protein for thyroid hormone and retinol-binding protein with vitamin A [17]. The plasma TTR concentration is reduced in conditions involving inflammation and protein malnutrition [18]. TTR causes two kinds of amyloidotic diseases. One is a hereditary systemic amyloidosis, FAP, which is induced by MT TTR [1-3]. The other type is senile systemic amyloidosis (SSA), which is an aging-related sporadic systemic amyloidosis that is induced by wild-type (WT) TTR [19,20]. Destabilization of TTR tetramers is widely believed to be a critical step in TTR amyloid formation (Figure 1) [21].

FAP

Until 20 years ago, FAP was believed to be a disease that was restricted to an endemic presence in those specific areas. However, progress in biochemical and molecular genetic analyses resulted in an understanding that this disease now occurs worldwide. To date, more than 130 mutations in the TTR gene have been reported [2,22]. Although 15 TTR mutations are nonamyloidogenic, other TTR mutations induce systemic amyloidosis, which can be classified into several phenotypes including peripheral neuropathy dominant type, commonly called FAP; cardiomyopathy dominant type, also known as familial amyloidotic cardiomyopathy; vitreous opacity dominant type, which is thought to be derived mainly from TTR synthesized from RPE cells; and leptomeningeal amyloidosis dominant type, which is believed to be derived mainly from the choroid plexuses of the brain and causes central nervous symptoms. Hereditary TTR amyloidosis manifests genotype-phenotype correlations [2,23,24], and some variation in clinical presentation is often observed in individual kindreds with the same mutation and even among family members. Of the pathogenic TTR mutations, Val30Met was the first to be identified and is the best known mutation found throughout the world, although the reason for this distribution is not known.

In Japan, Araki *et al.* first reported a group of patients with FAP ATTR Val30Met in Kumamoto [22]. Sensorimotor polyneuropathy, autonomic dysfunction, heart and kidney failure, gastrointestinal (GI) tract disorders, and other symptoms (Figure 2) that led to death, usually within 10 years of the onset of disease, have been documented in patients with FAP ATTR Val30Met [1]. In addition to the two endemic foci that were identified in Japan, many other FAP kindreds with TTR Val30Met and other mutations were found in Japan (Figure 3). That phenotypic differences exist among patients with the same Val30Met mutation and depend on geographic origin is well known [3,25-27]. Families originating from Portugal and two endemic areas in Japan (Arao city in Kumamoto Prefecture and Ogawa village in Nagano Prefecture) usually have early-onset and high-penetrance FAP, whereas other Japanese kindreds and Swedish families evidence late-onset and low-penetrance FAP [3,25-27]. Recent studies reported that certain Swedish patients with late-onset FAP ATTR Val30Met have amyloid deposits containing truncated TTR [28-31], which is usually found in SSA, a sporadic form of TTR amyloidosis [28,32]. Morphological ultrastructural studies showed that amyloid fibrils in those cases were tightly packed, haphazardly arranged, and fairly short compared with fibrils from other FAP ATTR Val30Met patients, usually with early-onset disease, who did not have truncated TTR in amyloid deposits [28]. The specific functional and pathological roles of truncated TTR remain to be determined, however.

SSA

SSA, in which WT TTR forms amyloid deposits in various tissues, is an age-related nonhereditary systemic amyloidosis

