Video Article

Genetic Analysis of Hereditary Transthyretin Ala97Ser Related Amyloidosis

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Abstract

Genetic testing is the most reliable test for hereditary transthyretin related amyloidosis and should be performed in most cases of transthyretin amyloidosis (ATTR). ATTR is a rare but fatal disease with heterogeneous phenotypes; therefore, the diagnosis is sometimes delayed. With increasing attention and broader recognition on early manifestations of ATTR as well as emerging treatments, appropriate diagnostic studies, including the transthyretin (TTR) genetic test, to confirm the types and variants of ATTR are therefore fundamental to improve the prognosis. Genetic analyses with polymerase chain reaction (PCR) methods confirm the presence of TTR point mutations much more quickly and safer than conventional methods such as southern blot. Herein, we demonstrate genetic confirmation of the ATTR Ala97Ser mutation, the most common endemic mutation in Taiwan. The protocol comprises four main steps: collecting whole blood specimen, DNA extraction, genetic analysis of all four TTR exons with PCR, and DNA sequencing.

Video Link

The video component of this article can be found at https://www.jove.com/video/57743/

Introduction

Transthyretin (TTR) amyloidosis (ATTR) is the most common form of hereditary systemic amyloidosis[1], and can be caused by an autosomal dominantly inherited mutation in the transthyretin (TTR) gene[2]. TTR mutations destabilize the tetrameric protein structure and lead to its dissociation into monomers that reassembles into amyloid fibrils[2]. More than 100 amyloidogenic TTR mutations have been reported worldwide[1]. Genetic analyses with polymerase chain reaction (PCR) methods confirm the presence of TTR point mutation and have advantages including avoiding the handling of radioactively labeled probes in comparison with southern blot[3]. PCR is a fast, easy, cheap, and reliable technique that has been applied to numerous fields in modern sciences[4].

The early diagnosis of this progressive and fatal disease is challenging given its phenotypic heterogeneity. With increasing attention and broader recognition on the early manifestations of ATTR as well as the emerging treatments[5], appropriate diagnostic studies including TTR genetic test are therefore critically fundamental to improve prognosis. Furthermore, different mutations are associated with different penetrance of the trait, age of onset, patterns of progression, disease severity, median survival, efficacy of liver transplantation, or TTR stabilizers[2,6], and variable degrees of neurological and cardiological involvement, which have great implications for genetic counseling[7,8,9]. Besides, a highly accurate genetic test is the only tool that differentiates the two distinct types of ATTR: hereditary (mutant) and wild type (non-mutant form, senile systemic amyloidosis, SSA)[7]. It is imperative to confirm the types of ATTR, because the therapies vary widely[2]. Therefore, there is an increasing necessity to describe the stepwise protocol of the TTR genetic test.

The molecular approach to detect the mutation will be illustrated using Ala97Ser, the most common endemic mutation in Taiwan, as an example. Modifications in the DNA extraction step reduce the amount of the three solutions used and yields a sufficient amount of DNA. In this protocol, all four TTR exons were analyzed, while regions including 5' upstream, 3' downstream, promoters, introns, and untranslated regions (UTR) were not sequenced.

Protocol

The testing performed in the laboratory was carried out in accordance with the requirements of the Clinical Laboratory Improvement Amendments (CLIA) of 1988, the regulations approved by the Institutional Review Board of Chang Gung Memorial Hospital and University (License no. 100-4470A3 and 104-2462A3). Informed consent was obtained from all patients.

1. Blood Specimen Collection

1. Collect whole blood into commercially available EDTA-treated tubes. Mix gently and store blood sample at 4 °C until processing.

2. DNA Extraction from Peripheral Blood

Use a DNA Extraction Kit for the genomic DNA extraction (see **Table of Materials**).

