an autonomic neuropathy in the absence of any central nervous system (CNS) symptoms.[1] This disease is progressive and results in death in approximately 10 to 20 years. FAP is most commonly associated with a single substitution of a methionine residue for valine at position 30 (V30M).[1]

Some FAP patients are known to have deposition of amyloid in the cerebral blood vessels and leptomeninges, but these deposits are not associated with any clinical symptoms.[4,5] In one kindred with FAP from Iiyama, Japan, some neurological symptoms such as ataxia have been reported, in addition to typical signs associated with peripheral nerve dysfunction; however, affected members of this family most likely have a combination of FAP linked to a TTR V30M mutation and a separate condition of spinocerebellar degeneration.[6] In this paper we describe a family made up of 56 individuals and span-

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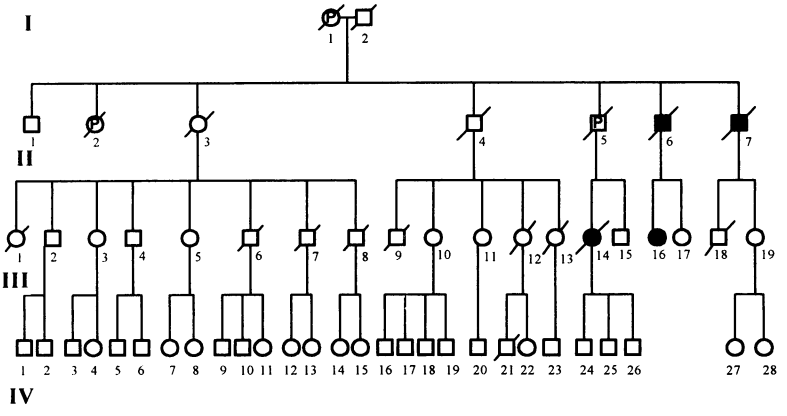

Figure 1. *Pedigree of the family, showing four generations (I through IV), including 56 members. □ and ○, deceased family members. ■ and ●, family members who are definitely affected with the disease by clinical history and autopsy studies. Living patient III-16 is labeled as definitely affected by clinical history, neuroradiological examination, and DNA analysis. Ⓟ, P, family members who are probably affected by clinical history. Immunohistochemical studies and extraction of DNA from paraffin-embedded tissue was done on affected patients III-14 and II-7 as well as unaffected patient III-18. DNA was extracted from living unaffected patient III-11 (aged 61 years) and affected patient III-16.*

ning four generations with predominantly CNS symptoms such as dementia, spasticity, ataxia, and hearing loss, in the absence of any clinical signs of peripheral nerve or cardiac dysfunction. Affected members of this family have a novel TTR mutation associated with extensive cerebrovascular and leptomeningeal amyloid deposition.

This TTR mutation is not found in control populations or in unaffected family members.

Materials and Methods

Subjects

The kindred contains 56 members and spans four generations. A pedigree is shown in Figure 1. Clinical and full autopsy data are available from 2 affected (Figure 1, III-14 and II-7) and 1 unaffected, deceased member (Figure 1, III-18). Clinical and gross autopsy data is known about deceased member 11-6 (Figure 1). Clinical data and neuroradiological studies are available from 1 affected, living member (Figure 1, III-16) and an unaffected, living 61-year-old, unaffected member (Figure 1, III-11). The onset of symptoms varied from age 36 to age 53, with death occurring between the ages of 51 and 60. The major clinical symptoms included short-term memory loss, hearing loss, signs of cerebellar dysfunction with ataxia, nystagmus, and intention tremor as well as bilateral pyramidal tract dysfunction with hyper-reflexia, bilateral Babinski signs, and a progressively increasing spasticity. In addition, most affected members had episodic confusion and hallucinations.