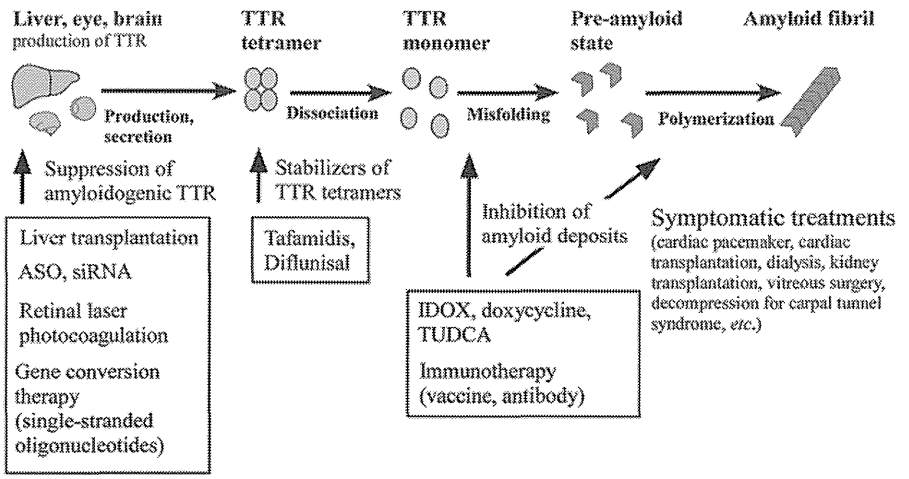

Figure 1 TTR amyloid formation and therapies for FAP.

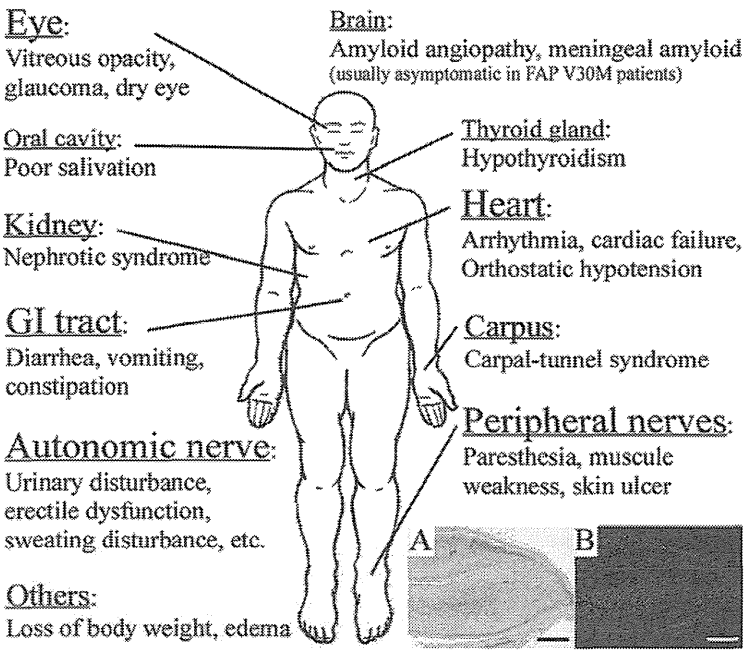

Figure 2 Clinical manifestations in FAP ATTR Val30Met patients. (A, B) Amyloid deposits in the sciatic nerve. **(A)** Congo red staining. **(B)** Polarized light. Scale bars: 500 μm.

and affects mainly cardiac functions in elderly people [19,20,33-35]. Postmortem studies demonstrated that the prevalence of SSA was 12–25% in patients older than 80 years [19,20]. Furthermore, recent studies determined that WT TTR amyloid may also cause several other disorders and conditions [12], such as radiculomyelopathy [36], tongue necrosis [37], hematuria [38], nodular amyloid deposits in the lung [39], and frequent amyloid deposition in ligaments and tendons such as carpal tunnel and spinal ligaments [40,41], to a degree not previously known. In addition to full-length WT TTR, truncated C-terminal WT TTR fragments starting at positions 46–52 are usually detected in amyloid deposits obtained from SSA patients [32,35].

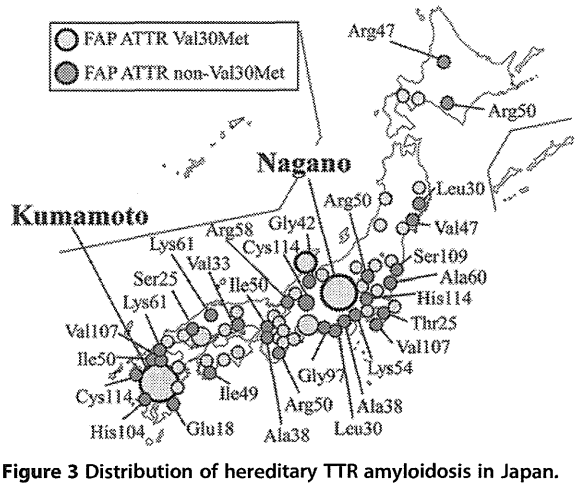

Figure 3 Distribution of hereditary TTR amyloidosis in Japan.

