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Identification of a novel transthyretin variant (Val^{30}→Leu) associated with familial amyloidotic polyneuropathy

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A novel variant transthyretin which contains a leucine-for-valine substitution at position 30 was isolated and identified in the serum of a patient with familial amyloidotic polyneuropathy (FAP). The amino acid substitution was proven to result from a guanine-to-cytosine change at the first base of codon 30 located in exon 2 in the mutated transthyretin gene by restriction fragment length analysis on the amplified transthyretin gene using *Cfr*13 I. The study indicates that the point mutation of the transthyretin gene is a cause of the disorder.

Familial amyloidotic polyneuropathy; Variant transthyretin; Amino acid sequence; Point mutation; DNA diagnosis

1. INTRODUCTION

Familial amyloidotic polyneuropathy (FAP) is an autosomal dominant, inherited disorder with systemic deposition of amyloid fibrils. Clinically, FAP is characterized by progressive sensory, autonomic, and motor polyneuropathy followed by fatal cardiac and renal failure [1]. Recent biochemical and molecular biological studies [2] have shown FAP to be a molecular disorder of transthyretin (also called prealbumin). The major protein constituent of FAP amyloid is a variant transthyretin which has one amino acid substitution resulting from one base change on the transthyretin gene. In type I FAP, the most widespread form of FAP, amyloid protein consists of a variant transthyretin with a methionine-for-valine substitution at position 30 [3–5].

We have studied a Japanese patient exhibiting symptoms of type I FAP. We identified a novel position 30 mutation in a variant transthyretin, being distinct from the methionine substitution. We also analyzed the transthyretin gene to find the base change causing the amino acid substitution.

2. MATERIALS AND METHODS

2.1. *Purification of serum transthyretin*

Five ml of the serum was obtained from a 53-year-old Japanese woman with FAP. She gradually developed dysesthesia in the legs at age 50, with subsequent progressive sensorimotor polyneuropathy and various autonomic dysfunctions including periodic severe anorexia and nausea. Her rectal and sural nerve biopsies revealed amyloid deposits which were specifically stained by anti-human transthyretin antiserum. Her serum was loaded on an Affi-Gel Blue resin column (1.5 × 25 cm, BioRad) pre-equilibrated with the above phosphate buffer as reported elsewhere [6]. Unretained fractions were subjected to immunoaffinity chromatography on an anti-transthyretin IgG Affi-Gel 10 column (0.8 × 6 cm, BioRad). After the column was washed with 50 mM sodium phosphate buffer (pH 7.4), bound transthyretin was eluted with 3 ml of 1% diethyl amine solution. Transthyretin was finally purified as a single peak by reverse-phase high performance liquid chromatography (HPLC) on a TSK ODS-Phenyl 5PW-RP column (4.6 × 25 mm, Tosoh Co. Ltd.). A linear gradient of acetonitrile (CH_3CN) from 10% to 60% in 0.1% trifluoroacetic acid (TFA) was employed for 40 min at a flow rate of 1.0 ml/min.

2.2. *Amino acid sequence analysis*

One-half of the purified serum transthyretin was treated with trypsin in 0.1 M Tris-HCl buffer (pH 8.0) for 2 h, and the resulting peptides were analyzed by reverse-phase HPLC on a TSK ODS SIL 120 A column (4.6 × 250 mm, Tosoh). A linear gradient of CH_3CN from 0% to 60% in 0.1% TFA was employed for 60 min at a rate of 1.0 ml/min. Tryptic peptides of normal human transthyretin were also analyzed by the same HPLC system. Sequence analysis was performed by a model 470A protein sequencer linked on-line to a model 120A PTH analyzer (Applied Biosystems). PTH-amino acids were measurable at concentrations as low as 0.5 pmol, and 5 pmol of a standard PTH-amino acid mixture (Pierce) was routinely used as a calibration mixture.

