

A transthyretin variant (alanine 71) associated with familial amyloidotic polyneuropathy in a French family

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Abstract

A transthyretin (TTR) mutation is described in a 44 year old French woman from Caen who presented at the age of 40 with neuropathy in all four extremities, diarrhoea, and orthostatic hypotension. Her father died with a similar syndrome including vitreous opacities. A nerve biopsy from the proband showed amyloid deposits which stained with anti-transthyretin. Direct genomic DNA sequencing of TTR exon 3 showed both thymine and cytosine in the position corresponding to the second base of codon 71. This codes for a variant alanine (GCG) as well as the normal valine (GTG), indicating that the proband is heterozygous for the substitution. Since this substitution does not result in the creation or abolition of a restriction endonuclease recognition site, a new technique (PCR-IMRA) was used to create an RFLP. Using a 24 bp nucleotide mutagenesis primer in the PCR reaction, a new *NspBII* site is created on amplification of the variant allele. With this method a 170 bp TTR exon 3 PCR product was generated for both the normal and the variant allele. On digestion of the PCR product with *NspBII*, DNA from a heterozygous subject showed both the 170 bp undigested product from the normal allele and a 146 bp digestion product from the variant allele. By PCR-IMRA, two of five children of the proband were positive for the variant allele. This non-radioactive technique gives a rapid method for testing subjects at risk for this mutation.

(*J Med Genet* 1993;30:120-2)

Familial amyloidotic polyneuropathy (FAP) can be associated with mutations in the plasma proteins transthyretin, apolipoprotein AI, and gelsolin.¹ While only one apolipoprotein AI (FAPIII) mutation and one gelsolin mutation (FAPIV) have been found associated with amyloidosis, there are numerous mutations in transthyretin. Although the majority of the transthyretin mutations are associated with systemic amyloidosis, three have been described without association with amyloidosis.²⁻⁴ It is obvious that many single amino acid substitutions in the transthyretin molecule may promote fibril formation; however, not all perturbations of TTR structure lead to this end. A new transthyretin mutation has been found in a French family with amyloidotic

polyneuropathy. Characterisation of transthyretin structural changes associated with this mutation may add to the understanding of amyloid fibril pathogenesis.

Materials and methods

A 42 year old French farmer's wife presented with an 18 month history of paraesthesiae in the lower extremities. At the age of 35 she had surgery for a left carpal tunnel syndrome. In addition to signs of neuropathy in the lower extremities, she had a one year history of constipation with rare episodes of diarrhoea and urinary incontinence associated with a decrease in sensation of the passage of urine. Physical examination showed a deficit in motor function and loss of reflexes in the lower extremities. Electromyography showed signs of neuropathy in the lower extremities and a nerve biopsy showed amyloid deposits within the nerve trunk. The proband's father, born in 1920, had a similar syndrome of paraesthesiae in the lower extremities and diarrhoea starting at about the age of 40 (fig 1). By the age of 45 he had decreased sensation to pin prick and temperature in the lower extremities. By 53 years the patient had orthostatic hypotension, severe diarrhoea, and impotence. There was neuropathy in all four extremities, muscle loss in the hands with flexion contractures of the fingers, anaesthesia to heat and cold, and loss of deep tendon reflexes. In addition, he was blind owing to vitreous opacities consistent with amyloidosis. He died at the age of 56 of bronchogenic carcinoma.

Paraffin embedded sections of a nerve biopsy from the proband were stained with anti-transthyretin, as previously described.⁵ DNA was isolated from the proband and her five

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Received 6 May 1992.
Revised version accepted
23 July 1992.

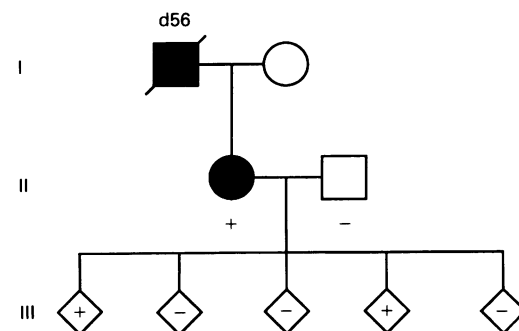


Figure 1 Family from Caen with FAP. The clinically affected proband has two of five children positive for the Ala71 mutation by PCR-IMRA. Her husband tested negative for the mutation.

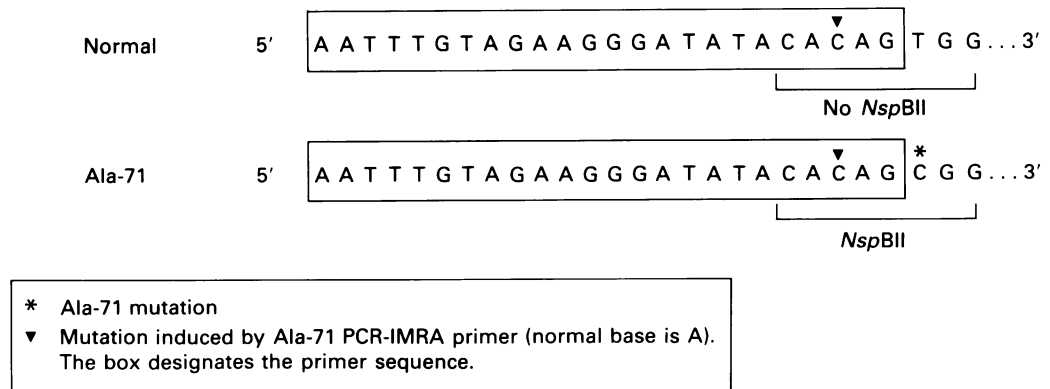


Figure 2 Ala71 PCR-IMRA amplification products.