1. Add 10 mL of Solution 1 to the 4.0 mL blood sample. Incubate the sample on ice for 10 min.
2. Centrifuge the sample at 2,000 x g for 5 min at 4 °C. Remove and discard supernatant carefully. Resuspend the pellet in 3 mL of Solution 1.
3. Centrifuge the sample at 2,000 x g for 5 min at 4 °C again and discard supernatant. Resuspend the pellet in 3 mL of Solution 2.
4. Add 10 µL of pronase stock solution (225 mg/mL) to get a final concentration of 100 µg/mL and mix well. Incubate at 37 °C overnight.
5. Chill the tube on ice for 10 min. Add 0.8 mL of Solution 3 to the sample and invert 3 - 5 times. Place sample on ice for 5 min.
6. Centrifuge the sample at 3,000 x g for 15 min at 4 °C. Carefully transfer the supernatant to a 15 mL sterile conical centrifuge tube.
7. Add 6 µL of RNase stock solution (10 mg/mL) to get a final concentration of 20 µg/mL. Incubate at 37 °C for 15 min.
8. Add 2.5 mL of isopropyl alcohol and mix thoroughly by gently inverting several times to precipitate DNA (strands of white, flocculent material will form). Using a large-bore pipet, transfer the DNA precipitant to a new 1.5 mL centrifuge tube.
9. Centrifuge at 12,000 x g for 3 min at 4 °C. Discard supernatant carefully. Wash DNA pellet by adding 500 µL of 70% ethanol.
10. Centrifuge at 12,000 x g for 1 min and discard supernatant. Air dry DNA pellet at room temperature. Add 100 µL of ddH_2O and incubate at 65 °C for 15 min to resuspend DNA.

3. Genomic DNA Quantification

Use a spectrophotometer (see **Table of Materials**) to detect the quantity and quality of the genomic DNA.

1. Log into the computer attached to the spectrophotometer machine. Open the software.
2. Initialize the spectrophotometer machine:
    1. Click "Nucleic Acid" on the spectrophotometer software.
    2. Clean the pedestal with a disposable paper wipe and purified water.
3. Blank the spectrophotometer:
    1. Load 2 µL of purified water on the pedestal.
    2. Lower the upper arm of the spectrophotometer and click "Blank" to calibrate it.
    3. When it is done, lift the upper arm and dry the pedestal with a wipe.
4. Measure the sample:
    1. Click "DNA-50" on Sample Type.
    2. Load 2 µL of DNA sample on the pedestal.
    3. Lower the upper arm and click "Measure" to measure the A260/A280 ratio and the concentration of the sample.
    4. Clean the pedestal in between each run with a wipe and purified water.
5. Click "Print Screen" to collect the data.

4. Genetic analyses of mutations

Amplify the target DNA with the PCR[4]. Perform PCR in a 25 µL reaction mixture using a DNA Polymerase kit (see **Table of Materials**). **Table 1** shows the TTR gene intronic primers.

gene	Primer sequence
Exon1	F: 5'-TCAGATTGGCAGGGATAAGC-3' R: 5'-GCAAAGCTGGAAGGAGTCAC-3'
Exon2	F: 5'-TCTTGTTTCGCTCCAGATTTC-3' R: 5'-TCTACCAAGTGAGGGGCAAA-3'
Exon3	F: 5'-GTGTTAGTTGGTGGGGGTGT-3' R: 5'-TGAGTAAAACTGTGCATTTCCTG-3'
Exon4	F: 5'-GACTTCCGGTGGTCAGTCAT-3' R: 5'-GCGTTCTGCCCAGATACTTT-3'

Table 1. TTR gene intronic primers. (NCBI Reference Sequence: NC_000018.10)

1. Add the reagents in **Table 2** to a 0.2 mL PCR tube.

Components	Volume (µL)	Final concentration
10x Buffer	2.5	1x
$MgCl_2$ (25 mM)	1.5	1.5 mM
360 GC Enhancer	1	-
360 DNA Polymerase	0.2	2 U/reaction
dNTP Mix (25 mM each)	2	2 mM each
Primer F (10 µM)	1	0.4 µM
Primer R (10 µM)	1	0.4 µM
DNA (100 ng)	2	
PCR-grade water	13.8	-
Total Volume	25	-

Table 2. PCR reaction conditions.

