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NOVEL VARIANT TRANSTHYRETIN GENE (Ser^{50} to Ile) IN FAMILIAL CARDIAC AMYLOIDOSIS

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Summary: We detected a point mutation in the transthyretin (TTR) gene in a patient with familial cardiac amyloidosis by using PCR-DCP (DNA conformation polymorphism) analysis that is based on the diversity in electrophoretic mobility of single-stranded DNAs and/or heteroduplex DNAs in PCR products. The PCR products of the transthyretin gene were denatured in the presence of formamide and electrophoresed in a non-denaturing polyacrylamide gel to detect an electrophoretic change due to a sequence variation. An unusual DNA fragment was visualized by silver staining in the PCR products of the exon 3 from the patient. Subsequent sequencing analysis revealed a T to A transversion and led to a replacement of Ser by Ile at codon 50 of the TTR gene.

Amyloidosis is characterized by extracellular deposition of insoluble protein fibrils, amyloid (1). Some clinical types of amyloidosis in diverse ethnic populations are inheritable and several mutations in the transthyretin (TTR) gene that is composed of four exons encoding for 3, 44, 45, and 35 amino acid residues, respectively, were reported in the patients with familial amyloid polyneuropathy (FAP) (2-11) or familial cardiac amyloidosis (FCA) (12-14). We report here a new variant TTR gene in a Japanese patient with familial cardiac amyloidosis (FCA) detected by the modified PCR-single-

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Abbreviations: DCP, DNA conformation polymorphism; FAP, familial amyloid polyneuropathy; FCA, familial cardiac amyloidosis; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; SSCP, single-strand conformation polymorphism; TTR, transthyretin.

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strand comformation polymorphism (SSCP) analysis that is designated PCR-DNA conformation polymorphism (DCP) analysis.

SUBJECTS AND METHODS

Subject: A 53 year-old Japanese male was referred with symptoms of exertional dyspnea and palpitation. He has 8 siblings and 2 of them were died with cardiac amyloidosis. An electrocardiogram showed first degree atrio-ventricular block and complete left bundle branch block. Two-dimensional echocardiogram revealed a symmetrical left ventricular hypertrophy (thickness of the interventricular septum:2.2 cm, thickness of the left ventricular posterior wall: 2.2 cm) with preserved systolic function. The thickened cardiac walls demonstrated a granular sparkling texture. Amiloid deposits were proved in biopsied specimen from rectum and skin of the patients. None of the three patients in the family showed apparent polyneuropathy.

Genomic DNA extraction and PCR: High molecular weight DNA was prepared from peripheral blood leukocytes of the patient or normal individuals by the SDS-proteinase K method and phenol/chloroform extraction. Eight primers flanking four exons of the TTR gene were synthesized by cyanophosphamidite method in Cyclon plus DNA synthesizer (MilliGen/Biosearch, Burlington) based on the reported sequences of the normal human TTR gene (15) (Table 1). Amplification of the TTR gene was done by using polymerase chain reaction (PCR) (16,17) in a reaction mixture of 50 μl composed of 100 ng genomic DNA, 10 pmole primers, 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 1.5 mM $MgCl_2$, 0.01 % gelatin, 0.8 mM dNTPs, and 1 unit of thermostable DNA polymerase (Ampli-Taq, Perkin Elmer Cetus) by 30 cycles of denaturation (96 °C, 1 min), annealing (60 °C, 30 sec), and extention (72 °C, 1 min) steps in a programmable heat block (DNA Thermal Cycler, Perkin Elmer Cetus Instruments, Norwalk).

PCR-DCP (DNA conformation polymorphism) analysis: Formamide dye (80 % formamide, 20 mM EDTA, 0.01 % bromophenol blue, pH 8.0) was mixed with an aliquot of PCR products (0.1 - 0.2 μg) to obtain a final concentration of 50 % formamide. Samples were heat-denatured at 96 °C for 5 min, rapidly chilled in ice-water, and electrophoresed in an 8 % polyacrylamide gel containing 10 % glycerol (14 x 14 x 0.1cm, 0.4 x TBE, acrylamide: bisacrylamide = 50:1) in 0.4 x TBE (1 x TBE: 89 mM Tris-borate, 89 mM boric acid, 2 mM EDTA, pH 8.0) at 13 V/cm for 8 h at room temperature. Single-stranded DNAs, heteroduplex DNAs, and double-stranded DNAs were visualized by silver staining using commercially available kit (Daiichi Chemical Co. Ltd., Tokyo) according to the manufacturer's instruction.

