

Original Article

Evidence of the presence of amyloid substance in the blood of familial amyloidotic polyneuropathy patients with ATTR Val30Met mutation

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Abstract: Transthyretin (TTR) is a major amyloid fibril protein found in patients with familial amyloidotic polyneuropathy (FAP) and senile systemic amyloidosis (SSA). Mainly synthesized in the liver, TTR is transferred in the form of tetramer bound with thyroxine, retinol-binding protein (RBP) and lipoprotein in the blood. The aim of this study was to demonstrate the presence of amyloid substances in the blood by investigating the hemocoelom amyloid in different tissue sections from autopsies such as brain, kidney, heart and aorta arch tissue. Congo red staining was employed following by application of polarized light examination, to verify the presence of amyloid deposition in the tissues. Immunohistochemical staining was then performed to identify the specific type of amyloid deposition. Matrix-assisted laser desorption-ionization/time of flight mass spectrometry (MALDI-TOF/MS) was also used to analyze TTR mutation in FAP patients. All subjects were FAP ATTR Val30Met patients. In FAP patients, TTR amyloid deposition was found mainly in the tunica intima of the aortic arch. Interestingly, amyloid substance was found in the blood of FAP patient. Our results suggest that amyloid substance was present in the blood of FAP ATTR Val30Met patients.

Keywords: Amyloid, transthyretin, familial amyloidotic polyneuropathy, immunohistochemistry, Congo red staining

Introduction

Transthyretin (TTR) is a problematic protein closely associated with familial amyloidotic polyneuropathy (FAP) and senile systemic amyloidosis (SSA). TTR is a protein of 127 amino acids in which four monomers are non-covalently bound to thyroxine or retinol binding protein (RBP) forming a tetramer of 56 kDa, and involved in transport of thyroid hormones in the blood [1, 2]. It has been demonstrated by immunohistochemistry and in situ hybridization that TTR is synthesized in the liver cells, choroid plexus cells, retina cells and islet cells. In human blood, more than 90 percent TTR proteins are synthesized and secreted by liver cells [3, 4].

It is well-known that FAP patients have been found with amyloid deposits in peripheral nerves, visceral organs, autonomic nervous system, gastrointestinal tract, heart, small and medium-sized vessels of the circulatory system, kidney and ocular tissues [5]. The amyloid deposition was appeared in extracellular and

as the formation of amorphous materials [6, 7]. Despite being tested positive for amyloid deposition in the above mentioned systems and organs in the FAP patient, the surrounding hepatocytes were clearly amyloid negative. This phenomenon suggested that the effect of TTR amyloid formation may play an important role in amyloidogenesis in FAP.

Recent reports have linked both TTR chemical modifications and mutations of TTR protein factor(s) of the TTR gene to amyloidogenesis [7-10]. However, it has not been clearly demonstrated that the modification of TTR is the target of amyloidogenesis. In current study, we explore the presence of amyloid substance in the blood of FAP ATTR Val30Met patients.

Materials and methods

Materials

Tissue samples were supplied by Department of Diagnostic Medicine, Graduate School of Medical Sciences, Kumamoto University. All

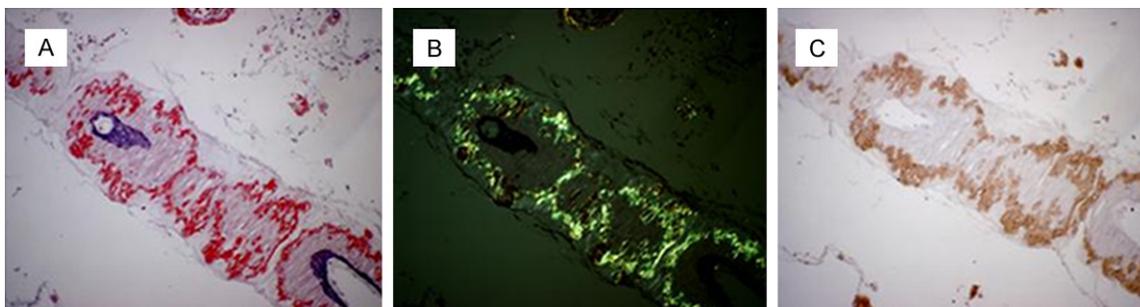


Figure 1. Analysis of TTR related amyloid deposition in the arteries of FAP ATTR Val30Met patients. Presence of amyloid deposits was assessed by Congo Red staining in adjacent sections (A) and the Congo red staining waved under polarized light examination (B). Immunohistochemical analysis of TTR deposition in Congo Red-positive, and specificity of anti-human TTR antibody immunoreaction was found in amyloid positive deposition (C).

