Vol. 180, No. 1, 1991 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1991 Pages 380-385

NEW MUTANT GENE (TRANSTHYRETIN Arg 58) IN CASES WITH HEREDITARY POLYNEUROPATHY DETECTED BY NON-ISOTOPE METHOD OF SINGLE-STRAND CONFORMATION POLYMORPHISM ANALYSIS

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Received September 2, 1991

Single-strand conformation polymorphism (SSCP) was analyzed to detect a mutation in the transthyretin (TTR) gene from the mother and son showing polyneuropathy with carpal tunnel syndrome. DNA segments containing TTR coding sequence were amplified by polymerase chain reaction, heat denatured and electrophoresed on a neutral polyacrylamide gel. The single-stranded DNA fragments in the gel were transferred to a nylon membrane and hybridized with biotinylated TTR cDNA probe, followed with chemiluminescent DNA detection. The mobility shift was found in the fragments of exon 3 from the patients' DNA. Sequencing analyses of the exon 3 confirmed a T → G base change, resulting in a Leu 58 → Arg substitution. TTR Arg 58 is the first mutant TTR gene that has been detected by SSCP analysis. The rapid and sensitive detection of new mutations at various sites on the TTR gene is hereafter possible by the present method in the facilities for non-radioactive experiments.

Nucleotide sequence polymorphisms in the human genome have been conventionally identified as restriction fragment length polymorphism (RFLP). RFLP, however, can be detected only when DNA polymorphisms are present in the recognition sequences for the corresponding restriction endonucleases or when deletion or insertion is present in the region detected by a specified probe. To identify DNA polymorphisms more efficiently, the limitations of RFLP analysis should be overcome by an alternative method.

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

0006-291X/91 $1.50

This is the first report showing the application of single-strand conformation polymorphism (SSCP) analysis for detection of a mutant transthyretin (TTR) gene. We have modified the SSCP detection method developed by Orita et al. (1, 2), in that radioactive primers and probes were omitted and DNA was detected by chemiluminescent reaction. In the mother and son affected with polyneuropathy, an electrophoretic mobility shift was found in the fragments of the exon 3 and sequence analysis confirmed a single base change, producing a new mutant gene (TTR Arg 58). In addition, this mutation provided an insight for the relationship between the mutation site on the TTR molecule and the clinical phenotypic expression in the hereditary polyneuropathy.

PATIENTS AND METHODS

Patients. The proband is a 39-year-old male with a 3-year history of weakness and dysesthesia in the hands. He presented muscular atrophy in the distal part of the four limbs. His other complaints included orthostatic hypotension, impotence and marked loss of weight. His 62-year-old mother also has been suffering from weakness and dysesthesia in the hands for 15 years and had surgical decompression of carpal tunnel without relief. Vitreous opacities were present since the age of 53.

Polymerase chain reaction (PCR). Nucleotide sequences and numbering of the TTR gene were based on published data (3). Genomic DNAs were prepared from peripheral leukocytes from the 2 patients and 30 controls including 2 whose TTR gene sequences were known to be normal. Four pairs of oligonucleotide primers were synthesized to amplify the coding sequence in each exon (Table 1). The primer sets previously reported (4) were also used to amplify the fragments including the entire length of each exon and exon-intron boundaries. PCR amplification conditions were as previously described (5).

Table 1. Summary of the primer sets for PCR-SSCP analysis

Primer	Primer sequence #	amplified##		
1-F	5'-CATCACAGAAGTCCACTCATTCTTGGCAGG-3'	-4-125	(exon 1)	129bp
1-R	5'-GTAGGAATGGGATGTCACAGAAACACTCAC-3'			
2-F	5'-TAACTTCTCACGTGTCTTCTCTACACCCAG-3'	990-1179	(exon 2)	190bp
2-R	5'-TCCTGTGGGAGGGTTCTTTGGCAACTTAC-3'			
3-F	5'-TAACTTAATCCAGACTTTCACACCTTATAG-3'	3213-3408	(exon 3)	196bp
3-R	5'-AAAACAACCCTCGAAGGTCTGTATACTCAC-3'			
4-F	5'-GGAAATGGATCTGTCTGTCTTCTCTCATAG-3'	6661-6828	(exon 4)	168bp
4-R	5'-GTCCTTCAGGTCCACTGGAGGAGAAGTCCC-3'			

The primer sequences and the numbering of the TTR gene are based on published data (3).
The amplified region of the TTR gene with each primer set and the fragment size.

