

# A new mutant transthyretin (Arg 10) associated with familial amyloid polyneuropathy

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## Abstract

**We report a new kindred with systemic amyloidosis presenting as peripheral neuropathy in the sixth and seventh decades of life. Polymorphism in exon 2 of the transthyretin (TTR) gene was suggested by single strand conformation polymorphism analysis and determined by direct DNA sequencing. We also developed restriction fragment length polymorphism analysis by polymerase chain reaction using a primer with an induced mutation. The point mutation (cytosine for thymine at position 1038 of the TTR gene) is responsible for substitution of arginine for cysteine at position 10 of the TTR molecule. It is hypothesised that the TTR molecules which have no cysteine have a unique structure in heterozygous TTR polymers and are responsible for amyloid fibril formation.**

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Familial amyloid polyneuropathy (FAP) is a late onset autosomal dominant disorder characterised by amyloid deposition in various organs including peripheral nerves. Recently, single amino acid changes in the amyloid precursor protein, transthyretin (TTR) in most cases, have been shown to relate to the disease, but the mechanism of the onset is not known.<sup>1</sup>

We examined TTR genes of patients with FAP by single strand conformation polymorphism (SSCP) analysis and direct DNA sequencing and found a mutant TTR gene, which codes for arginine at position 10 in place of cysteine. In addition, we developed a restriction fragment length polymorphism (RFLP) analysis to detect the mutant gene.

## Case reports

### CASE 1 (IV-13, FIG 1)

At the age of 59, the proband developed loss of appetite and dysgeusia leading to a 22 kg weight loss, and painful paraesthesiae in the fingertips followed by tingling and loss of sensation. He had urinary frequency, incomplete bladder emptying, and impotence. While he at first had increased flatulence, two years later he developed diarrhoea and subsequently had constipation. When first evaluated sensory deficits had ascended to the upper arms and upper thighs. By the age of 62 years he noted muscle cramping and distal weakness. On examination, there was diffuse distal weakness, moderate for hand intrinsic muscles and mild for arm and forearm muscles, as well as antero-lateral leg muscles. Pinprick and light touch were severely reduced to the level of the hips and shoulders. Vibration sense was mildly reduced in hands and absent in toes. All deep tendon reflexes were absent. He could walk on his toes, poorly on his heels, but could hop. The following laboratory investigation were normal or negative: urine analysis, haematological study, erythrocyte sedimentation rate, rheumatoid factor, antinuclear antibody, serum and urine protein electrophoresis and immunoelectrophoresis, and hepatorenal functions. The electrocardiogram showed a normal sinus rhythm and a probable inferior infarct. The echocardiogram showed normal left ventricular cavity size and ejection fraction, but increased left ventricular wall thickness and mild mitral regurgitation by Doppler. Nerve conduction studies showed absent sensory nerve action potentials in median, ulnar, and sural nerves and low amplitude compound muscle action potential in median, ulnar, peroneal, and posterior tibial nerves. A subcutaneous fat aspirate stained with Congo red was positive for amyloid. Sural nerve biopsy

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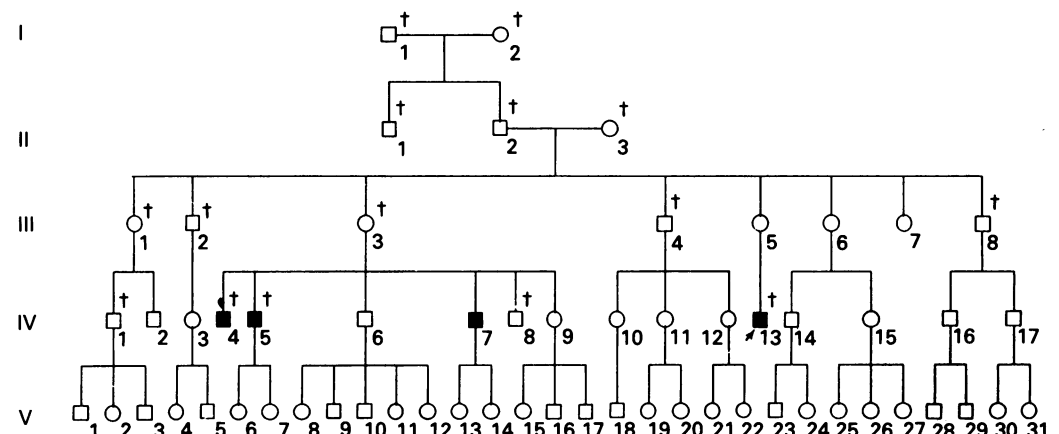


Figure 1 Family pedigree. Solid symbols indicate affected subjects. Arrow denotes proband.

showed severe reduction of the density of myelinated fibres in a diffuse fashion. Methyl violet stains showed unequivocal metachromasia around small endoneurial microvessels.