Diagnosis

Effective medical treatment of patients with TTR amyloidosis requires an accurate diagnosis based on various studies and techniques [42], such as histopathology, genetic testing, and mass spectrometry (Figure 4). Although diagnosis in the early stage of FAP is absolutely imperative for proper treatment, the diagnosis is sometimes delayed, especially in patients without a clear family history and typical clinical manifestations of FAP [43,44].

Histopathological examinations play a critical role in obtaining direct evidence of amyloid deposits and determining the type of amyloid-causing protein [45]. Systemic amyloidosis including FAP is usually diagnosed on the basis of biopsies of several tissue sites, such as subcutaneous adipose tissue of the abdominal wall [46], GI tract [47,48], and labial salivary gland [49], because biopsies of tissue sites with main clinical symptoms such as peripheral nerves, heart, and kidney are more invasive. Biopsy specimens are subjected to Congo red staining to detect amyloid deposits and are viewed with a microscope under polarized light. In patients with only a small amount of amyloid deposition, a confirmed diagnosis may require repeated biopsies. Most often, immunohistochemical determination of the chemical composition of amyloid

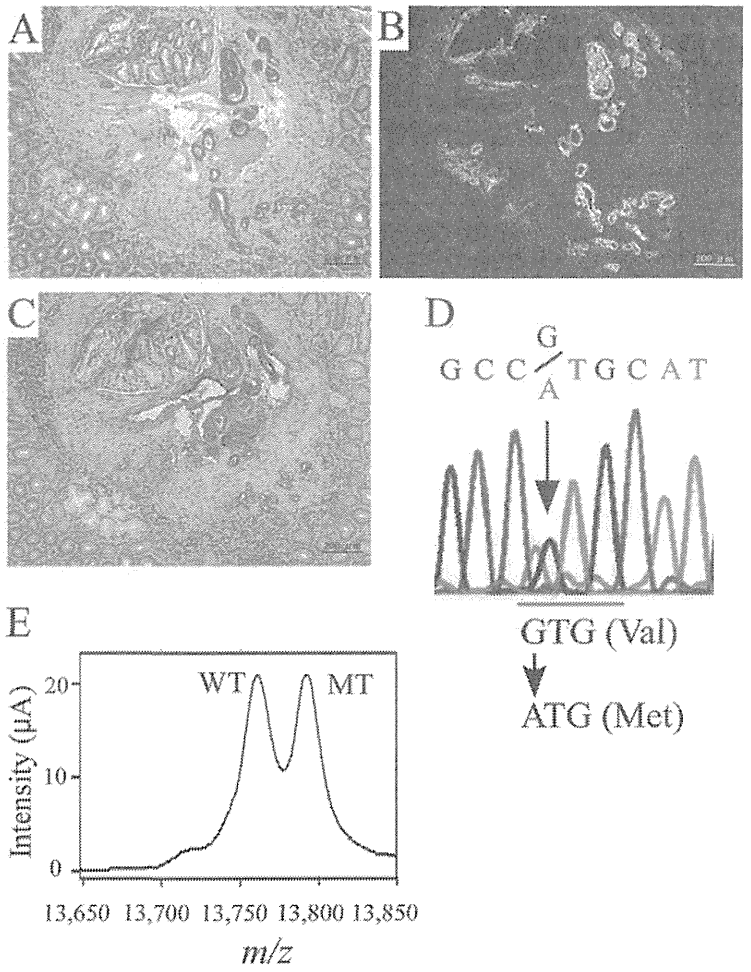

Figure 4 Diagnostic studies for FAP. (A-C) Histopathological images of a biopsy specimen of the duodenum obtained from an FAP ATTR Val30Met patient. **(A)** Congo red staining. **(B)** Polarized light. **(C)** Immunohistochemistry with an anti-TTR antibody. **(D)** Sequence analysis of the TTR gene. **(E)** SELDI-TOF MS analyses of serum TTR from an FAP ATTR Val30Met patient. The molecular weight of MT TTR is 32 *m/z* higher than that of WT TTR.

