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A NOVEL VARIANT OF TRANSTHYRETIN (Tyr^{114} to Cys) DEDUCED FROM THE NUCLEOTIDE SEQUENCES OF GENE FRAGMENTS FROM FAMILIAL AMYLOIDOTIC POLYNEUROPATHY IN JAPANESE SIBLING CASES

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A to G transversion was identified in exon 4 of transthyretin gene in familial amyloidotic polyneuropathy in two sibling cases living in Osaka. This transversion led to the replacement of tyrosine by cysteine residue at codon 114 of 127 residue molecule. This identification was achieved by randomly sequencing recombinant clones containing the entire length of each one of the four exons selectively amplified by polymerase chain reaction. Dot blot analysis with allele-specific oligonucleotides indicated the linkage of this mutation with the disease and confirmed the single base change.

Familial amyloidotic polyneuropathy (FAP) is a dominantly inherited disease associated with the genetic variants of plasma transthyretin (TTR) except for type III FAP which was recently shown to be associated with variant apolipoprotein A I (1). To date, eight different mutations in the gene for plasma TTR have been identified in autosomal dominant hereditary amyloidosis (2-11). Type I FAP, the most common form in various countries, is associated with the variant TTR with methionine substituted for valine at position 30 of the molecule (2-4).

Here we report a new FAP kindred from the Osaka area, possessing a novel variant of TTR. The two sibling cases belonging to this kindred had amyloid

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Abbreviations: familial amyloidotic polyneuropathy (FAP), transthyretin (TTR), polymerase chain reaction (PCR), Allele-specific oligonucleotide (ASO).

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depositions containing TTR, but mutant genes linked with the eight known types of hereditary amyloidosis were not detected in these cases. TTR is encoded by a unique, single copy gene consisting of four exons spread over seven kilo base pairs on chromosome 18 (12,13). We synthesized pairs of oligonucleotide primers, each of which was designed to amplify every one of the four exons of TTR gene by polymerase chain reaction (PCR). We cloned and randomly sequenced the amplified fragments of the TTR gene. This paper presents our findings on the genetic variation of TTR in the two siblings affected by FAP.

PATIENTS AND METHODS

Patients. In 1986, a 35-year-old man living in Osaka visited us with the complaints of numbness and cold sensation in the under-knee portion of the bilateral lower limbs. He had a 5 years' history of decreased libido, fecal incontinence, and pitting pretibial edema and numbness in his legs. Vitreous opacities were present. The touch and thermal senses were decreased in stock-globe distribution. The position sense was normal in the hands, but decreased in the toes. Sudden death occurred at age 38. The antiserum to human plasma TTR stained weakly the amyloid deposits in an autopsied kidney. Almost identical symptoms started in his cousin at age 34 and sudden death occurred 6 years after symptom onset. Four generations were traced back to 1835 when the family was in Nagasaki area of Japan.

PCR amplification and sequencing of TTR gene fragments. Genomic DNA was extracted from the peripheral-blood leukocytes of the present case and from the autopsied kidney of his cousin. Based on the published sequence of normal human TTR gene (12), the oligonucleotide primers flanking exons of TTR genes were prepared on Applied Biosystems model 381A DNA synthesizer, and purified by HPLC (Table 1). The four exons of the TTR gene were amplified from the genomic DNA using Gene Ampkit (Perkin Elmer Cetus). PCR run consisted of 30 cycles of denaturation at 94° C, annealing at 55° C, and extension at 74° C for 60 sec for each step. PCR products were electrophoretically separated on 3.7 % NuSieve GTG agarose (FMC Corp.). The gene fragments containing the entire length of each one exon were cloned into M13 vectors and sequenced by the dideoxy-chain termination method (14).

Allele-specific oligonucleotide (ASO) hybridization. Exon 4 of the TTR gene was amplified from genomic DNA of the patients and controls with oligonucleotide primers. Amplified products were denatured after dot-blotting onto nylon membranes. The filters were subsequently hybridized with (γ -32 P) dCTP endlabeled oligonucleotides: 19 bases of normal ASO containing A at position 6752 (5'-TGAGCCCCTACTCCTATTC-3') and mutant ASO containing G for A at the same position. Filters were washed in 5 X SSC (1 X SSC: 0.15M NaCl, 0.015M sodium citrate) at 54° C for 10 min.