One year later, he had vitreous opacities in his eyes. He became emaciated and died at the age of 64.

The family pedigree is shown in fig 1. The family is of Hungarian descent with all members at present living in the eastern United States. The proband's mother (III-5), who is 87 years old, has no symptoms of neuropathy. His maternal grandfather (II-2) died at 72 of heart disease and his grandmother (II-3) died at 69 of cancer of the liver. Three of his cousins, whose mother (III-3) died at 68 of heart disease, had similar symptoms.

#### CASE 2 (IV-4)

The oldest cousin noticed numbness in all four extremities at the age of 68, which progressed slowly and involved the arms below the elbows and the legs below the knees.

The general medical examination was remarkable for râles over the lower lobes of the lungs and a systolic murmur at the left lower sternal border. His lower extremities were markedly atrophic. There was muscle weakness, mild in the proximal upper extremities and moderate in the distal upper extremities and throughout the lower extremities. Sensation to pinprick, temperature, light touch, vibration, and proprioception was decreased in the lower extremities to mid-thigh and in the upper extremities to the elbows. Reflexes were decreased in the upper extremities and diminished in the lower extremities. Gait was wide based and unsteady and he was unable to walk on his heels or toes and unable to tandem walk.

A number of serum studies showed only a polyclonal increase in immunoglobulins on serum protein electrophoresis. Rectal and nerve biopsies were read as negative for amyloid deposition. However, reanalysis of these tissues with Congo red staining after the patient's death showed unequivocal deposits of amyloid.

He developed congestive heart failure and died at the age of 70.

#### CASE 3 (IV-5)

The second oldest cousin had a 27 kg weight loss and severe painful paraesthesiae in his lower extremities. He had a colectomy because of paralytic ileus. He died at the age of 67 with congestive heart failure.

#### CASE 4 (IV-7)

A younger brother of cases 2 and 3, who is 63 years old, has complained of hypaesthesiae to temperature in his feet since last year.

### Materials and methods

#### DNA ISOLATION

Total genomic DNA was isolated from peripheral blood cells of cases 1 and 4 and 10 other

family members of this FAP kindred and from a biopsied rectal specimen of case 2.<sup>2</sup>

#### SSCP ANALYSIS

SSCP was performed according to the method of Orita *et al.*<sup>3,4</sup> DNA fragments containing exons 2, 3, and 4 of the TTR gene (215 bp, 258 bp, and 190 bp, respectively) were amplified from the proband and normal control DNA by PCR with the primers described previously using GeneAmp PCR reagents (Perkin-Elmer Cetus, Norwalk, CT), 60 µmol/l dNTPs, and 30 nmol/l <sup>32</sup>P labelled dCTP (final concentration).<sup>5</sup> PCR conditions were: 35 cycles of denaturing at 94°C for one minute, annealing at 65°C for one minute, and extension at 72°C for one minute. PCR products were diluted with 100 volumes of the buffer containing 50% formamide, 0.05% SDS, 0.02% xylene cyanol FF, 0.02% bromophenol blue, and 10 mmol/l EDTA, heated at 95°C for five minutes, and loaded onto a 5% acrylamide gel (2% C, 40 cm × 20 cm × 0.04 cm). The gel was electrophoresed at 4°C for five hours at 40 W, dried, and exposed to Kodak X-Omat film.

#### DIRECT DNA SEQUENCING

Direct sequencing of exons 2, 3, and 4 of the TTR gene of cases 1, 3, and 4 was performed as described previously.<sup>5</sup>

#### RFLP ANALYSIS

PCR amplified DNA fragments containing exon 2 of the TTR gene of the proband and a normal control were digested with restriction enzyme *HgaI* (New England Biolabs, Inc, Beverly, MA) at 37°C overnight, electrophoresed through 4% Nusieve GTG agarose gel, stained with ethidium bromide, and photographed under UV light.