Immunohistochemical Studies

Six-micron paraffin sections of fronto-parietal cortex and from systemic organs such as kidney and peripheral nerves of two affected and one unaffected family member were subjected to immunohistochemical staining by deparaffinization followed by treatment with 100% formic acid for 20 minutes. The slides were blocked in phosphate-buffered saline with 0.1% Tween-20 and 10% fetal calf serum (PBST-FCS). After washing, sequential sections had the following primary antibodies applied: monoclonal 4G8 (1:200; anti- Aβ),[7] monoclonal 6E10 (1:15,000; anti-Aβ),[7] polyclonal anti-TTR,[8] monoclonal anti-apo E (Biodesign Int., Kennebunk, ME), polyclonal anti-amyloid P component (Dako Corp., Carpinteria, CA), polyclonal and monoclonal anti-PrP,[9] polyclonal anti-amyloid A (Dako), polyclonal anti-cystatin C,[10] polyclonal anti-gelsolin-related amyloid,[11] polyclonal anti-κ and -λ light chains (Chemicon Int. Inc., Temecula, CA), monoclonal anti-amylin (Peninsula Lab. Inc., Belmont, CA), and polyclonal anti-fibrinogen (Chemicon). The anti-TTR antibodies were raised to patient purified TTR amyloid.[8] The secondary antibodies used were biotin-conjugated (1:800; Sigma Chemical Co., St. Louis, MO) goat anti-rabbit IgG and biotin-conjugated (1:200; Sigma) goat anti-mouse IgG, followed by streptavidin-horseradish peroxidase conjugate (1:300; Amersham, Arlington Heights, IL) in PBST-FCS. The slides were developed in diaminobenzidine and H_2O_2, with cobalt chloride hexahydride (0.0005%) enhancement. Controls included preabsorption of the antibody to TTR with an excess of antigen and replacement of the primary antibody with nonimmune, species-specific serum.

DNA Amplification and Analysis

Total genomic DNA was isolated from peripheral blood leukocytes of one affected and one unaffected living family member, as well as from fixed, paraffin-embedded tissue from two affected and

one unaffected deceased family member. In addition, genomic DNA was isolated from the peripheral blood of 40 normal control individuals. All four exons of the TTR gene were amplified using the polymerase chain reaction (PCR) with oligonucleotide primer flanking individual TTR coding exons kindly provided by J. Herbert as described.[12] Aliquots of the PCR products were visualized on ethidium-stained 1% agarose gels to confirm successful amplification and lack of extraneous products. The amplified DNA fragments were then subcloned into plasmid pCRII (TA cloning kit, Invitrogen Corp., San Diego, CA). Recombinant plasmid DNA was isolated from at least five clones for each exon and sequenced by the dideoxy chain termination method.

Restriction Enzyme Analysis

Amplified samples of exon 2 from family members were digested with *Xba*I (GIBCO BRL, Gaithersburg, MD). As a negative control, amplified sample of exon 2 from human genomic DNA (BIOS Laboratories, New Haven, CT) was also digested with *Xba*I. All of the digested samples underwent electrophoresis on a 5.0% polyacrylamide gel followed by staining with ethidium bromide.

Slot-Blotting with Allele-Specific Oligonucleotides

Two kinds of 20-bp oligonucleotides were prepared, one containing the normal sequence and the other containing the mutation (5′-AAA GTT CTA GGT GCT GTC CG-3′). Oligonucleotides were 5′-end-labeled with [γ^{32}P]ATP using T4 polynucleotide kinase and [γ^{32}P]ATP. PCR products of amplification of exon 2 were denatured by addition of 0.6 mol/L NaOH and neutralized by addition of 2 mol/L ammonium acetate and applied to a nitrocellulose filter with a slot-blotting apparatus (Minifold II, Schleicher & Schuell, Keene, NH). The membrane was rinsed briefly in 2X standard saline citrate (SSC) and cross-linked at 0.3 J/cm^3. Prehybridization was performed in 5X SSC (750 mmol/L NaCl, 75 mmol/L sodium citrate, 20 mmol/L NaH_2PO_4), 5X Denhardt's solution (0.1 g/dl Ficoll, 0.1 g/dl polyvinylpyrrolidone, 0.1 g/dl bovine serum albumin), 20% formamide, and 100 μg/ml salmon sperm DNA for 2 hours at 42°C. A ^{32}P-labeled oligonucleotide probe was added and hybridized at 42°C for 4 hours. Filters were rinse with 2X SSPE (300 mmol/L NaCl, 20 mmol/L NaH_2PO_4, 2 mmol/L EDTA) and 0.1% sodium dodecyl sulfate at 42°C followed by a wash with the same buffer at 65°C and then autoradiography at −80°C for 1 hour.