2. Mix gently and briefly centrifuge on a benchtop centrifuge to collect all components to the bottom of the tube. Place tube in a thermocycler.
3. Perform PCR for 30 cycles. Each cycle consists of a denaturation step for 30 s at 94 °C, primer annealing for 30 s at 58 °C, and polymerase extension for 45 s at 72 °C (see **Table 3**).

Step	Temperature (°C)	Time	cycle
Initial denaturation	95	5 min	1
DNA denaturation step	94	30 s	30 cycles
Primer annealing step	58	30 s	30 cycles
Polymerase extension step	72	45 s	30 cycles
Post elongation step	72	10 min	1
End of PCR cycling	4	Indefinite	

Table 3. Cycling conditions for amplifying PCR products.

4. Validate the PCR product by visualizing with gel electrophoresis:
    1. Prepare a 1.2% agarose gel:
        1. Mix 1.2 g of agarose powder with 100 mL of 0.5x TBE in a microwavable flask. Microwave for 2 min until the agarose is completely dissolved. Cool down the agarose mixture to 55 °C.
        2. Add 2 µL of a ethidium bromide solution (10 mg/mL) to the agarose mixture and mix gently. Pour the agarose mixture into a gel tray to about 5 - 7 mm, then add the comb(s) in place. Allow it to solidify (at least 30 min) and carefully remove comb(s).
    2. Load samples and run an agarose gel:
        1. Place gel and tray into the electrophoresis cell. Fill the electrophoresis cell with 0.5x TBE until the gel is covered.
        2. Carefully load 2 µL of a 100 bp DNA ladder (see **Table of Materials**) into the one lane of the gel as molecular size marker. Load dye/sample mixture (5 µL of PCR product mix with 1 µL 6x loading dye) into the additional lanes.
        3. Turn on the power supply. Run the gel for 25 min at 100 V.
        4. Remove the gel from the electrophoresis cell. Visualize the DNA fragments under UV light in an imaging system.

5. DNA sequencing

1. Purify PCR products using a commercial kit and sequence commercially or with an automatic sequencer (see **Table of Materials**).
2. Submit nucleotide sequences to the NCBI BLAST server to compare the nucleotide query to the nucleotide databases[10]. After the results are displayed, perform sequence alignments and identify the expected mutation.

Representative Results

Agarose gel electrophoresis of two patients and one healthy individual revealed bands of the expected sizes, including a 454 bp PCR product for exon 4 of the TTR gene (**Figure 1**).

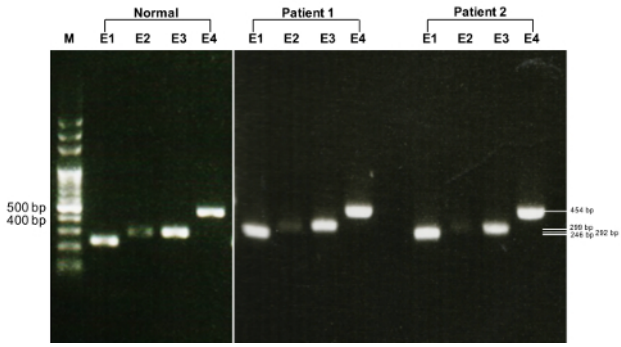

Figure 1. Gel electrophoresis depicting PCR amplified TTR gene. Normal: a healthy individual. Lane M: 100 bp DNA ladder as molecular size marker. Lanes 1: 246 bp (exon 1). Lanes 2: 292 bp (exon 2). Lanes 3: 299 bp (exon 3). Lanes 4: 454 bp (exon 4). Please click here to view a larger version of this figure.