DNA samples from 13 members of this kindred (three affected and 10 persons at risk) were examined by PCR induced mutation restriction analysis (PCR-IMRA). A new primer with a mutation arranged to give a recognition site of *BstUI* in the mutant gene (5'-AGGG-CACCGGTGAATCCACG-3') was synthesised. PCR was performed with this new primer and E2LP2 primer (5'-AGATCTG-CAGAAGTCCTGTGGGAGGGTTCT-3') under the same conditions as described above.<sup>5</sup> The PCR products were digested with *BstUI* (New England Biolabs, Inc, Beverly, MA) at 60°C for an hour and electrophoresed.

### Results

SSCP analysis showed an abnormally migrating band in exon 2 of the proband (fig 2). Direct sequencing of the exon 2 PCR products of cases 1, 3, and 4 showed both thymine and cytosine at position 1038 of TTR (fig 3).<sup>6</sup> Thus they were heterozygous with both a normal TGT (cysteine) and a variant CGT (arginine) codon at amino acid position 10 of mature TTR. The sequence data on exons 1, 3, and 4

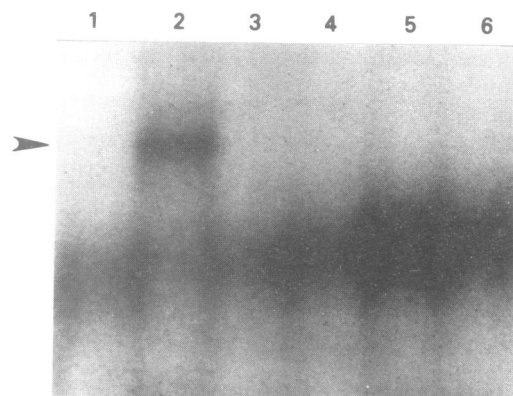


Figure 2 Autoradiograph of SSCP gel for TTR exon 2. Lane 2, proband; lanes 1, 3, 4, 5, and 6, controls. Abnormal migrating band in lane 2 is indicated by an arrow.

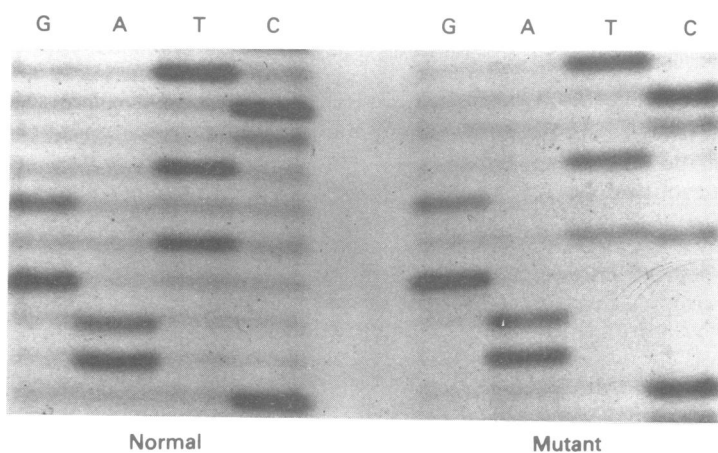


Figure 3 Autoradiograph of direct DNA sequencing of TTR exon 2. Both thymine and cytosine are present at position 1038 of the TTR gene of the proband (indicated by arrow).

of these cases were compatible with those of the normal TTR gene.

RFLP analysis with *Hga*I showed that the proband had the *Hga*I recognition site associated with the Arg10 mutation and therefore had digestion bands of 166 bp and 49 bp, while the normal control showed a band of 215 bp (fig 4). PCR-IMRA with *Bst*UI showed an extra band of 145 bp in the seven samples in addition to the normal band at 165 bp (fig 5). All three patients and four asymptomatic subjects in this kindred were positive for the TTR Arg10 gene and the other six asymptomatic subjects were negative.

#### Discussion

SSCP analysis is a rapid and sensitive method for the detection of DNA polymorphisms. In our preliminary experiments, all single nucleotide base changes of the TTR gene we examined were detected by SSCP, suggesting that it is the most useful method to screen for DNA polymorphisms.

