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A NOVEL TRANSTHYRETIN MUTATION AT POSITION 30 (Leu for Val) ASSOCIATED WITH FAMILIAL AMYLOIDOTIC POLYNEUROPATHY

Tatsufumi Murakami[1], Tetsushi Atsumi[2], Shuichiro Maeda[3], Sumio Tanase[3], Kuniko Ishikawa[2], Shuji Mita[1], Toshihide Kumamoto[1], Shukuro Araki[1], and Masayuki Ando[1]

[1]The First Department of Internal Medicine, Kumamoto University School of Medicine, Honjo 1-1-1, Kumamoto, Japan

[2]Seirei-Hamamatsu General Hospital, Sumiyoshi 2-12-12, Hamamatsu, Japan

[3]Department of Biochemistry, Kumamoto University School of Medicine, Honjo 2-2-1, Kumamoto, Japan

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Summary: A novel transthyretin (TTR) mutation associated with familial amyloidotic polyneuropathy was detected in a Japanese patient. Single-strand conformation polymorphism analysis and sequence analysis of polymerase chain reaction (PCR)-amplified exons of the patient's TTR gene revealed a point mutation resulting in a substitution of leucine for valine at position 30. As the mutation creates a *Cfr*13I site, it was confirmed by PCR and restriction analysis. Our finding indicates the importance of position 30 in TTR-derived amyloid fibril formation.

Familial amyloidotic polyneuropathy (FAP) is an autosomal dominantly inherited systemic amyloidosis (1). The amyloid fibrils derived from FAP consist of variant transthyretin (TTR) with single amino acid substitutions. Twenty-one distinct TTR point mutations have been found in association with FAP or cardiac amyloidosis (2-6), the most common being the Met-for-Val substitution at position 30. We reported that the main cause of the Japanese type I FAP is the presence of a point mutation in the first base of codon 30 of the TTR gene and which results in the Met-for-Val substitution (7, 8). The effect of several of the above single amino

Abbreviations: TTR, transthyretin; PCR, polymerase chain reaction; FAP, familial amyloidotic polyneuropathy; SSCP, single-strand conformation polymorphism.

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acid substitutions on the structure of TTR molecule was analyzed using a computer graphics system with the known tertiary structure of the molecule (9). The structure of the Met 30 variant TTR was analyzed by X-ray crystallography to 2.3 Å resolution (10). These data suggested that most of the substitutions may alter the outer surface of the TTR molecule (11). However, mechanisms by which single amino acid substitutions result in fibril formation are unknown.

Analysis of the effect of different FAP-associated substitutions at the same amino acid position may give further insight into the mechanisms of fibril formation. Herein we report a novel FAP-associated mutation at position 30 of the TTR molecule. Our finding suggests that even small conformational changes caused by mutation of Val 30 can lead to amyloid fibril formation.

Materials and Methods

Patient and family

The proband is a 53-year-old Japanese woman in whom weight loss and diarrhea occurred when she was 51. She was admitted to Seirei-Hamamatsu General Hospital at age 53 in 1991. Neurological examination revealed sensory disturbance in the lower extremities. Sural nerve biopsy revealed deposits of amyloid which stained positively with anti-human TTR antibody. There were no vitreous opacities and no family history suggestive of FAP.

DNA amplification

Total genomic DNA was isolated from peripheral blood leukocytes. All four exons of the TTR gene were amplified using the polymerase chain reaction (PCR) with four 20-base oligonucleotide primer sets. The sequence and position of the primers were previously described (3). We also used an oligonucleotide as a negative strand primer [5'-TATGCAGA TGATGTGAGCCT-3'; The nucleotide position of the 5' end of the primer is at position +1240 (12)] to amplify the 358 bp fragment containing exon 2 of the TTR gene for single-strand conformation polymorphism (SSCP) analysis. PCR was carried out as described (3).

SSCP analysis

SSCP analysis was performed essentially as described by Orita *et al.* (13). The PCR products of each exon of the TTR gene were purified by electrophoresis on a 4% NuSieve GTG agarose gel (FMC BioProducts), and precipitated by ethanol. The pellets were dissolved in 20 µl of H_2O and 0.5 µl was amplified by PCR (30 cycles) in a 5 µl volume containing 6.25 nmol of each dNTP, 5 pmol of each primer, 2 mCi of [α-^{32}P] dCTP (Amersham, 3000Ci/mmol), and 0.5 µl of the amplifying buffer. The PCR products were then added to 100 volumes of loading buffer containing 95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol. These samples were denatured by heating at 90°C for 10 min and 2 µl was immediately loaded on a 7.5% polyacrylamide gel (0.3 mm x 20 cm x 40 cm). Electrophoresis was done at 4°C at 30 W for 3 hrs, the gel was dried and autoradiographed at -80°C for 12 hrs.