Results

Immunohistochemistry

The amyloid deposits in the meningeal vessels and subpial areas were immunoreactive with antibodies to TTR, apo E, and amyloid P component (Figure 2). Antibodies against Aβ, prion protein, amyloid A, cystatin C, gelsolin-related amyloid, light chain, amylin, and fibrinogen did not immunoreact with the amyloid deposits. Slight amyloid deposition was also present in systemic organ, including the kidney, skin, ovaries, and peripheral nerves, which showed the same immunoreactivity (data not shown). However, these systemic amyloid deposits were not associated with any clinical symptoms. The TTR immunoreactivity was completely absorbed by preincubating the primary antibody with an excess of TTR. Both apo E and amyloid P components are known to be amyloid-associated proteins found in all types of cerebral and systemic amyloid deposits.[13,14] Faint immunoreactivity was also observed with anti-cystatin C antibodies. Cystatin C immunoreactivity is also known to occur in Aβ deposits, and its presence in these amyloid deposits is probably nonspecific.[15]

DNA Studies

We examined the entire coding sequence of the TTR gene after PCR amplification and subcloning as described in Materials and Methods of five family members (of whom three were affected and two were unaffected) as well as from 40 normal controls. Only one base difference was detected in the gene as compared with the previously determined normal TTR gene sequence[16] in the three affected family members. The presence of G instead of A at the second base of codon 18 (Figure 3) resulted in the conversion of Asp to Gly. To confirm that the mutation was present in all affected family members, we analyzed the genotype of the TTR alleles after PCR amplification of exon 2 DNA by slot-blot hybridization and restriction enzyme digestion. Two 20-mer oligos were used as probes for the hybridization, one with the normal sequence and the other incorporating the G-to-A substitution (see Materials and Methods). As shown in Figure 3, affected family members are heterozygous for this substitution, but the PCR-amplified exon 2 fragment from two unaffected members of the family as well as from a normal control hybridized only with the normal probe but not with the