Direct sequencing disclosed a nucleotide T substitution for G in exon 4 of the TTR gene. This missense mutation resulted in an alanine-to-serine substitution at amino acid position 97 in two patients. Sequence chromatograms of these two patients and one healthy individual are demonstrated in **Figure 2**.

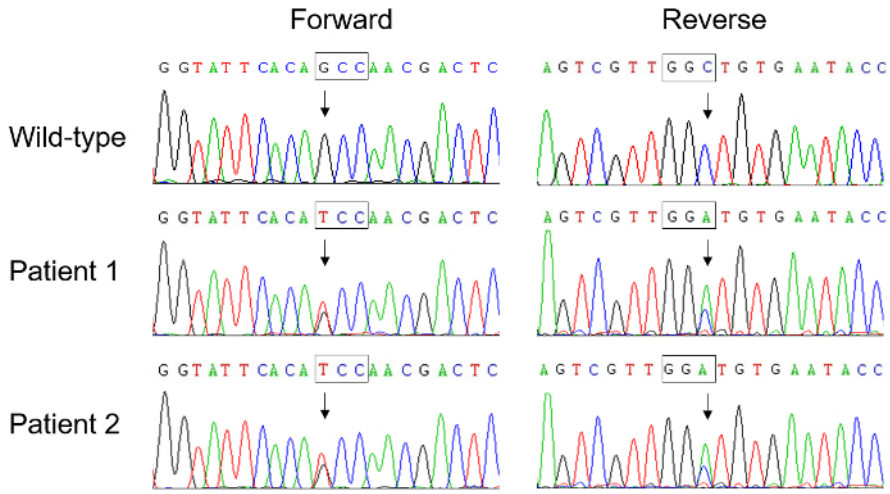

Figure 2. Sequencing of a healthy individual (wild-type) and a heterozygous G>T mutation in exon 4 of the TTR gene (patients 1 and 2). Arrows in the Patient 1 and Patient 2 chromatograms indicate two overlapping peaks of different colors. The two peaks are about half the height of the rest of the sequence. This heterozygous substitution, (G/T) is confirmed in the reverse sequence, (C/A). Parts of this figure have been modified from a figure in our previous publication[5]. Please click here to view a larger version of this figure.

Discussion

There are two critical steps within the protocol. First, in order to have sufficient number of white blood cells, a hemodiluted specimen should be avoided[11]. Second, the use of appropriate PCR primers is fundamental to obtain reliable results[12]. We used the Primer-BLAST web tool to design the primers[4,13]; a minimum of 40 base pairs on each side of the four TTR exons should be covered. We also run BLAST on NCBI to check the specificity of the primers.

Some modifications are made in the DNA extraction step. First, the amount of the three solutions used are reduced. This modification also yields a sufficient amount of DNA and saves solutions. Second, isopropanol or 100% ethanol are the two alternatives used in the precipitation of DNA[14,15]. Compared with ethanol, isopropanol precipitates DNA at room temperature, which reduces coprecipitation of salt[15]. Additionally, longer precipitation times at freezer temperatures may be needed to maximize the DNA yield[15]. Third, TE buffer is substituted for double distilled water (ddH_2O) to resuspend DNA. The DNA preparation in this protocol is used for subsequent PCR so protection on storage is not a concern. Also, EDTA will inhibit enzyme activity when the DNA is used in PCR[15].

For a lower cost and less waiting time, PCR product purification and sequencing were performed by a biotechnology company. The limitation in this protocol should be the manual methods, including repeated centrifugation steps. An automated nucleic acid extraction system has been developed, and is beneficial for reducing working time and increasing reproducibility and quality of the results[16].

In all patients with amyloid deposits, differential diagnosis of systemic amyloidoses should be performed. Mass spectrometry (MS) can be applied to determine the protein subunit and classify the disease as immunoglobulin light-chain amyloidosis (AL, with a median survival inferior to ATTR)[17] or transthyretin-related amyloidosis[18,19], which direct downstream genetic testing cannot do, especially for those patients for whom hereditary ATTR was not initially suspected[19].