Sequencing analysis: To identify a mutation in the exon 3, PCR products generated with primers, PALB3-5C (5'-GGGAAGCTTAATCCAGACTTTCACACCT-3') and PALB3-3C (5'-GGGTCTAGAAACAACCCTCGAAGGTCTG-3'), were digested with *Hin*dIII

Table 1. Nucleotide sequences of primers

Exon	Name	Sequence (5' to 3')	Length of PCR products
1	PALB1-5C	GGGAAGCTTGAAGTCCACTCATTCTTGGCA	177bp
	PALB1-3C	GGGTCTAGAAGGAGTCACTTCTACTTCA	
2	PALB2-5	AACTTCTCACGTGTCTTCTCT	190bp
	PALB2-3	GTCCTGTGGGAGGGTTCTT	
3	PALB3-5	TAATCCAGACTTTCACACCTTA	190bp
	PALB3-3	AAACAACCCTCGAAGGTCTG	
4	PALB4-5	GGATCTGTCTGTCTTCTCTCA	162bp
	PALB4-3	GTCCTTCAGGTCCACTGGA	

and *Xba*I and cloned into Bluescript SK$^-$ (Stratagene, obtained via Toyobo Co. Ltd., Osaka). DNA sequences were determined by the dideoxy chain termination method (18).

Dot blot hybridization: Oligonucleotide probes specific to normal and mutated sequences at the codon 50 of the TTR gene were 5'-AAAACCAGTGAGTCTGGA-3' and 5'-AAAACCATTGAGTCTGGA-3', respectively. The probes were end-labeled with [γ-^{32}P]ATP by T4 polynucleotide kinase. Amplified DNA fragments (3 μl) were dot blotted onto a nylon membrane (Hybond N plus, Amersham International, Tokyo) and hybridized with radio-labeled oligonucleotide probes (10 pmole/ml) in 3.0 M tetramethylammonium chloride, 50 mM Tris-HCl (pH 8.0), 2 mM EDTA, 0.1 % SDS, 5 x Denhardt's solution, and 100 μg/ml heat-denatured herring sperm DNA for 30 min at 54 ^{o}C. The filters were washed twice in 2 x SSPE (0.30 M NaCl, 0.02 M NaH_2PO_4, 2 mM EDTA, pH 7.4) and 0.1 % SDS at room temperature for 10 min, once in TMAC solution (3.0 M tetramethylammonium chloride, 50 mM Tris-HCl, 2 mM EDTA, 0.1 % SDS, pH 8.0) for 10 min at room temperature. Stringent wash was done twice in TMAC solution for 10 min at 58 ^{o}C. The filters were exposed to Kodak XAR5 film at room temperature for 30 min to detect hybridization signals.

RESULTS AND DISCUSSION

In PCR-DCP analysis of the exon 3 of TTR gene, normal subjects (Figure 1, lane 1, 3-6) showed four slow-migrating DNA fragments (single-stranded DNAs) and a fast-migrating DNA fragment (double-stranded DNA). These four slow-migrating fragments were suggested to be generated from two single-stranded DNAs, because the PCR was

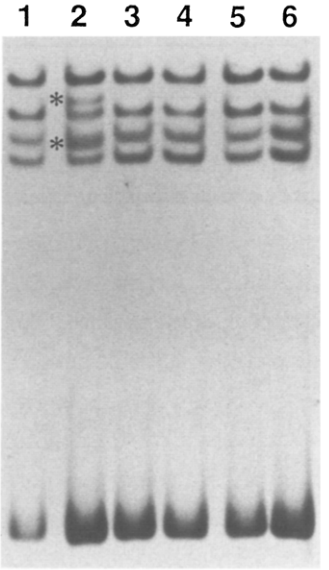

Figure 1. **PCR-DCP analysis of TTR gene.** PCR products of the TTR exon 3 were heat-denatured and electrophoresed in an 8% polyacrylamide gel. Two unusual DNA fragments indicated by asterisks were found in the patient's sample (lane 2) but not in the samples from healthy individuals (lanes 1, 3, 4, 5, and 6). An arrowhead indicates the position of double-stranded DNAs.

not affected by co-amplification of unknown gene as judged by the observation that no unexplainable restriction fragments were revealed by digestion of the PCR products with several restriction enzymes (data not shown). It was then suggested that one single-stranded DNA fragment could take two or more conformations under the specified conditions as was previously reported for the HLA-DP genes (19). The patient with FCA showed two additional DNA fragments (Figure 1, lane 2). Because the DCP patterns of PCR products from the other exons were not different from those of normal individuals (data not shown), the patient was suggested to have a sequence variation in the exon 3. PCR products from the exon 3 of the patient were then cloned and sequenced. Sequencing of six independent clones revealed that three clones had identical sequences and contained a one-base substitution at the codon 50 from AGT (Ser) to ATT (Ile) (Figure 2). The other three clones had normal sequences, indicating that the patient was heterozygous for the mutation. For further confirmation of the mutation, PCR products of the exon 3 were hybridized with allele-specific oligonucleotide probes. The PCR products from 95 controls were hybridized exclusively with the normal probe, whereas that from the patient showed hybridization with both mutant and normal probes (Figure 3).