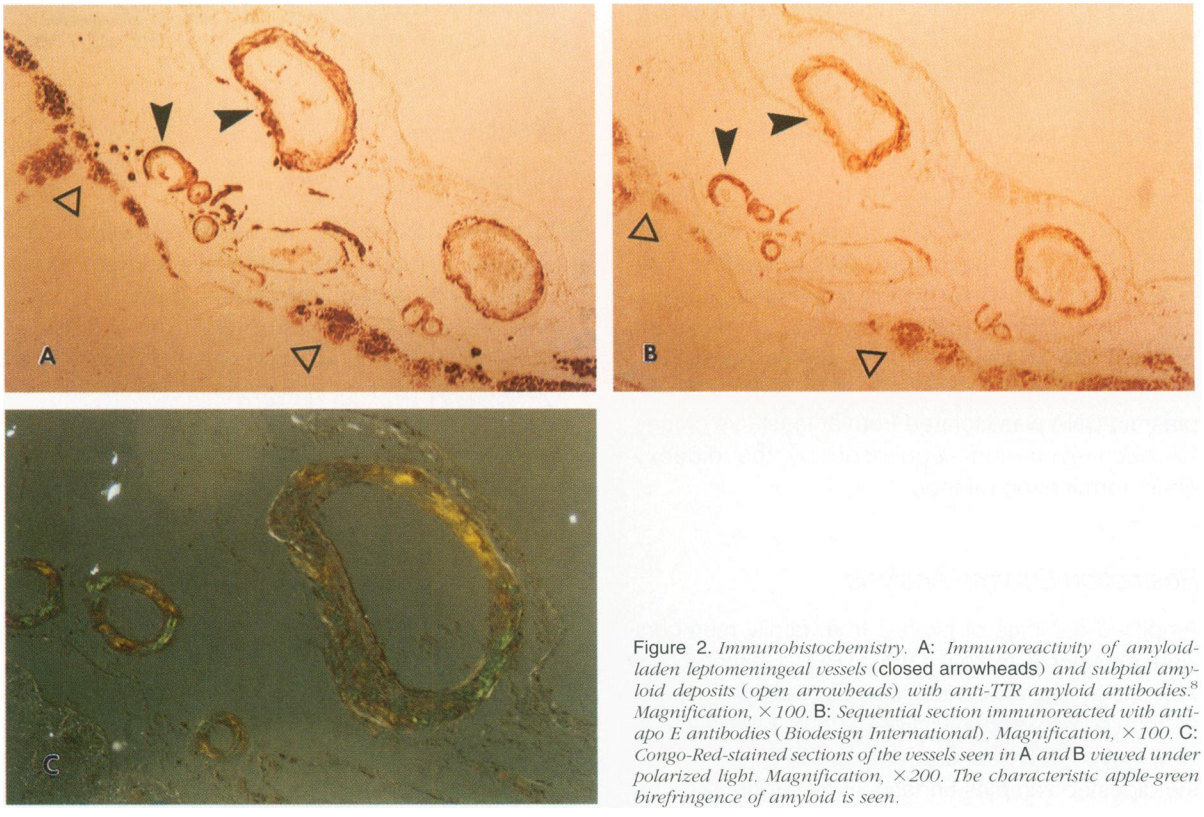

Figure 2. *Immunohistochemistry.* A: *Immunoreactivity of amyloid-laden leptomeningeal vessels* (closed arrowheads) *and subpial amyloid deposits* (*open arrowheads*) *with anti-TTR amyloid antibodies.*[8] *Magnification,* ×*100.* B: *Sequential section immunoreacted with anti-apo E antibodies* (*Biodesign International*). *Magnification,* ×*100.* C: *Congo-Red-stained sections of the vessels seen in* A *and* B *viewed under polarized light. Magnification,* ×*200. The characteristic apple-green birefringence of amyloid is seen.*

mutant one (Figure 4A). The single mutation abolishes an *Xba*I restriction site in the TTR gene. Loss of this site was detected by digestion of PCR-amplified exon 2 DNA with *Xba*I restriction endonuclease (Figure 4B). Two fragments of 129 and 86 bp were observed after digestion of DNA from unaffected individuals. An additional band of 215 bp corresponding to the intact molecule was found in affected patients in an almost equimolar amount. Linkage analysis studies could not be done as the family is too small.

Discussion

CNS amyloid deposition can be viewed as part of the brain's limited repertoire of response to injury.[2,17] Several biochemically distinct proteins can be de-

gttaacttctcacgtgtcttctctacacccag GGC ACC GGT GAA TCC AAG TGT CCT
Gly Thr Gly Glu Ser Lys Cys Pro

18
G
CTG ATG GTC AAA GTT CTA GAT GCT GTC CGA GGC AGT CCT
Leu Met Val Lys Val Leu Asp Ala Val Arg Gly Ser Pro
Gly

GCC ATC AAT GTG GCC GTG CAT GTG TTC AGA AAG GCT GCT
Ala Ile Asn Val Ala Val His Val Phe Arg Lys Ala Ala

GAT GAC ACC TGG GAG CCA TTT GCC TCT GG gtaagttgccaaagaaccctc
Asp Asp Thr Trp Glu Pro Phe Ala Ser Gl

ccacaggactt

Figure 3. *DNA sequence of exon 2 of the TTR gene. Capital letters, exons; lowercase letters, flanking introns; underlined, sequences of the oligonucleotides used for PCR amplification; broken line,* XbaI *restriction site.*