Subcloning and sequencing of PCR products

The amplified DNA fragments were purified by electrophoresis on a 4% NuSieve GTG agarose gel, phosphorylated by T4 polynucleotide kinase (Takara), rendered blunt-ended with Klenow fragment (Takara), and subcloned into *Hinc*II site of pUC18 plasmid. Recombinant plasmid DNA was isolated from 3 clones derived from exon 1, 5 clones derived from exon 2, and 6 clones each from exons 3 and 4. The DNA was sequenced according to the dideoxy chain termination method. Cloning procedures were carried out in accordance with guidelines for research involving recombinant DNA molecules, issued by the Ministry of Education, Science and Culture of Japan.

Detection of the point mutation using restriction analysis

The amplified samples of exon 2 from the patient and her mother were digested with *Cfr*13I (Takara). As negative controls, the amplified samples of exon 2 from 5 Japanese patients with FAP type I of different pedigree and 10 disease-free individuals were digested. As a positive control, the amplified sample of exon 2 from a Japanese patient with FAP who carries the TTR Gly 42 gene was digested. All the digested samples underwent electrophoresis on a 1.5% agarose gel and were stained with ethidium bromide.

Three-dimensional molecular model of TTR

A stereo-drawing of the human TTR dimer was carried out on a Silicon graphics Personal IRIS 4D/25 computer using Insight II/Discover program (Biosym Inc.) The X-ray crystallographic data of human TTR (9) deposited with the Protein Data Bank was used.

Results

For a rapid detection of the mutation-containing exon, we applied the SSCP technique (13) to all exons of the TTR gene. In the SSCP analysis of the amplified fragments containing exon 1, 3, and 4, the migration patterns were the same in the patient and the control subject (Fig. 1, lanes 1, 3, and 4). A difference in the band patterns was observed in exon 2. The fragment containing exon 2 from the patient showed one abnormal band in addition to normal bands (Fig, 1. lane 2).

We first examined the sequence of the exon 2 of the TTR gene after PCR amplification and subcloning, as described in Materials and Methods. The only difference detected in the patient's exon 2 was the presence of C instead of G in the position corresponding to the first base of codon 30 (Fig. 2); this substitution resulted in the conversion of Val to Leu. We then examined the sequence of the exons 1, 3, and 4. In accordance with the results of the SSCP analysis, no other mutations were detected in these exons.

The mutation creates a restriction endonuclease site for *Cfr*13I. To confirm that the mutation detected in the amplified DNAs is actually present in genomic DNA of the patient and to analyze the genotype of the TTR alleles of the patient and her mother, we digested the PCR-amplified exon

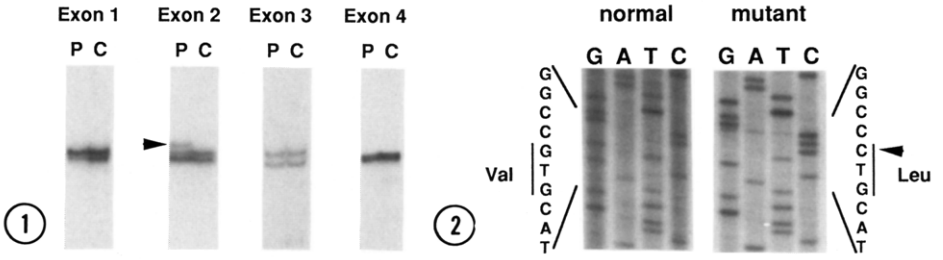

Figure 1. PCR amplification and SSCP analysis of exons of the TTR gene in the patient. The arrowhead marks an abnormal band in the patient. Lanes P; sample from a patient. Lanes C; sample from a control subject.

Figure 2. DNA sequence analysis of exon 2 of the TTR gene of the patient. Autoradiogram of the sequencing gels of the normal and mutant TTR alleles. The arrowhead indicates the position of the C-for-G substitution.

2 regions with *Cfr*13I. The amplified DNA derived from the patient yielded 212 bp and 300 bp fragments in addition to the uncleaved 512 bp fragment (Fig. 3, lane 1). This result shows that the patient is heterozygous for the TTR gene, carrying one normal and one mutant gene. On the other hand, the amplified DNA derived from her mother, 5 patients with FAP type I carrying the TTR Met 30 gene (Fig. 3, lanes 2-7), and 10 disease-free individuals (data not shown) could not been cleaved with *Cfr*13I. The amplified DNA from a patient with FAP carrying the TTR Gly 42 gene yielded 252 bp and 260 bp fragments in addition to the uncleaved 512 bp fragment as described previously (Fig. 3, lane 8) (14).