2.3. *Transthyretin gene analysis*

Total genomic DNA was isolated from the patient's peripheral blood leucocytes and from those of normal individuals. A 368 base pairs (bp) subsequence of the transthyretin gene including exon 2 was amplified with 2 kinds of 24 base oligonucleotide primers (5′-ATTGTCGACACTTACGTTCCTGAT-3′ and 5′-TGTAAATTCT-TTAGCAGATGATGT-3′) using Gene Amp PCR kit (Perkin Elmer Cetus). Amplified DNA was digested with endonuclease *Cfr*13 I (TaKaRa Shuzo Co. Ltd.), and the resulting DNA fragments were

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electrophoresed through 3% Nusieve 3:1 Agarose gel (FMC BioProducts).

3. RESULTS AND DISCUSSION

Serum transthyretin was efficiently purified by three-step chromatography. The amino acid sequence of the patient's serum transthyretin was determined by comparing its tryptic peptide map with that of normal human transthyretin as shown in Fig. 1. One extra peptide, peak T-4*, was observed in the patient's map. On the other hand, the T-4 peak area of the patient's transthyretin was decreased to 60% of that in normal transthyretin. Amino acid sequences of peptides T-4 and T-4* were determined with 100 pmol of each peptide by an automated gas-phase sequencer. Peptide T-4 was confirmed to correspond to the region (residues 22–34) of normal transthyretin. The amino acid sequence of peptide T-4* was determined to be: Gly-Ser-Pro-Ala-Ile-Asn-Val-Ala-Leu-His-Val-Phe-Arg, in which a leucine as the ninth amino acid residue was substituted for a valine in peptide T-4 from normal transthyretin. All the tryptic peptides except T-4* from the patient's transthyretin were observed at the same positions as those of the control on reverse phase HPLC. Thus, the transthyretin was clarified to be a variant transthyretin with a leucine-for-valine substitution at position 30. The ratio of T-4 and T-4* in the patient's map was 3:2, indicating that the patient's serum transthyretin consisted of a mixture of normal and the variant transthyretin. The relatively low ratio of T-4* in comparison to T-4 is probably due to amyloid deposition of the variant transthyretin in tissue.

The leucine-for-valine substitution can be ascribed to the change of GTG to CTG in codon 30 of the transthyretin gene. This mutation creates a new cleavage site for *Cfr*13 I (GGCCG→GGCCC), yielding two fragments of length 156 and 212 bps. After digestion with this endonuclease, the patient's DNA showed two extra bands of 156 and 212 bps in addition to a normal band of 368 bps (Fig. 2), indicating the presence of G→C transversion in the mutated transthyretin gene as shown in Fig. 3. This result shows that DNA diagnosis of the patients and carriers of the FAP can be done using restriction fragment length analysis on the amplified transthyretin gene.

Transthyretin is a stable and symmetrical tetramer composed of four identical subunits of 14 kDa. A transthyretin molecule has extensive β-sheet structure with monomers having eight β-strands [7]. This characteristic conformation is thought to predispose toward amyloid fibril formation, sometimes causing senile systemic amyloidosis by normal transthyretin in normal-aged individuals [8]. A conformational modification produced by an amino acid substitution would lead to more formation of amyloidogenic conformation in the variant transthyretin molecule. More than 15 kinds of variant