Mass spectrometry-based proteomic analysis has been used for screening and typing of TTR amyloidosis[19,20,21]. Nevertheless, MS alone is not sufficient to exclude a pathogenic mutation in patients with hereditary ATTR[22], because some of the atypical or rare TTR variants may not be separated by MS-based analysis of amyloid deposits specimen or serum samples in a clinical laboratory[19,23]. Furthermore, possible sampling errors, and the uneven distribution of amyloid fibrils may lead to a false negative tissue biopsies[5,21,24], making the downstream proteomic analysis difficult. Therefore, DNA testing, the most reliable test for ATTR, should be performed in most cases of ATTR[5,22], as well as in any idiopathic progressive axonal peripheral neuropathy or distal symmetric painful small-fiber neuropathy[25,26].

Other factors that may related to phenotypic variation for ATTR and guide future directions include post-translational modification (PTMs) variants present in serum and ATTR fibril composition[27,28]. Although hereditary ATTR is a monogenetic disease, considerable variability even among those with the same mutation or within the same family has been observed[27]. Genetic heterogeneity alone fails to elucidate the diverse onset and pathology of the ATTR[28]. Therefore, both genetic and proteomics methods should be utilized when these factors are taken into account.

Disclosures

The authors have nothing to disclose.

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References

1. Planté-Bordeneuve, V., Said, G. Familial amyloid polyneuropathy. *The Lancet Neurology.* **10** (12), 1086-1097 (2011).
2. Planté-Bordeneuve, V.*, et al.* Long-term treatment of transthyretin familial amyloid polyneuropathy with tafamidis: a clinical and neurophysiological study. *Journal of Neurology.* **264** (2), 268-276 (2017).
3. Nichols, W.C., Benson, M.D. Hereditary amyloidosis: detection of variant prealbumin genes by restriction enzyme analysis of amplified genomic DNA sequences. *Clinical Genetics.* **37** (1), 44-53 (1990).
4. Lorenz, T.C. Polymerase Chain Reaction: Basic Protocol Plus Troubleshooting and Optimization Strategies. *Journal of Visualized Experiments.* (63), e3998 (2012).
5. Hsu, H.C.*, et al.* Phenotypic expressions of hereditary Transthyretin Ala97Ser related Amyloidosis (ATTR) in Taiwanese. *BMC Neurology.* **17** (1), 178 (2017).
6. Barroso, F.A.*, et al.* Long-term safety and efficacy of tafamidis for the treatment of hereditary transthyretin amyloid polyneuropathy: results up to 6 years. *Amyloid.* **24** (3), 194-204 (2017).
7. Rapezzi, C.*, et al.* Disease profile and differential diagnosis of hereditary transthyretin-related amyloidosis with exclusively cardiac phenotype: an Italian perspective. *European Heart Journal.* **34** (7), 520-528 (2013).
8. Mariani, L.L.*, et al.* Genotype-phenotype correlation and course of transthyretin familial amyloid polyneuropathies in France. *Annals of Neurology.* **78** (6), 901-916 (2015).
9. Hammarström, P., Jiang, X., Hurshman, A.R., Powers, E.T., Kelly, J.W. Sequence-dependent denaturation energetics: A major determinant in amyloid disease diversity. *Proceedings of the National Academy of Sciences of the United States of America.* **99** (suppl 4), 16427-16432 (2002).
10. Johnson, M., Zaretskaya, I., Raytselis, Y., Merezhuk, Y., McGinnis, S., Madden, T.L. NCBI BLAST: a better web interface. *Nucleic Acids Research.* **36** (Web Server issue), W5-9 (2008).