Non-isotope SSCP analysis. SSCP analysis was performed by the method as described by Orita (2), except that radioactive oligonucleotide primers and probes were omitted. The PCR products were added with 4-10 volumes of loading buffer containing 0.1 % SDS, 20 mM EDTA, 95 % formamide, 0.05 % bromophenol blue and 0.05 % xylene cyanol. The DNA samples were heated at 90 ℃ for 10 min to denature. Next, 2 μl of each sample was loaded onto a 5 % nondenaturing polyacrylamide gel (20 cm X 55 cm X 0.3 mm), containing 0.5 X TBE. Electrophoresis was carried out at 15 ℃, 2000 V for 3 hrs (Macrophor system, Pharmacia LKB). DNA fragments in the gel were then transferred to a nylon membrane (Hybond N+, Amersham) by capillary transfer blotting in 10 X SSC overnight at room temperature. The membrane was then dried and baked at 80 ℃ for 2 hrs. TTR cDNA was labeled with biotin-11 dUTP by the random primer method using Smilight DNA Labeling Kit (Sumitomo Metal Industry LTD.) and hybridized to single-stranded DNAs immobilized on the membrane. After hybridization in 4 X SSPE at 65 ℃, the membrane was washed for 30 min at 55 ℃ in 0.1 X SSC. DNA on the membrane was detected by the chemiluminescent DNA detection method using Smilight DNA Detection Kit (6, 7). Biotinylated alkaline phosphatase was bound to the biotinylated probe via streptavidin bridge and catalyzed the light reaction by cleaving off phosphate group from an added chemiluminescent substrate. A 10-minute exposure to Kodak Omat AR film was usually sufficient to allow detection of the single strand DNA.
Nucleotide sequence analysis. PCR products amplified were cloned into M13 vectors and sequenced by the dideoxytermination method (8).
RFLP analysis. The single base change predicted a new restriction site for HhaI (GCGC). The digested amplified fragments were run on a 12 % polyacrylamide gel, stained with ethidium bromide and observed over UV .
Allele-specific oligonucleotide (ASO) hybridization. PCR-amplified exon 3 fragments from the patients and 30 controls were dot-blotted onto nylon membranes (Hybond N+) and denatured with NaOH. The filters were subsequently hybridized with ^{32}P-endlabeled ASOs: 19 base of normal ASO containing T at position 3275 (5'-TGCATGGGCTCACAACTGA-3') and mutant ASO containing G at the same position. Filters were washed in 5 X SSC at 60 ℃ for 10 min.

RESULTS AND DISCUSSION

SSCP analysis of amplified fragments containing coding sequence of exon 1, 2 and 4 showed the same patterns in the patients and controls. As shown in Figure 1, the fragments carrying exon 3 from the patients showed one extra band in addition to 2 normal bands. This pattern suggested that the patients were heterozygous for the TTR gene, and one strand of the mutant allele took a novel conformation with a different electrophoretic mobility. The other strand, possessing the complementary sequence change, did not take an aberrant conformation causing a mobility shift, and so migrated with the corresponding strand of the normal allele. As the folded conformation of single-stranded DNA is expected to be influenced by environmental factors such as the temperature and presence of glycerol in gels (1), various conditions at different temperatures (5-22 ℃) were employed for the electrophoresis in this study. The aberrant shift of the DNA fragments of the exon 3 was clearly observed at 15 ℃ without glycerol. The mobility shift was reduced when the

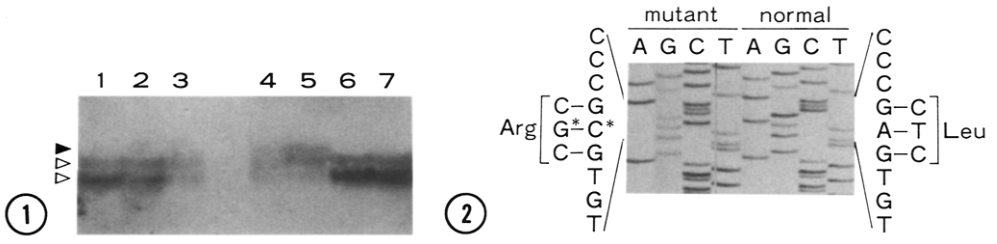


Fig.1. SSCP analysis of PCR-amplified exon 3 from the proband (lane 4), his mother (lane 5) and controls (lanes 1, 2, 3, 6, 7). The positions of the aberrantly migrating fragments in the patients (lanes 4, 5) are shown by a closed arrow. The bands indicated with open arrows are seen in all individuals.

Fig.2. Sequencing analysis of PCR-amplified exon 3 detected the two allelic segments in the patient's TTR gene. There is a T to G transversion, resulting in a Leu to Arg substitution in one allele and the other allele has normal sequence.

electrophoresis was performed with the presence of 10 % glycerol in the gels or at lower temperatures (data not shown).