is the first step in classifying the type of amyloid: a panel of antibodies recognizing different amyloid types is used to reveal the origin of deposited protein [50,51]. However, misdiagnoses have occurred in some cases, especially when immunohistochemical staining is performed in the absence of standardized antibodies and appropriate positive controls [52]. Extraction of amyloid fibril proteins from frozen and formalin-fixed tissues or from amyloid-containing histopathological tissue sections followed by immunostaining or amino acid sequence analyses is another useful way to characterize amyloid deposits [53,54], especially when immunohistochemical data are negative or inconclusive. A novel rapid test to determine the type of amyloidosis based on tandem mass spectrometric analysis, with specific sampling of clinical biopsy specimens by means of laser microdissection, was recently reported [55,56]. With this method, the authors successfully identified the amyloid proteins and classified the types of amyloidosis including TTR amyloidosis [39,57]. This method may be a useful clinical tool for aiding the accurate typing of amyloidosis.

To obtain accurate results for the TTR mutation in FAP patients or carriers of variant TTR genes, both genetic and proteomic methods should be applied to compensate for the disadvantages and possible pitfalls of each technique (Figure 4). Mass spectrometric analysis allows detection of variant TTRs in serum, because an amino acid substitution results in a change in the molecular weight of TTR circulating in the bloodstream [58-60]. We recently applied surface-enhanced laser desorption/ionization time-of-flight mass spectrometry (SELDI-TOF MS) to rapid detection of variant TTR in a patient's serum [61]. This technique allows the analysis of a variant form of TTR in a one-step procedure.

FAP treatments

The sections that follow provide details of conventional and potential treatments of TTR amyloidosis (Figure 1).

Liver transplantation

With regard to treatment of FAP, liver transplantation (LT) has reportedly halted the progression of clinical manifestations [62,63]. Exchange of an FAP patient's diseased liver with a healthy liver causes MT TTR in the body to be replaced by WT TTR, except for cerebrospinal fluid and eyes, into which MT TTR is secreted from the choroid plexus and the retina, respectively, even after LT [64,65]. Since 1990, FAP patients have undergone LT as FAP treatment [66,67]. According to data in the FAP World Transplant Registry [66], approximately 120 orthotopic LTs are performed worldwide each year. LT reportedly prolonged the survival of FAP ATTR Val30Met patients who were carefully selected for the procedure [68,69]. The modified body mass index, disease duration, age, type of TTR mutation, and degree of cardiac involvement are thought to be important prognostic factors for the disease course after LT [62,67,69]. The survival rate of FAP ATTR non-Val30Met patients after LT was reportedly less than that of FAP ATTR Val30Met patients after LT [66,70,71]. LT could be less effective for the patients with ATTR non-Val30Met who have cardiomyopathy or leptomeningeal dominant symptoms.

Our criteria for performing LT for FAP patients were as follows: age younger than 60 years, duration of disease from onset <5 years, creatinine clearance >70 ml/min, modified body mass index >500, no cardiomegaly, and no gait disturbance [72]. The main causes of death in patients after orthotopic LTs involved cardiac problems (24%), sepsis (23%), and liver-related complications (14%) [66,70,71]. Although clinical evaluations indicated that progression of other clinical symptoms such as peripheral neuropathy [73,74], GI symptoms [75], and renal involvement [72,76] usually stopped after LT in FAP ATTR Val30Met patients, other studies suggested that LT failed to prevent progression of cardiac amyloidosis in FAP ATTR Val30Met patients after LT [69,77], with this failure reportedly being due to continued formation of amyloid mainly derived from WT TTR secreted from the transplanted normal liver graft [29,30,78]. However, why WT TTR amyloid deposits, which are usually found in elderly people with SSA, occur in some tissue sites of FAP patients after LT remains to be clarified. It may be that older amyloid deposits formed by MT TTR before LT may act as a nidus, which is well known to enhance polymerization of proteins [79], and that additional WT TTR amyloid fibrils may form because of nucleation-dependent polymerization after LT, although TTR amyloid formation *in vitro* reportedly did not depend on nucleation [80].