11. Fox, A.J.*, et al.* Next generation sequencing for the detection of actionable mutations in solid and liquid tumors. *Journal of Visualized Experiments.* **2016** (115), (2016).
12. Date, Y., Nakazato, M., Kangawa, K., Shirieda, K., Fujimoto, T., Matsukura, S. Detection of three transthyretin gene mutations in familial amyloidotic polyneuropathy by analysis of DNA extracted from formalin-fixed and paraffin-embedded tissues. *Journal of the Neurological Sciences.* **150** (2), 143-148 (1997).
13. Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., Madden, T.L. Primer-BLAST: a tool to design target-specific primers for polymerase chain reaction. *BMC Bioinformatics.* **13** 134 (2012).
14. Green, M.R., Sambrook, J. *Molecular cloning: A laboratory manual.* Cold Spring Harbor Laboratory Press. Cold Spring Harbor, N.Y. (2012).
15. Buckingham, L., Flaws, M.L. *Molecular diagnostics: Fundamentals, methods, and clinical applications.* F.A. Davis Co. Philadelphia. (2012).
16. Tan, S.C., Yiap, B.C. DNA, RNA, and Protein Extraction: The Past and The Present. *Journal of Biomedicine and Biotechnology.* **2009** (2009).
17. Rapezzi, C.*, et al.* Systemic cardiac amyloidoses: disease profiles and clinical courses of the 3 main types. *Circulation.* **120** (13), 1203-1212 (2009).
18. Gertz, M.A., Dispenzieri, A., Sher, T. Pathophysiology and treatment of cardiac amyloidosis. *Nature Reviews Cardiology.* **12** (2), 91-102 (2015).
19. Vrana, J.A.*, et al.* Clinical diagnosis and typing of systemic amyloidosis in subcutaneous fat aspirates by mass spectrometry-based proteomics. *Haematologica.* **99** (7), 1239-1247 (2014).
20. Nakamura, M.*, et al.* Identification of a new transthyretin variant (Ile49) in familial amyloidotic polyneuropathy using electrospray ionization mass spectrometry and nonisotopic RNase cleavage assay. *Human Heredity.* **49** (4), 186-189 (1999).
21. Ueda, M., Ando, Y. Recent advances in transthyretin amyloidosis therapy. *Translational Neurodegeneration.* **3**, 19 (2014).
22. Brown, E.E.*, et al.* Genetic testing improves identification of transthyretin amyloid (ATTR) subtype in cardiac amyloidosis. *Amyloid.* **24** (2), 92-95 (2017).
23. Tasaki, M.*, et al.* Rapid detection of wild-type and mutated transthyretins. *Annals of Clinical Biochemistry.* **53** (4), 508-510 (2015).
24. Plante-Bordeneuve, V.*, et al.* Diagnostic pitfalls in sporadic transthyretin familial amyloid polyneuropathy (TTR-FAP). *Neurology.* **69** (7), 693-698 (2007).

25. Hsu, J.L., *et al.* A prospective, observational study of patients with uncommon distal symmetric painful small-fiber neuropathy. *PLoS ONE.* **12** (9), e0183948 (2017).
26. Salvi, F., Pastorelli, F., Plasmati, R., Bartolomei, I., Dall'Osso, D., Rapezzi, C. Genotypic and phenotypic correlation in an Italian population of hereditary amyloidosis TTR-related (HA-TTR): Clinical and neurophysiological aids to diagnosis and some reflections on misdiagnosis. *Amyloid.* **19** (suppl 1), 58-60 (2012).
27. Suhr, O.B., Lundgren, E., Westermark, P. One mutation, two distinct disease variants: Unravelling the impact of transthyretin amyloid fibril composition. *Journal of Internal Medicine.* **281** (4), 337-347 (2017).
28. Vilà-Rico, M., *et al.* Quantitative analysis of post-translational modifications in human serum transthyretin associated with familial amyloidotic polyneuropathy by targeted LC-MS and intact protein MS. *Journal of Proteomics.* **127**, 234-246 (2015).