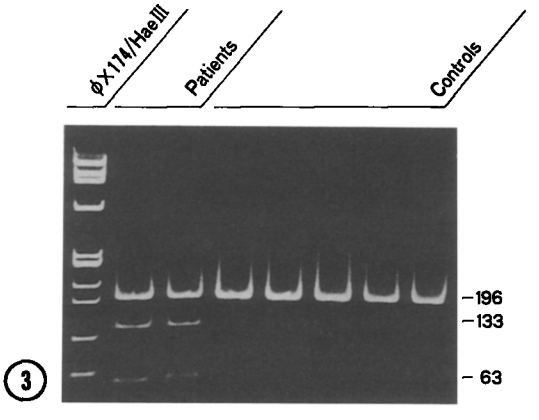
In the present study, the chemiluminescent DNA detection method (6, 7) was used for the SSCP analysis because of its several advantages over the radio-isotope detection method. The biotinylated DNA probes and chemiluminescent substrate are stable for more than 6 months. The phosphatase catalyzed light reaction shortens the detection time; only a 10-minute exposure to X-ray film is usually sufficient to obtain the signals.

To determine the nucleotide change responsible for the mobility shift, the DNA fragments coding for exon 3 were amplified from the proband's DNA, cloned into M13 vectors and sequenced. The sequence analysis of 10 independent clones showed that the sequences of 6 clones had a single base change from T to G at position 3275 in the TTR gene. This mutation corresponded to the codon change from CTC coding for Leu to CGC coding for Arg at position 58 of the 127-residue molecule. The other 4 clones contained gene fragments with the normal sequence (3) (Figure 2). The same mutation was confirmed to exist in the clones established from the genomic DNA of the affected mother. To rule out the aberrant sequences over the entire length of each exon and exon-intron boundaries, the gene fragments were amplified using the primer sets previously reported (4) and sequenced. The same mutation was identified and sequences of exon-intron boundaries were normal in

the 2 patients. All these results effectively excluded cloning and amplification artifacts and established the mutation. The detection of the 2 different allelic segments endorsed that the patients were heterozygous for the TTR gene.

The T to G transversion was expected to produce a new restriction site for HhaI (GCGC) within exon 3. HhaI digestion of the amplified gene fragments (196 bp:3213-3408) containing the exon 3 from the patients yielded two extra fragments (63 and 133 bp) in addition to the normal fragment (196 bp) (Figure 3). To ascertain the linkage of the base change with the disease, the amplified DNA was subjected to ASO hybridization analysis. DNA samples from the patients were hybridized with both variant and normal ASOs and those from 30 controls exclusively with a normal ASO (Figure 4). As a result, it was confirmed that the patients were heterozygous for the TTR gene, thus strongly suggesting that this mutation was responsible for the disease.

TTR Arg 58, identified in this study, may provide an insight for the relationship between the mutation site and the clinical phenotypic expression. Twelve TTR variants have been hitherto reported in the different kindreds with familial amyloid polyneuropathy (9), and the clinical manifestations in cases



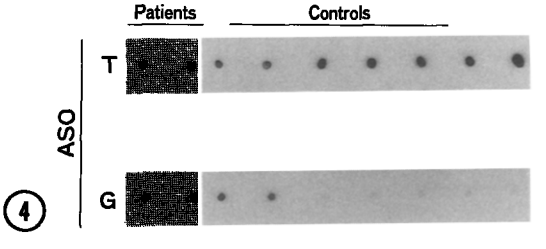


Fig.3. PCR-amplified exon 3 was digested with HhaI and electrophoresed on a 12 % polyacrylamide gel. The samples from the patients were heterozygous for the HhaI restriction site. The samples from controls had no restriction site for HhaI.

Fig.4. Autoradiograph of ASO hybridization. Exon 3 from the proband, his mother and 5 controls, when probed with ^{32}P-ASOs complementary to the normal (specific for T) or the mutant (specific for G) allele sequences.

of an individual kindred vary considerably. However, carpal tunnel syndrome is commonly seen and a distinguishing feature in the present cases (Arg 58) and two other independent families, the Maryland/German family (His 58) (10, 11) and the Appalachian family (Ala 60) (12). Interestingly, all the three mutations are located closely with one another in the D-E loop of the TTR molecule, in an area joining two beta-plated sheet regions (13). This fact implies that the conformation changes of the region including, at least, residues 58-60 seem to be responsible for the unique clinical expression, carpal tunnel syndrome.

In summary, the non-isotope SSCP analysis method has been used successfully for the detection of a single base change in the TTR gene, and eventually a new mutant gene encoding TTR Arg 58 has been identified in the two cases with hereditary polyneuropathy. Hereafter, SSCP in a large number of sample DNAs can be analyzed by our present method even in the facilities for non-isotope experiments.

ACKNOWLEDGMENT

This work was supported in part by a grant from the Senri Life Science Center (S.U.).

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