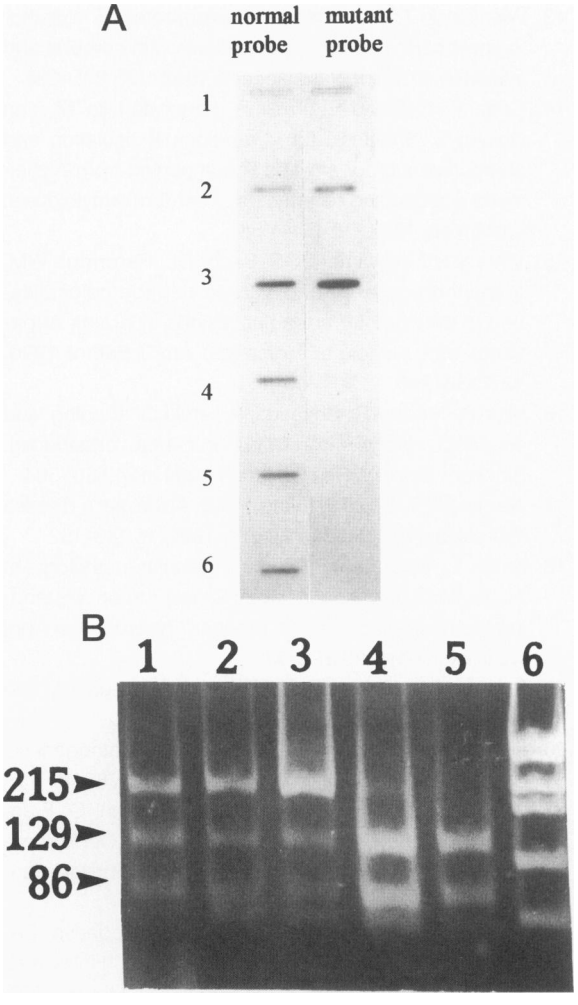

Figure 4. A: *Genotype analysis of PCR-amplified DNA using synthetic oligonucleotide probes.* Slots 1 *to* 3, *from affected family members hybridized to normal and mutant probes.* Slots 4 *and* 5, *from two healthy family members, and* slot 6, *from a normal control, hybridized only to the normal probe.* B: *Restriction pattern of PCR-amplified exon 2 DNA digested with XbaI.* Lanes 1 *to* 3, *affected subjects;* lane 4, *unaffected family member;* lane 5, *normal control; and* lane 6, *φX174 RF DNA* HaeIII *fragments marker. The figures on the left denote the size of the bands in base pairs.*

posited in the CNS as amyloid.[2] The most common cerebral amyloidosis is Alzheimer's disease, in which Aβ is deposited in the form of senile plaques and cerebrovascular amyloid.[17,18] Alzheimer's disease is also characterized by the deposition of abnormally phosphorylated tau as neurofibrillary tangles.[17,18] In prion-related diseases, prion-related protein (PrP) can also be deposited in brain parenchyma and in the cerebrovasculature as amyloid.[19,20] In certain kindreds, PrP amyloid deposition is also associated with neurofibrillary tangle formation.[21] Both PrP and Aβ amyloid deposits can occur in connection with mutations in the PrP and amyloid β precursor protein genes[17–20] as well as in association with linkage or mutations on different genes such as the S182 gene in many familial early onset Alzheimer's disease kindreds[22] and a locus on chromosome 17 in the PrP-related condition of familial progressive subcortical gliosis.[23] However, both PrP and Aβ deposition can occur in the absence of any mutations, similar to TTR-related amyloid.[3,17,19] An additional protein that can form cerebral amyloid is cystatin C, which is found in hereditary cerebral hemorrhage with amyloidosis, Icelandic type.[10] This protein is also known to co-deposit with leptomeningeal amyloid associated with either Aβ or TTR.[15]

In this paper we clearly establish an additional protein that can be associated with CNS amyloid deposition, producing a clinical picture dominated by a progressive neurological decline. The TTR amyloid deposition in our kindred occurs primarily in the leptomeninges and cerebral vessels as well as in subpial and subependymal areas. Interestingly, another kindred has been recently reported, with a mutation at residue 30, where valine is replaced by glycine, associated with similar amyloid deposition in the leptomeninges and periventricular brain tissue; however, in this family the retina and vitreous are affected.[24] Hence, both CNS parenchymal and cerebrovascular amyloid deposition can be related to either Aβ, PrP, or TTR.

TTR, Aβ, PrP, and cystatin C are each expressed in tissues throughout the body.[1,2,10,19] In the diseases mentioned above, the reason for the predominant or exclusive deposition of amyloid in the CNS is not clear. In the family that we are reporting, the exclusive presence of CNS symptoms is likely to be dependent on both the position of the mutation in the TTR gene and the type of amino acid substitution. In our Hungarian kindred, a substitution of the aspartic acid to glycine at codon 18 is found. This codon is in the DAGH β-sheet of TTR.[25] Glycine residues are known to break β-sheet structures; hence, this particular mutation is likely to produce a conformational alteration of the TTR protein. The role of glycine at this position is illustrated by another kindred who also have a mis-sense mutation at codon 18, but with glutamate substituting for aspartate.[1] This family has the typical symptoms of FAP, without CNS dysfunction. In addition to the type and site of the TTR amino acid substitutions, the importance of other genes and local brain-specific factors is illustrated by different kindreds who have similar TTR genotypes, but with disparate phenotypes. For example, the TTR methionine codon 30 mutation (V30M) in northern Portuguese kindreds is associated with a typical onset of symptoms at age 30, whereas in Swedish kindreds with the same mutation, the onset is not until the seventh decade.[1] Additional studies are

needed before the reasons for the predominant, cerebral deposition of amyloid in our kindred and in other CNS amyloidoses will be known.

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