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A novel mutant (transthyretin Ile-50) related to amyloid polyneuropathy

Single-strand conformation polymorphism as a new genetic marker

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DNA sequence polymorphisms in transthyretin (TTR) genes were investigated by single-strand conformation polymorphism (SSCP) analysis of polymerase chain reaction products. The amplified DNA fragments that encode each exon of the normal TTR gene showed two bands, representing the two complementary single strands of DNA. In one patient with amyloid polyneuropathy, the exon 3 DNA showed a unique, aberrant migration pattern. Direct sequencing analysis of the amplified exon 3 revealed a single base change (G-to-T), resulting in a novel amino acid substitution (Ser-50 → Ile). We also present the SSCP patterns for five known Japanese TTR variants.

Familial amyloid polyneuropathy; Transthyretin variant; Single-strand conformation polymorphism analysis; Japanese variant

1. INTRODUCTION

The human transthyretin (TTR) is a 55 kDa plasma protein composed of four identical subunit molecules, each consisting of 127 amino acids. Only a single amino acid substitution in the molecule can contribute to amyloid fibril formation in familial amyloid polyneuropathy (FAP). Although FAP had been estimated to be very rare in Japan, using molecular cloning techniques we have disclosed four different genotypes in Japanese FAP [1–3]. Further screening and characterization of TTR variants may facilitate an understanding of the molecular basis for the amyloidosis associated with mutant TTR.

We here report that single-strand conformation polymorphisms (SSCP) provide novel genetic markers for variant TTR genes and identify a novel TTR mutant in a patient with amyloid polyneuropathy.

2. MATERIALS AND METHODS

2.1. *DNA samples*

Genomic DNAs were prepared from peripheral blood leukocytes from 50 healthy volunteers and 50 patients without peripheral neuropathy. The DNA samples obtained from a patient with TTR Met-30 and the four reported patients [1–3], each having one of the following Gly-42, Arg-50, Arg-58 or Cys-114, were also tested.

Abbreviations: TTR, transthyretin; FAP, familial amyloid polyneuropathy; SSCP, single-strand conformation polymorphism(s); PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; ASO, allele-specific oligonucleotide.

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2.2. *Patient*

A 56-year-old Japanese woman, living in Oita Prefecture, had a 7-year-history of sensory disturbance and muscular atrophy in the lower limbs. The autonomic dysfunction, especially orthostatic hypotension, limited here ambulation. Amyloid deposition was proven by a sural nerve biopsy. Genomic DNA was prepared from peripheral blood leukocytes.

2.3. *SSCP analysis of polymerase chain reaction (PCR) products*

Four pairs of oligonucleotide primers described previously [3] were used to amplify the coding sequence in each exon. Genomic DNA (50 ng) was amplified for 30 cycles (94°C for 30 s, 56°C for 45 s and 72°C for 60 s) using the Gene Ampkit (Perkin Elmer Cetus) as recommended by the supplier except for the addition of [α-^{35}S]dCTP (0.35 μl, 1,000 Ci/mmol) in a final volume of 5 μl. Each product was diluted 10-fold with loading dye (95% formamide, 20 mM EDTA, 0.05% Bromophenol blue and 0.05% xylene cyanol), and heated at 90°C for 10 min. 2 μl of each sample was electrophoresed on 6% non-denaturing polyacrylamide gels containing 45 mM Tris-borate (pH 8.3), 1 mM EDTA and 5% glycerol at 26°C for 3 h at 2,000 V. The gel was dried and exposed to Kodak X-Omat AR film with intensifying screen at −70°C for 24 h.

2.4. *Direct sequencing analysis*

The DNA fragments containing exon 3 of the TTR gene were amplified and isolated by agarose gel electrophoresis. The purified DNA was used as a template for the 2-step PCR amplification to obtain a single-stranded DNA suitable for direct sequence analysis. The first step was 20 cycles of symmetrical amplification in 100 μl of reaction mixture, using 10 ng of the purified DNA and 5 pmol of primers of both ends. The second step was 18 cycles of asymmetric amplification (94°C for 15 s, 57°C for 15 s and 72°C for 30 s) from 20 μl of the first-step PCR product using 50 pmol of primer from either end. The amplified single-stranded DNA was dialyzed using the Centricon-30 microconcentrator (Amicon) and was sequenced by modification of the techniques of Nichols et al. [4]. Restriction fragment length polymorphism (RFLP) analysis (see legend to Fig. 3) and allele-