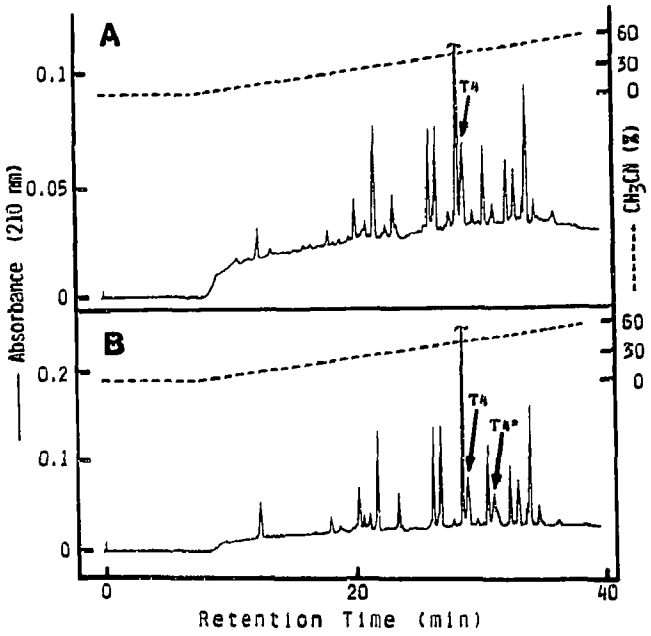


Fig. 1. HPLC profile of tryptic peptides from (A) normal serum transthyretin and (B) patient's serum transthyretin. Tryptic digests were applied to a column of TSK ODS SIL 120A (4.6 × 250 mm) and eluted with a linear gradient of CH_3CN (0–60%) in 0.1% TFA for 60 min at a flow rate of 1.0 ml/min.

transthyretins have been found in FAP of different ethnic origins. Four variant transthyretins are known to be involved in hereditary amyloid cardiomyopathy, and four others are apparently non-amyloidogenic [9]. The patient studied here had amyloid deposits in the rectum

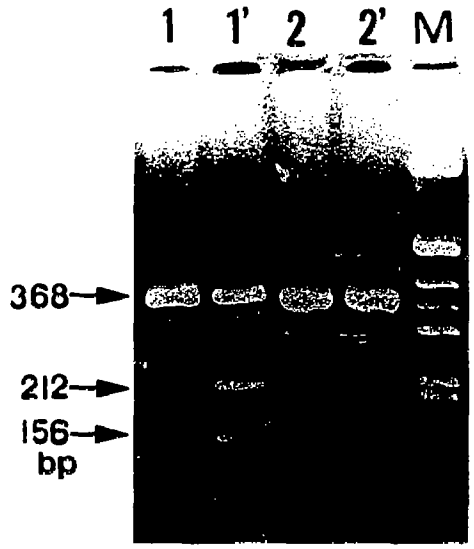


Fig. 2. Analysis patterns of amplified transthyretin DNA digested with *Cfr*13 I. (Lane 1) Non-digested patient's DNA; (1') digested patient's DNA; (2) non-digested control DNA; (2') digested control DNA; (M) 1 kb DNA Ladder (Gibco BRL Life Technologies Inc.).

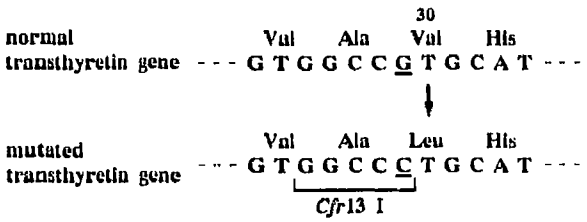


Fig. 3. Partial amino acid and nucleotide sequences of Leu[30] variant transthyretin. A new cleavage site (GGCCC) for *Cfr*13 I is produced in the mutated transthyretin gene.

and sural nerve which were confirmed by alkaline Congo-red staining. The amyloids were specifically stained by anti-human transthyretin antiserum. Thus, the leucine-for-valine substitution is concluded to be a pathogenic one, not a polymorphic substitution.

Leucine at position 30 identified in this study is the third mutation at the position found to be associated with FAP. A methionine-for-valine substitution at position 30 has been found in type I FAP, the subclass of FAP most widely distributed throughout the world [3–5]. An alanine-for-valine substitution at the same position was reported in FAP of German origin [10]. These three types of FAP with different amino acid substitutions have remarkably similar clinical manifestations. Valine-30 is situated at the beginning of one β-strand, being placed into the subunit core located between the two β-sheets. The alteration to hydrophobic amino acids at the position would favor aggregation and amyloid fibril formation of variant molecules, causing clinical development of FAP.

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