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Molecular Analysis of a Variant Type of Familial Amyloidotic Polyneuropathy Showing Cerebellar Ataxia and Pyramidal Tract Signs

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Abstract

A Japanese family with atypical type I familial amyloidotic polyneuropathy (FAP) in Iiyama, Japan was studied. Most of the family members have dysfunctions of the central nervous system, in addition to typical symptoms of type I FAP. The transthyretin (TTR, also called prealbumin) gene of the atypical FAP (FAP-IY) was analyzed with recombinant DNA techniques and a RIA method. FAP-IY was found to have the mutation responsible for the methionine-for-valine substitution at position 30 of TTR, as in the case of typical type I FAP. However, analysis of DNA polymorphisms in the TTR locus showed that FAP-IY has a genetic background differing from that of the typical type I FAP. These observations lead to the consideration that a genetic factor(s) involved in the dysfunction of the central nervous system may locate in a chromosome region in close proximity to the TTR gene.

Introduction

Familial amyloidotic polyneuropathy (FAP)1 is a dominantly inherited disorder characterized by systemic deposition of amyloid fibrils, and is clinically classified into four types (1). Type I FAP is the most common type and is manifested by sensory dissociation and autonomic involvement followed by motor neuropathy. Amyloid fibrils of type I FAP of Portuguese (2, 3), Japanese (4, 5), and Swedish (6, 7) origins contain a variant of transthyretin (TTR, also called prealbumin) with an amino acid substitution at position 30 (methionine-for-valine, 30Val→Met) as a major component. Analysis of normal and patient types of TTR gene revealed that the 30Val→Met substitution is caused by a single base change G→A and that this is the only base change specific for type I FAP in the TTR gene (8–10). These findings led to the establishment of diagnostic methods for FAP using RIA (11), immobiloblotting procedure (12), and recombinant DNA techniques (8, 13). It became clear that type I FAP is a molecular disorder of TTR.

The disease process of type I FAP is not well understood at the molecular level. One approach to this issue may be a careful analysis of atypical FAP compared with the typical form. Such a study should provide a clue to genetic or other factors involved in the disease process. FAP families with variant TTR having amino acid substitutions other than the 30Val→Met change have been reported (14–17). An atypical FAP family has been detected in Iiyama, Japan (18, 19). In the course of the disease, patients with atypical FAP (FAP-IY) often show cerebellar signs of dysarthria, incoordination in the limbs, ataxic gait, and pyramidal tract signs, in addition to typical clinical features of type I FAP. The symptoms of central nervous system (CNS) involvement and type I FAP appear to be linked each other (18, 19). These observations raise the possibility that FAP-IY is a new phenotype of FAP (18).

We analyzed FAP-IY with recombinant DNA and RIA techniques and found that patients of FAP-IY carry the mutation responsible for the 30Val→Met substitution in the TTR gene, just as in the case of typical type I FAP. However, the family has a different genetic background from that of typical FAP families of Japan. The results are discussed in terms of the factor(s) that may be involved in dysfunction of the CNS in case of FAP-IY.

Methods

Subjects. Data on a family with FAP-IY have been reported (18, 19). The pedigree of this family and the clinical features are given in Fig. 1. Information was obtained for the 30 members, and 18 were available for clinical examination. Details of the neurological signs are summarized in Fig. 1. Peripheral neuropathy was present in 11, and there were 11 with signs of CNS involvement, such as cerebellar signs (dysarthria, impaired finger-to-nose testing, dysdiadochokinesia, and ataxic gait) and hyperreflexia. Babinski’s sign was present in five (II-6, II-9, III-1, III-10, III-11).

Preparation of high-molecular weight DNA and blotting experiments. Peripheral blood samples (10–20 ml) were obtained from available members of the FAP-IY family (IV-1, IV-2, IV-3, III-10, III-11, V-1), and high-molecular weight DNA was prepared from white blood cells by the procedure of Ryan et al. (20) with minor modifications. Southern blotting was carried out as described by Maniatis et al. (21). Unless noted, the cDNA clone pH64-1 was used as the probe. A polymorphic base change that does not result in restriction site polymorphism was identified using a pair of oligonucleotide probes (5’ TGGTATAGTACTCCCTCT 3’ and 5’ TGGTATAGTACTCCCTCT 3’) after the specific DNA region had been enzymatically amplified using a set of primers (5’ GGGTCTGGATGTTCTGGA 3’ and 5’ GTAATGACCATACATAGGG 3’), according Saiki et al. (22).