children using the method of Gautreau *et al.*⁶ Genomic DNA (1 µg) was amplified using the polymerase chain reaction (PCR) and primers flanking the TTR exons 2, 3, and 4.⁷ Conditions for amplification included 30 cycles at 94°C for one minute, 65°C for one minute, and 72°C for one minute. PCR products were separated on 4% NuSieve gels and a Pasteur pipette was used to remove a plug of agarose containing a portion of the amplification product. The agarose plug was melted at 75°C for 15 minutes in 1 ml 10 mmol/l Tris pH 8.0, 1 mmol/l EDTA. Forty cycles of asymmetrical amplification were used with the above conditions to generate single stranded template for direct DNA sequencing. Then 40 mmol/l PCR primer 2 and 0.8 pmol PCR primer 1 were used to amplify 1 µl of the 1 ml agarose extracted samples. Amplification products were extracted with phenol and chloroform and subjected to spin dialysis using centricon 30 microconcentrators. Sequencing of the single stranded templates was conducted as previously described using 7 µl of each retentate with 1 µl of a 5 mmol/l solution of PCR primer 1 and 2 µl of 5 × reaction buffer.⁸ Labelled reactions were electrophoresed for 2 hours at 60 watts, dried overnight, and exposed to X-omat Kodak film for 48 hours at -70°C.

Since no restriction enzyme recognition site is created by the thymine to cytosine transition, a new procedure called PCR-IMRA

(polymerase chain reaction-induced mutation restriction analysis) was used to detect carriers of the TTR variant allele. For this RFLP analysis, a 24 nucleotide mutagenesis primer (A71 PCR-IMRA, fig 2) was used in the PCR reaction. This primer anneals immediately 5' to the site of the mutation, contains a single mismatch near its 3' end, and creates an *Nsp*BII site in the PCR product derived from the mutant allele. To reduce non-specific background, an initial amplification of exon 3 was performed. The PCR products were electrophoresed on 4% NuSieve gels and agar plugs collected in 0.5 ml 1 × TE as described above. The agarose plugs were melted and 1 µl was used as the template for a subsequent PCR reaction using 150 ng each of primers A71 PCR-IMRA and E3LP2. Thirty-five amplification cycles were performed using the same conditions as above. Completed reactions were extracted with chloroform and a 10 µl aliquot digested with 5 U of *Nsp*BII using conditions recommended by the supplier to distinguish between the amplification product of the normal allele and that of the variant allele. Genomic DNA samples from seven members of the family (the proband, her husband, and five children) were tested.

Results

Paraffin embedded sections of a nerve biopsy from the proband showed amyloid deposits within the substance of the nerve. Immunohistochemistry with anti-transthyretin showed positive staining. Direct DNA sequencing of genomic DNA from the proband showed the presence of both thymine and cytosine in the position corresponding to the second base of TTR codon 71 (fig 3). This indicates that the subject is heterozygous for both the normal valine and a variant alanine at position 71 of transthyretin. No other mutation was found in exon 2, 3, or 4 of transthyretin from the proband. RFLP analysis using PCR-IMRA was performed on DNA from the proband and her five children. Using the 24 bp nucleotide mutagenesis primer, all subjects gave the expected 170 bp TTR exon 3 PCR product. Digestion with *Nsp*BII showed the proband and two of her five children to be heterozygous for the variant allele with both the 170 bp undigested product of the normal allele and

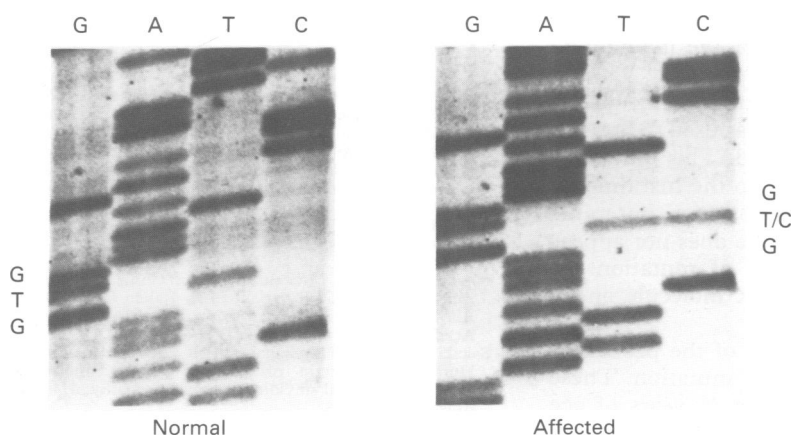


Figure 3 DNA sequence of transthyretin exon 3. An affected subject shows both thymine and cytosine in the second position of codon 71 giving both the normal sequence GTG (valine) and the variant GCG (alanine).