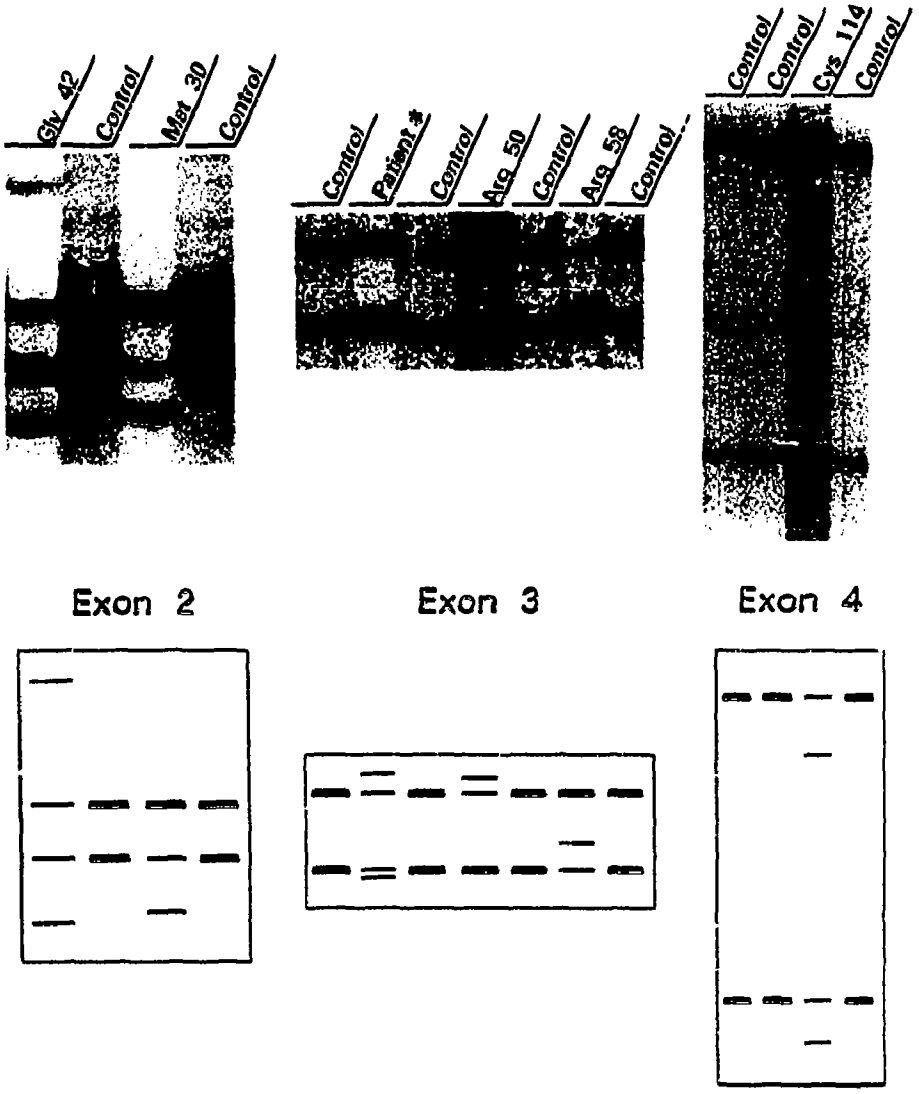

Fig. 1. SSCP analysis of exon 2, 3 and 4 of TTR genes. Electrophoresis was performed on 6% non-denaturing polyacrylamide gels containing 5% glycerol at 26°C. The five known variants (Gly-42, Met-30, Arg-50, Arg-58 and Cys-114) showed electrophoretic mobility shifts. Exon 3 DNA from the patient (•) presented an aberrant SSCP pattern distinguishable from controls, Arg-50 and Arg-58.

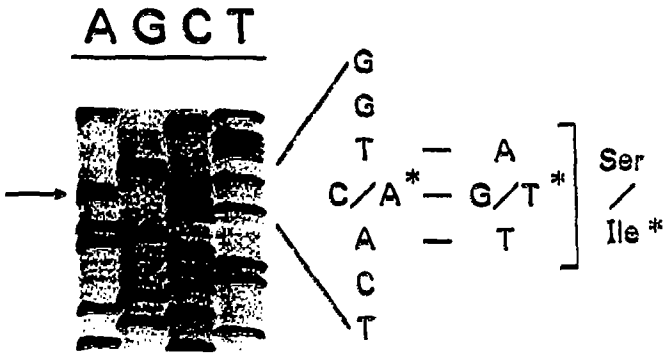

Fig. 2. An autoradiogram of a direct sequencing gel for TTR exon 3 from the patient using an asymmetric PCR product. Guanine and thymine at the same nucleotide position (indicated by arrow) showed the presence of two codons at position 50. AGT coding for Ser and ATT coding for Ile.

specific oligonucleotide (ASO) analysis was done to confirm the detected mutation.