Construction and screening of a genomic DNA library. High-molecular weight DNA of a FAP-IY patient (III-10) was completely di-

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1. Abbreviations used in this paper: FAP, familial amyloidotic polyneuropathy; SCD, spinocerebellar degeneration; TTR, transthyretin; 30Val→Met, methionine-for-valine substitution at position 30.

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gested with Eco RI and DNA fragments of 4–6 and 8–10 kb long were isolated by 5–20% sucrose gradient centrifugation. Exons were found on 9 kb and 4.89 kb Eco RI fragments (Fig. 2 A). These fragments were ligated to λgtWES arm DNA and packaged into phage particles by the in vitro packaging reaction (21). Phages were grown on L-agar plates, with *Escherichia coli* K12 (LE392) as the host. ~ 6.0 × 10² independent clones were obtained. The DNA library was screened by the plaque hybridization technique (23) using ³²P-labeled DNA fragments from human cDNA (pHH64-1) (8) as the probe, and five clones carrying Eco RI 9- kb fragment and three clones of 4.89-kb fragment were obtained.

**Nucleotide sequence analysis.** Recombinant phage DNA was digested with restriction endonucleases, and DNA fragments to be sequenced were subcloned into the plasmid pUC 9. The nucleotide sequence was determined by the dideoxy chain termination method described by Hattori and Sakaki (24).

**RIA of variant TTR.** Variant TTR with the 30Val → Met substitution in serum was measured by the RIA based on FAP-specific nonapeptide (position 22–30) of the TTR variant (11). 5 μl of serum were treated with cyanogen bromide followed by trypsin before RIA, as described by Nakazato et al. (11).

**Results**

**Southern blotting analysis.** Several different types of amino acid substitution of variant TTR have been noted in FAP: 30Val → Met (2–7), isoleucine for phenylalanine at position 33 (33Phe → Ile) (15), glycine for threonine at position 49 (49Thr → Gly) (17), alanine for threonine at 60 (60Thr → Ala) (16), and serine for isoleucine at 84 (84Ile → Ser) (14). Mutations responsible for these amino acid substitutions (except 49Thr → Gly) can be detected by Southern blotting, using appropriate restriction enzymes: 30Val → Met by Nsi I, Bal I (8, 13) and 33Phe → Ile by Bcl I using pHH64-1 as a probe, and 60Thr → Ala by Pvu II (16) and 84Ile → Ser by Alu I using the 0.88-kb Alu I fragment (from position 3,844 to 4,728 [10]) as a probe. DNA from FAP-IY patients were digested with these restriction enzymes and subjected to Southern blotting analysis. As shown in Fig. 2 B, DNAs of III-10, 11, IV-1, and V-1 digested with Nsi I showed two additional bands of 4.90 and 1.45 kb, indicating that patients with FAP-IY carry the mutation responsible for 30Val → Met, heterozygously, just as in the case of type I FAP (8, 13). No significant difference was observed in the blotting profiles of FAP-IY DNAs digested with Bcl I, Pvu II, and Alu I (data not shown). The absence of the 49Thr → Gly substitution in FAP-IY became evident by the sequence analysis described below.

**Nucleotide sequence analysis of FAP-IY type TTR gene.** We then asked whether the FAP-IY TTR gene has additional mutations in exons and possible regulatory regions. The human TTR gene consists of four exons, and some possible regulatory signals have been identified in the 5' flanking region and introns (10). The structure of the TTR gene is schematically shown in Fig. 3. Clones carrying the 9.0-kb and 4.89-kb Eco RI fragments that cover most of the TTR gene (10) were isolated from a genomic DNA library of a FAP-IY patient (III-10), as described in Methods. As the patient is heterozygous for the mutation, the Eco RI fragments can be derived from both normal and mutant chromosomes. Two types of the 4.89-kb Eco RI fragments were cloned, based on the polymorphisms of Fnu 4HI site at position 5,610 (for position numbers, see references 9 and 10). Two types of the 9.0-kb fragment (with and without Nsi I site at exon 2) were also cloned. The nucleotide sequences of these four fragments (two 9.0 kb and two 4.89 kb) were determined. Sequenced regions are indicated at the bottom of Fig. 3. The results show that except for the mutation responsible for the 30Val → Met change in exon 2, no base change was present in the coding and possible regulatory regions of FAP-IY TTR gene in comparison with the normal TTR gene (10) cloned from Maniatis' human genomic DNA library (25).