RESULTS AND DISCUSSION

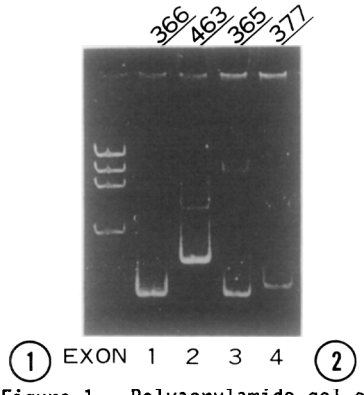
The gene fragments containing entire length of each one exon were effectively amplified (Fig. 1). Six clones were randomly selected and sequenced for each of the four exons of TTR gene. The sequence data on the

Table 1. Summary of TTR gene amplification primer sets

Exon and size	Primer sequence #	amplified ##
Exon 1	5'-AGAATCAGCAGGTTTGCAGTCAGAT-3'	-91-275
95 bp	5'-AGCTCAGTAAGCTCAGTGGAACTT-3'	
Exon 2	5'-TGTCGACACTTACGTTCCTGATAAT-3'	885-1347
131 bp	5'-ATGCTCAGGTTCCTGGTCACTT-3'	
Exon 3	5'-ATCTACAGTGAGCTTTTCAAAA-3'	3034-3398
136 bp	5'-TCGAAGGTCTGTATACTCAC-3'	
Exon 4	5'-GAAATGGATCTGTCTGTCTTC-3'	6659-7035
253 bp	5'-CAGCGAATTCCTTTGATTCTTTGTAA-3' ###	

The primer sequences and the numbering of TTR gene are based on the published data (12).
The amplified region of TTR gene with each primer set.
Extra bases (CAGC) are added 5' to the EcoRI recognition sequence to ensure that the efficiency of restriction enzyme cleavage is maintained.

exons 1, 2, and 3 were compatible with the published data of normal human TTR gene (12). Upon examining the six independent clones containing entire length of exon 4, the sequences of three clones were identical and contained a single base change of G for A at position 6752 in the TTR gene. This mutation corresponded with the change of codon TAC coding for tyrosine to TGC coding for cysteine at position 114 of the 127-residue TTR molecule. The other three clones contained gene fragments with the normal sequence (Fig. 2). We



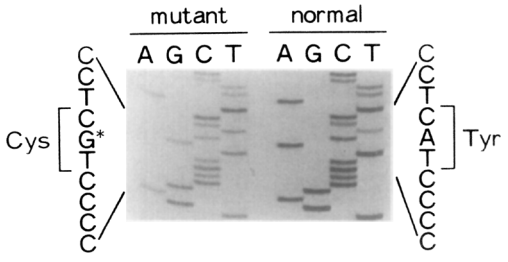


Figure 1. Polyacrylamide gel electrophoresis of PCR-product containing each one of the four exons of the TTR gene. The markers are Hae III-digested ϕ X174-RF. The lengths of the products are indicated at the top of each lane.

Figure 2. Nucleotide sequence of a portion of PCR-amplified exon 4. The two allelic segments are detected in the patient. There is a single base change of G for A at position 6752 in the TTR gene, which may result in replacement of tyrosine (TAC) by cysteine (TGC) at codon 114.

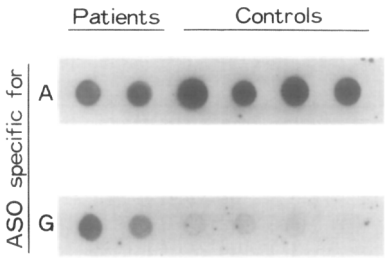


Figure 3. Allele-specific oligonucleotide (ASO) hybridization of PCR-amplified exon 4 from the two patients and four controls. The present case (right) and his affected cousin (left) hybridize with both the normal (specific for A) and the mutant (specific for G) ASOs, while the controls hybridize only with the normal ASO.

repeated the same procedure on the genomic DNA from his affected cousin, and found the identical base change at the same position in three of the five independent clones sequenced. For further confirmation of this base change, we sequenced gene fragments containing a portion of the exon 4 amplified from a new DNA specimen using a new primer pair. The genomic DNA was extracted from the autopsied sympathic ganglion tissue of his cousin. 204 base pair segments (6659-6862) of TTR gene were obtained using two primers : the forward primer complementary to the antisense strand shown in table, and the reverse primer, 5'-AATGGAATACTCTTGGTTACATG-3', which was newly synthesized complementary to the sense strand 87 base apart from the locus of the mutation. Sequence of the PCR products proved the same mutation at the same position. All these results effectively excluded the cloning and amplification artifacts and established the mutation. The detection of the two different allelic segments endorsed that the patients were heterozygous for the TTR gene mutant.

To ascertain the linkage of the base change with this type of FAP, the amplified DNA was tested for allele-specific oligonucleotide hybridization. DNA samples from 22 controls were hybridized exclusively with a normal probe and those from the patients with both variant and normal probes. The representative hybridization was shown in figure 3. This test repeatedly confirmed the base change, strongly suggesting that this mutation was responsible for the disease. In addition, this procedure is considered of diagnostic value, because the base change detected in this kindred created no

new restriction sites in the computer-assisted search. No other base changes seemed present in the TTR gene of these two patients according to the sequencing analysis. Alterations of the nucleotide sequences of exon-intron junctions were not recognized.

In summary, we identified a novel variant of TTR in Japanese kindred with FAP. The two siblings referred to here were heterozygous for mutant TTR gene, which was responsible for tyrosine-to-cysteine substitution in TTR.

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