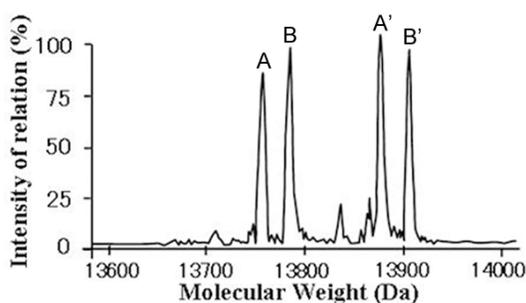


Figure 2. Identification of TTR mutation by MALDI-TOF/MS analysis. A serum sample from a 58 year old male FAP ATTR Val30Met patient was analyzed by MALDI-TOF/MS. A: Free form of wild-type TTR; B: Cystine (Cys)-conjugated form of wild-type TTR; A': Free form of variant TTR (ATTR Val30Met); B': Cys-conjugated form of variant TTR.

FAP patients in this study had a definitive diagnosis of FAP on the basis of genetic investigations and clinical manifestations of FAP. The research followed the guidelines of the Kumamoto University Ethical Committee.

Seven patients with ATTR Val30Met (five men and two women, age 31-58 years, average age 41.7 ± 10.7 years) were involved in study. All of them have been diagnosed with TTR mutation based on matrix-assisted laser desorption-ionization/time of flight mass spectra (MALDI-TOF/MS) analysis and genetic investigation. Whole blood were collected from these 7 patients and stored at -80°C for protein and gene mutation analysis. Venous blood was drawn into vacutainer tubes (Becton-Dickinson, Rutherford, NJ) containing disodium-EDTA (1.5 mg/dl). Immediate identification of TTR protein mutations were made separately for collected plasma and blood cells, respectively. Brain, kidney, heart and aorta arch tissue from autopsies were seri-

ally sectioned to 4-5 μm thickness and processed for Congo red staining and immunohistochemical analysis.

Congo red staining

The presence of amyloid in tissue sections of FAP Val30Met patents was investigated after staining with Congo red via observation under polarized light [9]. Deparaffinized tissues sections were incubated with 80% ethanol saturated with NaCl for 30 min followed by 0.5% Congo red in 80% ethanol saturated with NaCl, then analyzed under polarized light. Amyloid was identified by its characteristic green birefringence.

Immunohistochemistry

Immunohistochemistry testing was performed using polyclonal rabbit anti-human transthyretin antibodies (DAKO, Glostrup, Denmark), and the same tissues examined by Congo red staining were incubated in blocking buffer (1% bovine serum albumin). Anti-human TTR antibody diluted 1:500 in blocking buffer and a 1:100 dilution in blocking buffer of horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (DAKO, Glostrup, Denmark) were used as the primary and secondary antibodies respectively. Reactivity visualization was made with the DAB liquid System (DAKO, Glostrup, Denmark) as described by the manufacturer. The primary antibody on parallel control sections was replaced by blocking buffer [9].

Immunoprecipitation of TTR and subsequent analysis by MALDI-TOF/MS

500 μl serum was mixed with 30 μl of polyclonal rabbit anti-TTR antibody (DAKO) and