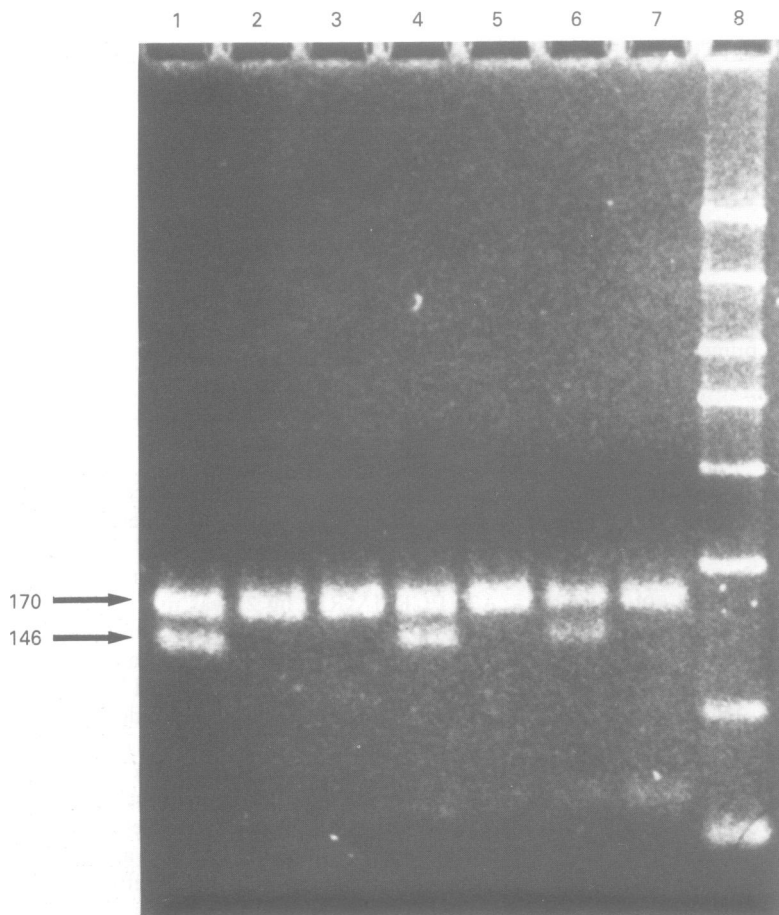


Figure 4 Ethidium bromide stained agarose gel of *NspBII* digested PCR products. Lanes 1, 4, and 6 show both the normal 170 bp and the digested variant products indicating heterozygosity for the Ala71 allele. Lane 8 contains *biolow* (Bioventures) size markers.

the 146 bp digestion product of the variant allele on agarose electrophoresis (fig 4).

Discussion

This French family from Caen represents another kindred with FAP similar to the syndrome seen in Portuguese, Swedish, and American kindreds. This syndrome has fairly early onset (in the 30s or 40s) and includes peripheral neuropathy in all extremities, carpal tunnel syndrome, orthostatic hypotension, diarrhoea, and vitreous opacities. After the proband had been tested by RFLP analysis and found to be negative for the methionine 30, tyrosine 77, serine 84, isoleucine 33, and alanine 60 variants of transthyretin, direct genomic DNA sequencing was used to find the new mutation in this kindred. This mutation, alanine 71, is in proximity to the histidine 69 which also causes vitreous opacities, but minimal neuropathy. So far there does not appear to be any clear association of mutations in specific regions of the TTR molecule and a particular clinical syndrome.

In the present study, two of the proband's children carry the alanine 71 mutation. These children are between 15 and 20 years of age

and so far are asymptomatic for the syndrome. It should be noted that the father of these children was tested and found to be homozygous normal by the PCR-IMRA for alanine 71.

Familial amyloidotic polyneuropathy associated with transthyretin mutations has been found in a number of kindreds in France and, to date, four separate transthyretin variants have been found in French families. These include the methionine 30, tyrosine 77, alanine 49, and alanine 71 variants.^{9,10} While the methionine 30 TTR mutation is presumed to be of Portuguese origin, haplotype analysis has not been performed to confirm this hypothesis. The alanine 49 variant has been found in an Italian family in addition to a French family from Bordeaux.¹¹ Again, a common ancestry is not known. On the other hand, the tyrosine 77 family from Amiens does not share the haplotype of the German/Illinois family nor other subjects in the United States who have the tyrosine 77 mutation.¹² It will be of interest to see if the French alanine 71 mutation is found in other parts of the world.

This work was supported by Veterans Affairs Medical Research (MRIS 583-0888), and grants from RR-00750 (GCRC), United States Public Health Service, NIDDK-34881, NIDDK-42111, National Institute of Arthritis Metabolism and Digestive Diseases (AM205-82), The Arthritis Foundation, and The Marion E Jacobson Fund.

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