Because of the shortage of livers for transplantation to patients with malignant or end-stage liver diseases, the method of using sequential LT with resected livers from FAP patients was developed [81]. Some patients who underwent sequential LT with livers from FAP patients reportedly started to evidence TTR amyloid deposits less than 10 years after the surgery [82-84]. However, we do not know whether all such second recipients will eventually have symptoms of FAP.

Stabilizers of TTR tetramers: tafamidis and diflunisal

The working hypothesis of amyloid formation established by a substantial number of studies led to the idea that stabilizing tetrameric TTR would be a promising method to prevent amyloid formation [85]. Tetrameric TTR itself is thought to be nonamyloidogenic, but dissociation of the tetramer into compact non-native monomers with low conformational stability can lead to amyloid fibril formation [86]. Baures *et al.* reported that, on the basis of *in vitro* experiments, various nonsteroidal anti-inflammatory drugs have potential for stabilizing tetrameric TTR [87]. These efforts thoroughly established this possibility of stabilization of the tetrameric form of TTR as a therapeutic strategy.

Two drugs—tafamidis, which is a novel TTR stabilizer, and diflunisal, which is a nonsteroidal anti-inflammatory drug developed in 1971 and can stabilize TTR tetramers—are undergoing clinical development throughout the world. Tafamidis, a potent and selective stabilizer of tetrameric TTR, has been approved in Europe and Japan for treatment of adult FAP patients with early symptomatic polyneuropathy to delay neurological impairment [88,89]. In the clinical trial, patients treated with tafamidis had less neurological deterioration than patients who began tafamidis later; they had some preservation of function as measured by the Neuropathy Impairment Score-Lower Limb [88,89]. Recently, Berk et al. also reported that diflunisal reduced the rate of progression of neurological impairment and preserved quality of life [90]. Therapeutic effects of those TTR stabilizers on long-term outcomes, cardiac functions and ophthalmic symptom remain to be elucidated. Moreover, a number of structurally diverse small molecules that bind to TTR, increase its stability, and thereafter inhibit amyloid fibrillogenesis have been tested.

Gene therapies to suppress TTR expression

Knowledge gained by using LT as therapy for FAP suggested that inhibition of amyloidogenic TTR may prevent progression of the disease. Antisense methods and small interfering RNAs (siRNAs) are effective gene-silencing tools. The antisense oligonucleotides (ASO), which were recognized as a therapeutic tool in the 1970s, cause enzymatic degradation of mRNA. RNA interference, first discovered in *Caenorhabditis elegans*, is sequence-

specific post-transcriptional gene silencing [91]. Specific gene expression has also been inhibited by RNA interference in mammalian cells by skipping the Dicer step [92]. These methods should be powerful tools for FAP gene therapy.

Early studies in this field aimed to selectively inhibit production of MT TTR. Different kinds of siRNA selectively silenced TTR gene expression both *in vitro* and *in vivo* [93]. Recently developed siRNA and ASO therapies, however, inhibit both MT TTR and WT TTR [94,95], because WT TTR also contributes to the formation of amyloid in FAP, especially after LT. Benson *et al.* demonstrated that ASO suppressed TTR mRNA levels in the liver and in the choroid plexus of the brain [96,97]. Researchers completed a phase I study evaluating the safety and activity of ASO in healthy volunteers [95]. ASO reduced plasma TTR protein levels up to 80% without causing clinically significant adverse reactions. A phase II/III study evaluating the efficacy of ASO in patients with FAP is ongoing in 2014. Also, a phase I clinical study with siRNA for FAP was completed [94], and a phase II/III study is ongoing in 2014.