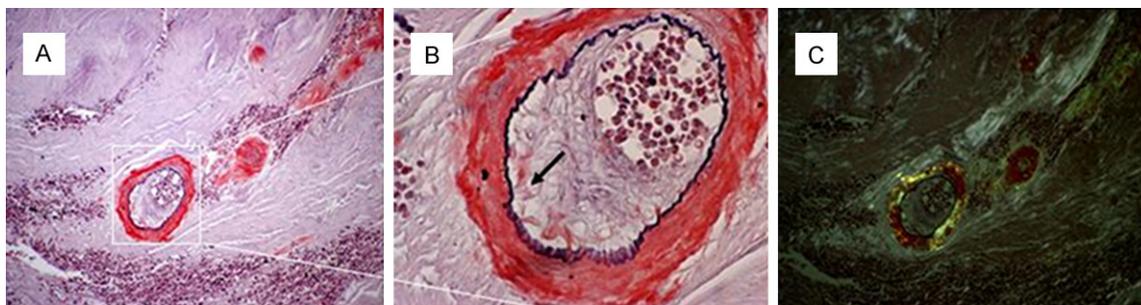


Figure 3. Identification of amyloid substance in a FAP Val30Met patient with Congo red staining and polarized light. (A, C) Congo red staining. (C) The Congo red staining under polarized light microscopy. (B) It is magnification of (A). Original magnifications: $\times 10$ (A, C); $\times 50$ (B).

incubated for 24 h at 4°C. After centrifugation at 9000 g for 15 min, the supernatant was removed and the precipitate washed twice with 100 μ l of saline and twice with 100 μ l of distilled water (DH₂O). The precipitate was dissolved in 30 μ l of solution containing 4% acetic acid and 4% acetonitrile. This solution was then passed through a 1000 kDa TTR centrifugal concentrator (Pall Filtron Northborough, MA). The pass-through fraction was mixed with sinapinic acid in a saturated diluent of 0.1% trifluoroacetic acid. The mixtures were deposited onto the sample probe assembly.

All experiments were performed with a Bruker Reflex mass spectrometer (Bruker Franzen Analytik GmbH, Bremen, Germany) operated at a wavelength of 337 nm. The best spectra of TTR were obtained at an ion accelerating voltage of 27.5 kV and a reflection voltage of 30 kV. The spectra were calculated using external calibration and horse myoglobin (m/z 16952.27) [11].

Distribution of amyloid deposition in the FAP patient tissues

The renal tissues and aorta arch tissues in all patients with ATTR Val30Met were Congo red stained. In the renal section, ratio of amyloid deposits glomerular positive to glomerular negative was calculated for seven FAP ATTR Val30Met patients. The midline of the wall of the aortic arch was used to create two visible regions under light microscopy, one being the region of tunica intima, the other the region of tunica adventitia. The case number of amyloid positives was investigated on the tunica intima region and/or tunic adventitia region in the aorta arch wall for all patients.

Results

Assessment of TTR-related amyloid deposition by Congo red staining and immunohistochemistry

For all subjects of this study, we firstly identified the amyloid deposition by Congo red staining and polarized light examination. Immunohistochemical staining was then performed to identify the specific type of amyloid deposition was TTR TTR-related amyloid. In FAP patients, TTR related amyloid was examined in organs such as the heart, small intestine, kidney, brain and the wall of the aorta arch. All of these locations were found amyloid positive. The positive results of TTR immunoreaction and Congo red staining were completely consistent (**Figure 1**).

Analysis of TTR mutation by MALDI-TOF/MS

Using the combination of immunoprecipitation and subsequent MALDI-TOF-MS, TTR mutation could be shown as well as the monozygosity or heterozygosity. In serum of all the patients, the mass spectra results have shown four principal peaks which molecular mass were corresponded to the native, unmodified TTR (**Figure 2A**), ATTR Val30Met (**Figure 2B**), Cys adducts for S-cysteine (TTR-Cys-S-S-Cys) (**Figure 2A**) and ATTR Val30Met-Cys-S-S-Cys (**Figure 2B**), respectively. The relative abundance of total Wild-type transthyretin (W-TTR) was approximately equal to total ATTR Val30Met (**Figure 2**).

Investigation of blood containing amyloid substance

Amyloid substance-containing blood was investigated in the following organs in FAP ATTR

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Table 1. Assessment of TTR deposition in aorta for FAP patients

Aortic arch	Congo red	Polarized light examination	TTR immunoreaction
Site of tunica intima	7/7	7/7	7/7
Site of tunica adventitia	1/7	1/7	1/7

Val30Met patients: heart, peripheral nerves, gastrointestinal tract, kidney and vascular wall of aorta arch by Congo red staining and polarized light examination. Amyloid substance in the blood was proved both by Congo red staining and polarized light examination positive in intra-artery and/or intravenous blood coagulation. Among the seven FAP patients, amyloid substance was found in the brain arterial cavity of one ATTR Val30Met patient (**Figure 3**).