Various mutations in the TTR gene have been reported in association with familial amyloidosis and most of the patients showed clinical symptoms of polyneuropathy (1). We found a mutant TTR gene in a patient with familial cardiac amyloidosis (FCA), who

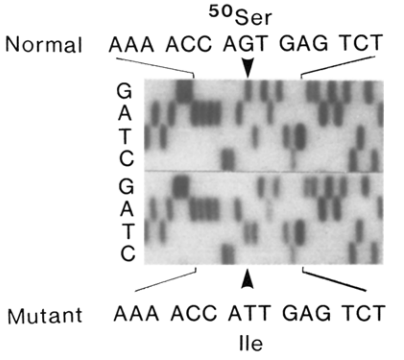

Figure 2. **Nucleotide sequences of normal and mutant TTR alleles from the patient.** The PCR products of the exon 3 were cloned and sequenced. The codon 50 of a normal allele is AGT and that of a mutant allele is ATT, showing that the patient is heterozygous for the G to T substitution at codon 50.

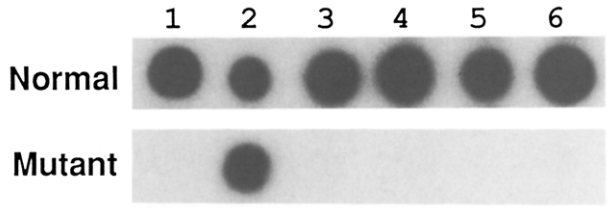

Figure 3. **Hybridization with allele-specific oligonucleotide probes.** PCR samples from the patient and normal subjects were hybridized with normal and mutant probes. Only part of the analysis is shown. The patient (no. 2) showed positive hybridization with both normal and mutant probes.

showed no apparent polyneuropathy. Cardiomyopathy is often associated with the variant TTRs in amyloidosis manifesting polyneuropathy. However the cardiac amyloidosis that predominantly affect cardiac tissue is rare and only three mutant TTR genes have been reported, *i.e.* $Ala^{45}\rightarrow Thr$ in Italian (12), $Leu^{111}\rightarrow Met$ in Danish (13), and $Val^{122}\rightarrow Ile$ in Black (14). This is the first report of a variant TTR, $Ser^{50}\rightarrow Ile$, found in Japanese. On the other hand, four variant TTRs were reported in Japanese familial amyloid polyneuropathy (FAP), i.e. $Val^{30}\rightarrow Met$ (2), $Glu^{42}\rightarrow Gly$ (5), $Ser^{50}\rightarrow Arg$ (5), and $Tyr^{114}\rightarrow Cys$ (10). Another variant TTR, $Gly^{47}\rightarrow Arg$ (20), was also reported in a Japanese patient with amyloid polyneuropathy. It is of interest that one variant TTR in FAP was reported for the codon 50 (from Ser to Arg). This missense mutation is found at the same codon as is the case reported here (from Ser to Ile), although the clinical symptoms of the patients are apparently different. The Arg^{50} variant was reported to manifest the polyneuropathy, while the Ile^{50} variant mainly affected the cardiac tissue. It is then suggested that the difference in the nature of the TTR variants and/or its interaction with other proteins determines the preferencially depositing tissues.

The use of restriction enzymes is a useful method for diagnosis of the familial amyloidosis with a known mutation if the mutation creates or destroys a restriction cleavage site, because the mutation could be detected by Southern blot analysis or by digestion of PCR products with the restriction enzymes (PCR-RFLP analysis). For example, $Val^{30}\rightarrow Met$ mutation generates a new restriction site of *Nsi*I and *Bal*I (21), $Phe^{33}\rightarrow Ile$ mutation creates a new *Bcl*I and *Mbo*I site (4), $Val^{122}\rightarrow Ile$ mutation lead to the loss of a *Mae* III restriction site (22). The restriction analysis however is not applicable to detection of mutations which do not change a restriction site, e.g.

Ala^{45}→Thr (12), Gly^{47}→Arg (20), and Try^{114}→Cys (10). We confirmed the mutation in the TTR gene of the patient by dot-blot hybridization with an allele-specific oligonucleotide probe (ASOP) because the mutation was not associated with change in restriction sites. It is important from genetical and clinical view points to know whether a patient with hereditary disease such as familial amyloidosis and its relatives have a mutation in the causative gene. A "definite" or "presymptomatic" diagnosis could be however achieved by the ASOP or RFLP analyses only for the known mutations. Therefore another efficient and accurate screening method will be require for unidentified mutations. The SSCP analysis (23) was originally introduced to detect sequence variations in the human genome based on the finding that the electrophoretic mobility of single-stranded DNA in a non-denaturing polyacrylamide gel depends not only on size but also on sequence. We have modified the original SSCP methods so that neither radioactive materials nor special apparatus to cool-down the gel are required, and proved the modified method, PCR-DCP method, was enough effective to detect polymorphisms including one base substitutions in HLA-DP genes (19). The PCR-DCP analysis is a sensitive, simple and rapid method to detect a mutation, and then expected to be applicable to the screening of causative defects in other inheritable diseases.

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