**RIA of variant TTR in serum.** Variant TTR with 30Val → Met in serum can be quantitatively measured by RIA after treatment with cyanogen bromide followed by tryptic digestion (11). Using this method, we asked whether the FAP-IY shows differences in serum levels of the variant TTR, in comparison

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2. These have not been experimentally demonstrated, rather were predicted from the cDNA sequence.
with typical type I FAP. As indicated at the bottom of Fig. 2B, three FAP-IY patients showed values of 8.10 mg/dl (IV-1), 10.91 mg/dl (III-10), and 9.47 mg/dl (III-11), respectively, for the serum levels of the variant TTR. These levels are close to the value in typical type I FAP patients (9.18±2.69 mg/dl) (26). A family member carrying the mutation (V-I) also showed a similar level of the variant TTR long before the onset of the disease (Fig. 2B) as in the case of typical type I FAP. These findings indicate that there is no significant difference in expression of the variant TTR gene between typical FAP and FAP-IY.

**Haplotype analysis of FAP.** Our findings suggested that in FAP-IY, there is a certain factor(s) other than the TTR gene that is responsible for the clinical feature characteristic of FAP-IY, that is, dysfunction of the CNS. To determine the genetic background of the FAP-IY family, we analyzed two polymorphic markers in the TTR gene (9, 27). Our previous work showed that there are six polymorphic base changes in the TTR gene region (9). Among them, the association of T or G change at nucleotide position 1,218 and G or A (Msp I site + or –) change at position 2,537 to the G or A change at position 1,679 responsible for the 30val→Met substitution in exon 2 was examined. At first, DNA was digested with both Bal I and Msp I, which makes the four types of haplotypes distinguishable by length of the restriction fragments (Fig. 4A). The blots were done with probes specific sequencing primers. The results of Southern hybridization using probe DNA show similar restriction profiles (Fig. 4B). Therefore, we determined that the 30val→Met substitution with Bal I site (+), geno-
type B) was found to be associated with the Msp I site (+) (genotype M) in patients of FAP-IY. On the contrary, all 28 patients with typical type I FAP showed the Bal I site (+) (genotype B) with Msp I (−) (genotype m) (details will be published elsewhere).

The nucleotide at position 1,218 (G or T) was determined either by nucleotide sequencing or by dot blot hybridization using a pair of 19 mer nucleotide probes, as described in Methods. The results showed that the genotype of Bal I site (+) (genotype B) is associated with a G residue at position 1,218 in all five typical type I FAP patients examined, but FAP-IY patients (III-10, 11, IV-1) had a T residue at 1,218 associated with Bal I (+). These results are summarized in Table I. It is evident that FAP-IY and typical type I FAP families in Japan have different haplotypes, the former being ‘TBM’ and the latter being ‘GBm’. These findings strongly suggest that the family with FAP-IY has a genetic background differing from that of the typical type I FAP families in Japan.

**Discussion**

Although the most common type of hereditary generalized amyloidosis in Japan is type I FAP (28, 29), a family of atypical type I FAP (FAP-IY) with CNS involvement has been found in Iyama. The most remarkable feature of FAP-IY is the disorder of the CNS: cerebellar signs of dysarthria, incoordination in limbs, ataxic gait, and pyramidal tract signs. Although the patient IV-1 has at present a peripheral neuropathy (Fig. 1), the symptom of CNS involvement may appear in the future. For example, the Babinski’s sign has recently become evident in patients III-10 and III-11 (unpublished data).