Characterization and distribution of amyloid deposition in the aortic arch of FAP ATTR Val30Met patients

In all seven FAP ATTR Val30Met patients, amyloid deposits were found in the wall of the aorta arch. This type of amyloid was deposited following smooth muscle fibers as conglomeration. More specifically, in all seven subjects, amyloid deposits appeared in the region of tunica intima, but not in the tunica adventitia (**Table 1**). And in kidney, the amyloid deposition appeared in the some glomerulus whereas other areas were negative (**Figure 4**).

Discussion

By examining amyloid deposition in FAP ATTR Val30Met patients, amyloid deposits were found in the peripheral nerves, gastrointestinal tract, heart, kidney, and aorta arch. Immunohistochemical staining was then performed to identify the specific type of amyloid depositions were TTR immunoreaction-positive ones. We found that amyloid substance was present in the blood and hemocoelom as demonstrated by Congo red staining and polarized light examination. In one particular study, there was simultaneous finding of amyloid substance in the hemocoelom of small arteries in the brain of FAP ATTR Val30Met patients and around a thrombus.

To classify whether Wild type transthyretin (W-TTR) or mutated transthyretin (M-TTR) was associated with amyloidosis. We first identified amyloid deposits in the tissues by Congo red staining and polarized light examination, then

analyzed TTR mutation by MALDI-TOF/MS. In our investigation, amyloid-positive tissues include: cardiac muscle, aorta arch, gastrointestinal tract and kidney (**Figure 1**). For all amyloid-positive patients, the selected TTR

mutation was ATTR Val30Met, characterized by four peaks in serum TTR and the ratio of W-TTR to mutant transthyretin (M-TTR) equaling approximately 50 percent (**Figure 2**). Mutation of the TTR gene was identified by PCR (data not shown) so as to guarantee that all participated subjects in this study were indeed FAP ATTR Val30Met patients.

In order to determine whether amyloid substances exist in circulation, our experiment focused on hemocoelom amyloid substance in organs such as the brain, visceral organs, gastrointestinal tract, heart, kidney and aorta arch. The amyloid deposits were detected by Congo red staining and polarized light examination in FAP ATTR Val30Met patients. In one case, both Congo red staining and polarized light examination was found positive in hemocoelom of small arteries in brain f (**Figure 3**). According to the above evidence, the presence of amyloid substance in blood should be found when patient brain tissue and small vessels are studied (**Figure 1**). As supporting evidence, Congo red-positive staining was localized in the white thrombi in the hemocoelom of small arteries. More specifically, amyloid deposits existed in blood condensation. This phenomenon revealed that amyloid substance was fixed with blood agglomeration so that they can be stained by Congo red. It has been well known, that Congo red staining and polarized light examination are powerful methods for the identification of amyloid deposition. However, it was difficult to find amyloid deposits in blood due to false negatives produced when amyloid substance is lost during the dyeing process.

It has been reported that the TTR formation is significantly augmented by TTR fibril seeding in acidic condition. TTR fibril seed can therefore promote TTR amyloidogenesis [8, 9]. In circulation, the form of TTR in blood contained both free TTR and lipoprotein-bound TTR [10]. The exact form of TTR associated with TTR amyloidogenesis is in need of more study.