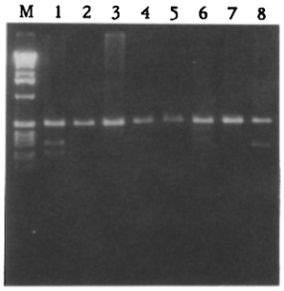

Figure 3. Detection of the TTR Leu 30 gene by restriction analysis of amplification products. Lane 1; patient; Lane 2; patient's mother; Lanes 3-7; patients with type I FAP carrying the TTR Met 30 gene. Lane 8; patient with FAP carrying the TTR Gly 42 gene. Lane M; size marker (1kb ladder).

Discussion

We found a novel point mutation in exon 2 of the TTR gene in a Japanese patient with FAP. The C-for-G substitution resulted in conversion of Val to Leu at position 30. Because other affected family members were not available, we could not demonstrate a direct link between the mutation and FAP. However, TTR-derived amyloid deposition in the affected tissue, plus the fact that no mutation was detected in the other sequences of exons of the patient's TTR gene, and the well-documented link between other TTR mutations and FAP, taken together, support the conclusion that the Leu 30 mutation is the cause of FAP, in this patient.

The first base (G) of the Val 30 codon (GTG) is part of the CpG dinucleotide mutation hot spot sequence and it is suggested that the high frequency of the Met-for-Val substitution at position 30 is due to the high mutation rate of the CpG sequence to TpG (15). On the other hand, the C-for-G substitution which causes the Leu-for-Val substitution in this patient cannot be explained by the mutation hot spot hypothesis. This may explain why the Leu 30 mutation is less common than the Met 30 one.

This is the third FAP-associated mutation to be found at position 30 of the TTR molecule. TTR is a tetramer composed of four identical subunits (9). Residue 30 is located in the B strand of the TTR subunit and in the core of the subunit (9). The side chain of Val 30 makes contact of 5 Å or less with non-polar side chains of Val 14, Val 16, Val 28, Val 32, Leu 55, Leu 58, Val 71, and Ile 73 (Fig. 4). The main chain of B strand makes hydrogen bonding to C and E strands to construct the ß-sheet (9). The larger side chain of Met at position 30 expands the TTR tetramer (10) and the Ala 30 mutation may reduce hydrophobic interaction in the core (4). The hydrophobicity and accessible surface area of the Leu side chain is similar to

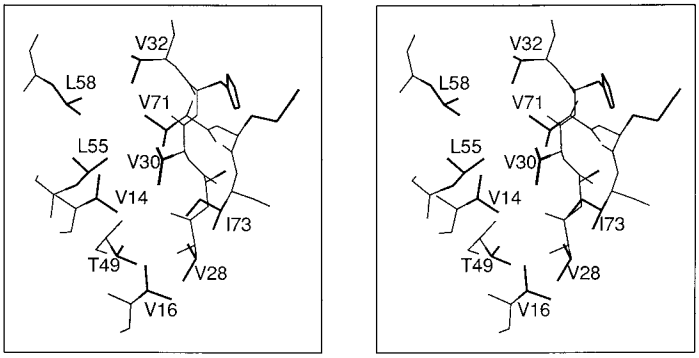

Figure 4. Stereo-drawing of the cluster of residues which make close contact with the side chain of Val 30. Thick lines represent side chains.

that of the Val side chain, as compared with bulky Met side chain. The Leu 30 mutation appears to cause only small conformational changes in the TTR molecule. Because the subunits are compactly arranged in the tetramer (9), it is likely that slight conformational changes caused by mutations of Val 30 or amino acid residues which make close contact may alter the outer surface of the TTR tetramer and predispose it to precipitation as amyloid fibrils. Characterization of the conformational change caused by the Leu 30 mutation is expected to elucidate common mechanisms responsible for TTR-derived amyloid fibril formation.

Interestingly, among the twenty-two TTR point mutations known to be associated with FAP or cardiac amyloidosis, seven (Met 30, Ala 30, Leu 30, Ala 49, Pro 55, His 58, and Arg 58) have been located on the residues shown in Fig. 4. On the other hand, a comparison of the primary structure of human and mouse TTR subunits revealed that both are composed of 127 amino acid residues and that the overall homology is 80% (16). Nine of the ten amino acid residues shown in Fig. 4 are conserved at exactly the same positions in the human and mouse TTR's, the only exception being the substitution at position 73. Here, Ile is substituted by a chemically similar Leu in the mouse TTR. Because all these residues make close contact with Val 30, it may well be that point mutations at residues 14, 16, 28, 32, 71, or 73 are associated with amyloidosis.

Acknowledgments

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