**Table I. Nucleotide Substitutions in TTR Genes**

<table>
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<tr>
<th>Positions</th>
<th>In reference 10</th>
<th>1,218</th>
<th>1,679</th>
<th>2,422</th>
<th>2,537</th>
<th>5,198</th>
<th>5,610</th>
<th>5,708</th>
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<td></td>
<td>(In reference 33)</td>
<td>(637)</td>
<td>(1,098)</td>
<td>(1,841)</td>
<td>(1,956)</td>
<td>(4,618)</td>
<td>(5,030)</td>
<td>(5,128)</td>
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<tr>
<td>Normal</td>
<td></td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>G</td>
<td>A</td>
<td>C</td>
<td>G</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>(Bal I−)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Msp I+)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Typical type I FAP</td>
<td>G</td>
<td>A</td>
<td>C</td>
<td>A</td>
<td>C</td>
<td>G</td>
<td>T</td>
<td>9</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Bal I+)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>(Msp I−)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAP-IY</td>
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<td>A</td>
<td>G</td>
<td>G</td>
<td>(Msp I+)</td>
<td></td>
<td></td>
<td>This work</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Bal I+)</td>
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<td></td>
</tr>
<tr>
<td>Normal</td>
<td>G*</td>
<td>G</td>
<td>G</td>
<td>A</td>
<td>C</td>
<td>G</td>
<td>(Fnu 4H+−)</td>
<td>33</td>
<td></td>
</tr>
<tr>
<td></td>
<td>(Bal I−)</td>
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</table>

The nucleotide sequence of TTR gene has been published by our group (10) and by others (33). The numbering system for base positions by reference 10 is employed in this work. Although the sequence in reference 10 was revised after its publication (filed in GenBank DNA data base), the original numbering system has been employed to avoid confusion. Even after reversion, there are several differences (including deletions and insertions) between the data. The two numbering systems cannot be simply converted. * It is not clear whether the sequenced fragment is from the Maniatis’s library or a Japanese genomic library.
We analyzed the structure and expression of FAP-IY TTR gene using recombinant DNA techniques and an RIA method and found that the FAP-IY patients carry the mutation responsible for the $30^{\text{Val}} \rightarrow ^{\text{Met}}$ substitution as in the case of typical type I FAP. No significant difference was observed in quality and quantity of the variant TTR in serum between typical type I FAP and FAP-IY. These findings suggested that a certain factor(s) other than the TTR gene is involved in the dysfunction of the CNS characteristic for FAP-IY. As shown in Fig. 1, seven of 11 patients with peripheral neuropathy had obvious signs of CNS involvement, that is, the cerebellar signs and/or Babinski’s sign (II-6, 9, III-1, 3, 8, 10, 11). The remaining three (III-6, 9, 12) had a hyperreflexia. Thus, symptoms of type I FAP appear to be closely associated with dysfunction of the CNS in this family. The association of peripheral neuropathy with CNS involvement has not been detected in other FAP families in Japan. These findings suggested that such a factor(s) is genetically determined.

It is conceivable that FAP-IY is a combination of type I FAP and some hereditary disorders of the CNS. FAP-IY shares clinical features (cerebellar ataxia and pyramidal tract signs) with familial cerebellar ataxia and cerebrovascular amyloid (30), and also with hereditary spino-cerebellar degeneration (SCD). Ikeda et al. (18) pointed out that the computerized tomography scan imaging of FAP-IY patients shared findings similar to those with SCD, although no patient with FAP-IY has been autopsied. FAP patients in Japan number $\sim 1,000\sim 2,000$ and the prevalence of SCD (containing hereditary type and sporadic type) is roughly $1\sim 7/100,000$. Thus, the probability of a combination of these two diseases in one family would be extremely low, but not zero. Alternatively, the variant TTR with the $30^{\text{Val}} \rightarrow ^{\text{Met}}$ substitution may directly cause dysfunction of the CNS in the presence of different genetic backgrounds. Further clinical and pathological studies will be required to determine the molecular basis of the dysfunction of the CNS. The genetic factor(s) involved in the dysfunction of CNS, if present, is probably linked to the TTR gene, which is assigned to $q11.2\sim 12.1$ of chromosome 18 (31, 32).

In conclusion, we obtained evidence that FAP-IY has the same mutation as type I FAP in the TTR gene. A genetic factor(s) involved in the dysfunction of the CNS may locate in a chromosomal region in close proximity to the TTR gene.

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