When investigated amyloid deposits in the kidney of FAP ATTR Val30Met patients, all subjects

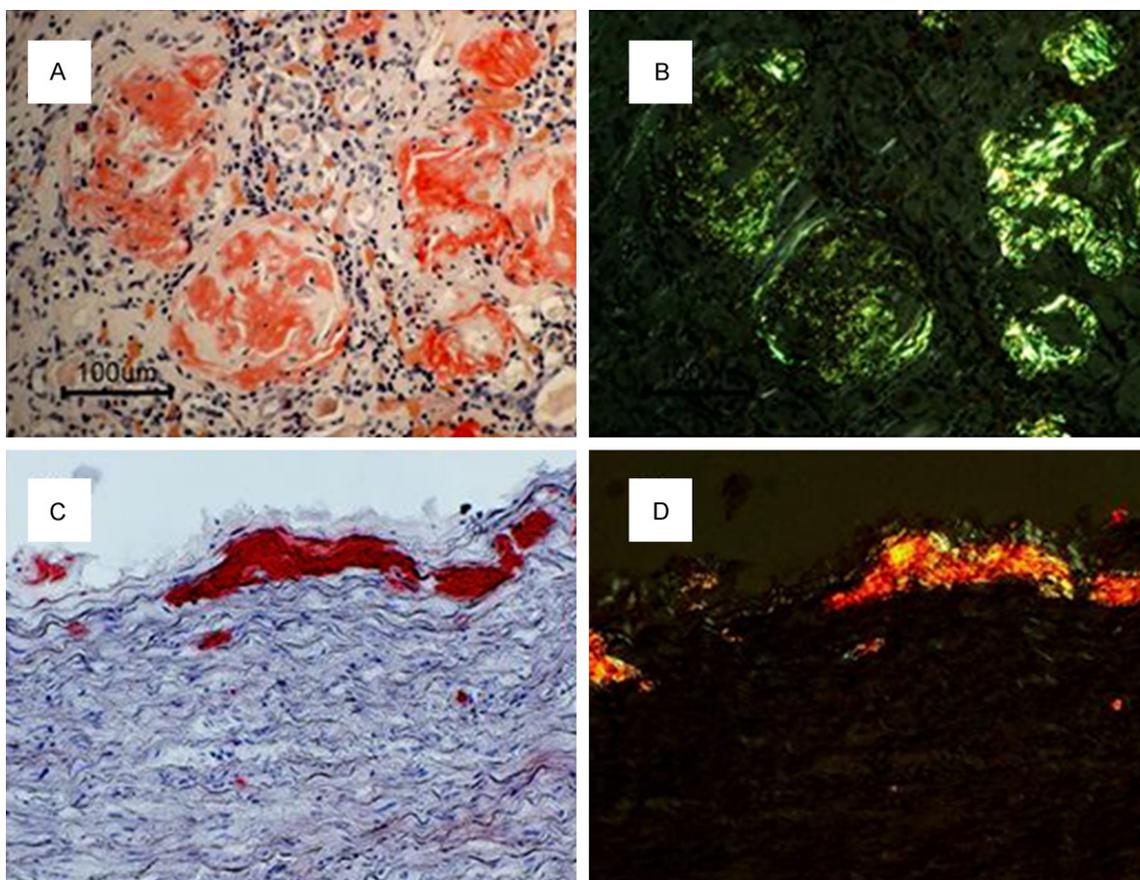


Figure 4. The character of amyloid in FAP ATTR Val30Met patient with Congo red staining and polarized light examination. A, C: Congo red staining; B, D: Polarized light examination. A, B: Kidney. C, D: Large artery.

were found amyloidical depositing in some glomeruli whereas other areas were negative (**Figure 4**). We speculated that, whether there is blood inside the deposition of amyloid components, which small molecules of TTR amyloid fibers can go through the filtration membrane and retain in the interstitial glomeruli where the formation of amyloid deposition was promoted.

Amyloid deposits were mainly distributed in medium-sized and/or small arterial wall tissues of FAP patients. Aortic arch amyloid deposits were found to be non-continuous and arranged along the muscle-fibers of aorta. An outer surface consisting of non-amyloid deposits was found (**Figure 4C, 4D**). H&E stained muscle fibers did not display obvious morphological changes (results not shown). TTR in blood circulation may exist as monomers, tetramers, and apolipoprotein-bound TTR, the same parts of the vessel wall present within the TTR will not have significant differences in content, but the density of amyloid formation was so differently

in the artery, suggesting that amyloid fibers may be involved in the formation and deposition of intramural aortic arch.

Amyloid substance was found in the blood of FAP ATTR Val30Met patients with arterial thrombosis. The form of serum TTR may be associated with TTR related amyloidogenesis.

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Disclosure of conflict of interest

None.

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