3. RESULTS AND DISCUSSION

SSCP analysis of the normal homozygous samples presented only two bands, representing the two complementary single strands of DNA. The DNA fragments from the five variant TTR genes showed various electrophoretic patterns (Fig. 1). The heterozygous samples (TTR Gly-42 and Cys-114) presented two additional bands corresponding to the two complementary strands of the mutant allele. On the other hand, TTR Met-30, Arg-50 and Arg-58 had three distinct bands when only one strand of the mutant allele was expected to have a novel conformation with a different electrophoretic mobility. The other strand, possessing the complementary

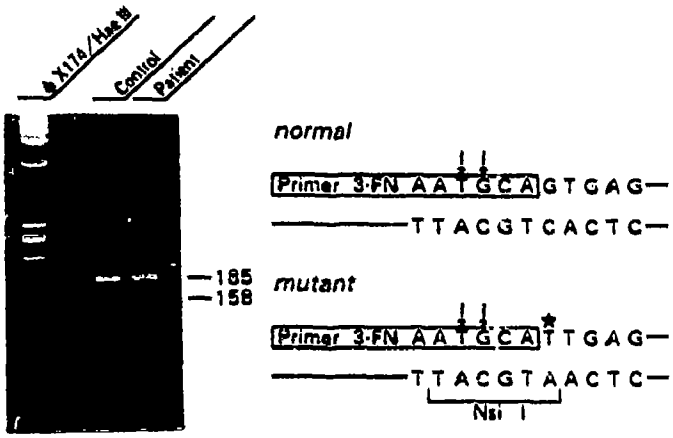

Fig. 3. RFLP analysis of the TTR Ile-50 gene. Forward primer (3-FN: 5'-AGACTTTCACACCTTATAGGAAATGCA-3') with two base substitutions (A-to-T and C-to-G: indicated by arrows), and reverse primer (3-R: 5'-AAAACAACCCTCGAAGGTCTGTATACTCAC-3') were synthesized to amplify the two different allelic exon 3 fragments (185 bp). The genomic DNAs from the patient and a control were amplified for 30 cycles (94°C for 1 min, 54°C for 1 min and 72°C for 1 min). The normal allele had two primer-introduced base changes, while the mutant had a third base change (G-to-T: indicated by ★). Only the latter allelic sequence could produce a new restriction site for *Nsi*I, and thereby the mutant allelic segment could be digested into two fragments (158 and 27 bp). The digested products were electrophoresed on a 3.7% Nusieve agarose gel. The patient was heterozygous for the *Nsi*I site, while a control had no sites in the TTR gene.

base substitution, did not have a new conformation to cause a mobility shift, and continued to migrate with the corresponding strand of the normal allele.

SSCP analysis of exons 1, 2 and 4 from the patient with amyloid polyneuropathy (see section 2) showed the same pattern as controls, but exon 3 presented an aberrant migration pattern, suggesting sequence alterations in the exon (Fig. 1). Direct sequence analysis revealed both G and T at nucleotide position 3,251 [5]. This result indicated that the patient was heterozygous for the TTR gene, Ser-50 and Ile-50 (Fig. 2). The sequence of the entire length of each exon and exon-intron boundaries, as determined by the method described previously [1,2], had the same base change and there were no other sequence alterations. The base change was confirmed by RFLP analysis using a mismatch primer (Fig. 3). The mutation was further confirmed by ASO hybridization analysis. The normal complementary ASO probe (5'-CCAGACTCACTGGTTTTCC-3') and the mutant one with a C-to-A base change at the underlined position were synthesized. Amplified exon 3 from the patient and 100 controls were tested as described previously [1-3]. Exon 3 of the patient hybridized with both the normal and the mutant probes, whereas controls hybridized only with the normal probe (data not shown).

The mechanism by which variant TTR molecules are deposited is not fully understood. We cannot rule out the possibility that a mutation at phylogenetically conserved sites of the TTR molecule is important for the amyloid formation. Met-30, Gly-42, Arg-47 and Arg-58 occurred at the conserved sites among human, rabbit, rat and mouse [6,7], but the other Arg-50, Ile-50 and Cys-114 occurred at the non-conserved sites. All these mutations were located around the junction areas connecting the loop and the β-pleated sheet [8]. It is speculated that point mutations in the junction areas may produce critical conformational changes of the molecule, thereby causing amyloid deposition.

Among 17 TTR variants reported from a number of countries [1–3,9–13], the six variants are restricted within the Japanese race and only Met-30 is widely distributed. This fact shows the striking heterogeneity of Japanese FAP at the molecular level and suggests that it has a diverse origin with each mutation possibly originating from different ethic groups.

In summary, SSCP of the six variant TTR genes have been illustrated, and we have reported a new mutant gene, TTR Ile-50, in a patient with amyloid polyneuropathy.

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