The TTR Arg10 gene has a recognition site of *Hga*I, so it can be detected by RFLP with *Hga*I. However, the enzyme activity of *Hga*I is low and can easily be affected by conditions of

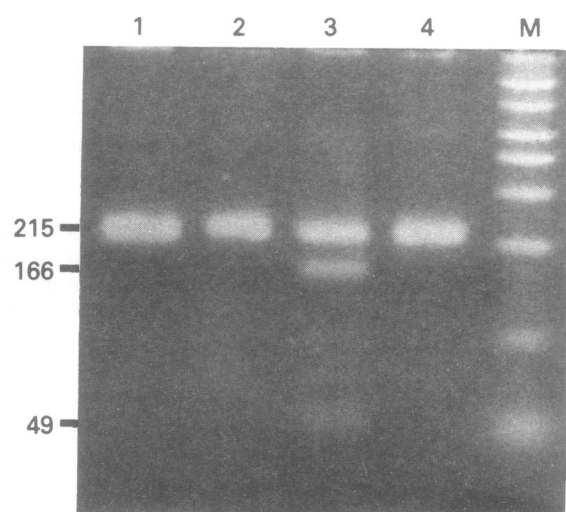


Figure 4 RFLP analysis of *Hga*I digest of the PCR amplified TTR exon 2. Lane 1, *Hga*I digested PCR product of control; lane 2, undigested PCR product of control; lane 3, *Hga*I digested PCR product of the proband; lane 4, undigested PCR product of the proband. Lane M, DNA size marker (BioMarker LOW, Bio Ventures, Inc). The figures on the left denote the sizes of the bands in base pairs.

digestion. The use of restriction enzyme analysis for detection of DNA mutation requires complete and reproducible DNA digestion to prevent false negative results caused by poor digestion. In this respect, PCR-IMRA with *Bst*UI is superior to *Hga*I digestion for detection of the Arg10 mutation. *Bst*UI digestion is less affected by conditions and, in addition, this enzyme is about 100 times less expensive than *Hga*I.

We examined the DNA of 13 subjects in this pedigree and found seven who carry this mutant gene. It is noticeable that none of three female mutant gene carriers, who are 87, 85, and 76 years old, have symptoms of the disease, while four of five male carriers, including one patient whose DNA was not available for testing, developed the disease in their 50s or 60s. It has been observed in other types of FAP that males are affected predominantly or affected at younger ages than females.<sup>7-10</sup> This might suggest that one factor which enhances amyloid deposition relates to male sex.

Affected subjects in this kindred showed sensory and motor neuropathy, bowel disorder, sexual impotence, cardiomyopathy, and vitreous opacity, but no kidney dysfunction. Clinical features of the neuropathy are similar to those of type I FAP, except for age of onset which is later than the mean age of onset of type I.<sup>11,12</sup> Study on a larger number of subjects over a longer period is necessary before reaching definite conclusions on the clinical features of FAP associated with this molecular abnormality.

About 30 different amino acid substitutions of TTR have been reported to cause amyloidosis. The mechanisms whereby variant TTR proteins are involved in deposition in tissues as amyloid fibrils have not been determined. Normal TTR monomer has one free cysteine at position 10. It has been suggested from *in vitro* experiments that disulphide bond formation between TTR molecules might play some

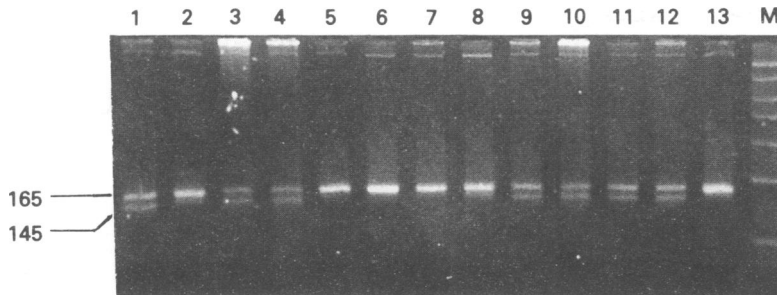


Figure 5 PCR-IMRA of *Bst*UI digest of the *TTR* exon 2. Lane 1, IV-4; lane 4, IV-7; lane 10, IV-10; lanes 2, 3, 5, 6, 7, 8, 9, 11, 12, and 13, subjects at risk in this kindred. Lane M, DNA size marker.

role in amyloid fibril formation.<sup>13,14</sup> It is hypothesised that the conformational modification of TTR protein induced by an amino acid substitution results in the resistance to proteolytic digestion and solubilisation. This variant TTR, which has no cysteine, may cause structural changes in the heterozygous TTR dimer and tetramer that lead to polymerisation of TTR molecules.

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