Retinal laser photocoagulation

Even after LT, ocular complications have reportedly persisted and worsened, because RPE cells of the eye continued to synthesize MT TTR in FAP patients [64,98]. To suppress TTR synthesis and ocular symptoms, we have evaluated retinal laser photocoagulation. This operation, which is commonly used to treat many retinal diseases, damages the retinal pigment epithelium, the main location of synthesis of ATTR in ocular tissues. To date, we discovered that retinal laser photocoagulation clearly prevented progression of amyloid deposition in the vitreous and on the retinal surface in certain FAP patients, without causing any adverse effects [99]. Retinal laser photocoagulation may thus be a new procedure for mitigating ocular manifestations in FAP patients.

4′-Iodo-4′-deoxydoxorubicin, doxycycline, tauroursodeoxycholic acid, and cyclodextrin

Other candidate therapeutic compounds, including 4′-iodo-4′-deoxydoxorubicin (IDOX), doxycycline, tauroursodeoxycholic acid (TUDCA), and cyclodextrin (CyD), have also been studied.

Merlini *et al.* first reported IDOX as an agent that would bind to amyloid fibrils found in five different types of amyloidosis [100]. Sebastiao *et al.*, in an *in vitro* study, noted the interaction of IDOX and ATTR Leu55-Pro and reported the rapid dissociation of monoclinic ATTR Leu55Pro crystals soaked with IDOX [101].

Doxycycline influences many functions of mammalian cells such as proliferation, migration, apoptosis, and matrix remodeling [102]. Cardoso and colleagues investigated the effects of doxycycline treatment *in vivo* by using ATTR Val30Met transgenic mice [103,104].

TUDCA is a unique natural compound that is a potent antiapoptotic and antioxidant agent, as it reduces cytotoxicity in a number of neurodegenerative diseases. Macedo *et al.* studied the possible therapeutic application of TUDCA in FAP [105] and found that TUDCA treatment significantly decreased the amount of TTR toxic aggregates.

CyDs are cyclic oligosaccharides composed of 6–8 glucose units [106]. Because CyDs contain a central hydrophobic cavity, which can serve as an inclusion site for hydrophobic molecules, CyDs are now used as multifunctional drug carriers [107]. Jono *et al.* reported that 6-*O*-α-(4-*O*-α-D-glucuronyl)-D-glucosyl-β-CyD (GUG-β-CyD), a branched β-CyD derivative, may inhibit TTR misfolding by stabilizing the conformation of TTR by means of interacting with hydrophobic amino acids, especially the Trp residue of TTR, which thereby suppresses TTR amyloid formation [108]. CyDs are safe and already widely used in many fields, especially pharmaceuticals, so GUG-β-CyD may become a curative drug for TTR amyloidosis.

Gene conversion therapy

As described above, LT was originally suggested as a treatment that would halt production of variant TTR in the liver. Most FAP symptoms do not progress after LT, when the MT TTR gene is replaced by the WT TTR gene in the liver. This finding led to the suggestion that gene therapy to correct the TTR gene mutation may ameliorate the clinical symptoms of FAP. Nakamura *et al.* demonstrated gene conversion by single-stranded oligonucleotides in rabbit eyes expressing rabbit WT TTR and in transgenic murine liver in which the intrinsic WT TTR gene was replaced by a TTR Val30Met gene [109].

Immunotherapies

Immunotherapies are also major candidates for TTR amyloidosis treatment. Gustavsson *et al.* used various antigenic mapping methods to find out whether major antigenic sites differed for normal TTR, ATTR, and *in situ* amyloid fibrils [110]. Their data suggested that antigenic sites on normal plasma TTR included the AB and CD loops, with the associated amino acid sequences occurring on the outside of the TTR molecule. An antiserum against β-strand H (anti-TTR115-124), which establishes the dimer's monomer-to-monomer interaction areas, reacted with only ATTR in amyloid fibrils, not with normal TTR in plasma. These findings suggested an altered TTR configuration in amyloid fibrils compared with the TTR configuration in plasma. Thus, anti-TTR115-124, which seems to be amyloid specific, may be valuable as a probe and in antibody therapies.

An MT TTR Tyr78Phe that was designed to destabilize the native structure of TTR tetramer has exposed a cryptotope

recognized by a monoclonal antibody that reacts only with amyloid fibrils or with highly amyloidogenic MTs that present the amyloid fold [111]. Terazaki *et al.* demonstrated that immunization with TTR Tyr78Phe effectively reduced TTR deposition and cleared amyloid deposits in an FAP rodent model transgenic for human TTR Val30-Met with amyloid deposition in the GI tract [112]. This therapy may be applied to FAP ATTR Val30Met patients with amyloid deposition in tissues. In addition, this treatment may be useful for vaccination of healthy variant TTR gene carriers to prevent TTR amyloid deposition in tissues.

Serum amyloid P component (SAP) is a major component of amyloid deposits in all types of amyloidoses. Recently, Bodin, et al. investigated therapeutic effects of anti-human SAP antibodies on AA amyloid deposition using a human SAP transgenic mouse model [113]. The antibodies removed AA amyloid deposits in the mouse model. This antibody therapy might be applicable to FAP.

Conclusions

Although LT and TTR stabilizers became practical treatments for FAP, there remain many clinical issues we have to improve. Several clinical trials using other new methods are ongoing. These novel therapies may prove to prevent progression of FAP.

Abbreviations

ASO: Antisense oligonucleotides; ATTR: Amyloidogenic TTR; FAP: Familial amyloidotic polyneuropathy; GI: Gastrointestinal; GUG-β-CyD: 6-*O*-α-(4-*O*-α-D-glucuronyl)-D-glucosyl-β-CyD; IDOX: 4′-iodo-4′-deoxydoxorubicin; LT: Liver transplantation; MT: Mutant; RPE: Retinal pigment epithelial; SELDI-TOF MS: Surface-enhanced laser desorption/ionization time-of-flight mass spectrometry; siRNAs: Small interfering RNAs; SSA: Senile systemic amyloidosis; TTR: Transthyretin; TUDCA: Tauroursodeoxycholic acid; WT: Wild-type.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

MU and YA drafted and revised the manuscript. Both authors read and approved the final manuscript.

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Long-Term Outcomes and Complications of Trabeculectomy for Secondary Glaucoma in Patients with Familial Amyloidotic Polyneuropathy

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Abstract

Objective: Secondary glaucoma is a serious complication in patients with transthyretin (TTR)-related familial amyloidotic polyneuropathy (FAP). We assessed the long-term outcomes and complications of trabeculectomy with mitomycin C (MMC) for secondary glaucoma associated with FAP.

Methods: Medical case records of Kumamoto University Hospital were retrospectively reviewed. Twenty-one eyes of 13 patients (10 with FAP ATTR Val30Met; 3 with FAP ATTR Tyr114Cys) underwent trabeculectomy with MMC and follow-up of at least 2 years. The primary outcome measure was Kaplan-Meier survival, with failure of this treatment being defined as an intraocular pressure (IOP) of ≤5 mm Hg or ≥22 mm Hg on two consecutive visits or as additional operations needed to reduce IOP. Secondary outcome measures included complications, bleb characteristics, and additional postoperative interventions required.

Results: The mean postoperative follow-up period was 5.7 years (range, 2.2–12.7 years). Kaplan-Meier analysis indicated probabilities of success of 0.76, 0.67, and 0.53 at 1, 2, and 3 years after operation, respectively. Significant complications included ocular decompression retinopathy in 7 eyes (33%) and bleb encapsulation in 10 eyes (48%). Twelve eyes (57%) needed additional surgery, such as bleb revision or trabeculectomy with MMC, to reduce IOP.

Conclusions: Trabeculectomy with MMC may not be optimal for patients with FAP-related glaucoma and may have several significant complications.

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Introduction

Transthyretin (TTR)-related familial amyloidotic polyneuropathy (FAP), which is characterized by systemic accumulation of mutant amyloidogenic TTR (ATTR) in organs and peripheral nerves, is a fatal amyloidosis that is inherited in an autosomal dominant fashion [1]. Of more than 100 ATTR mutations, FAP ATTR Val30Met is highly prevalent in Portugal, Sweden, Japan, and other countries. In Portugal and Japan, the penetrance is higher and symptoms typically develop before the age of 40. In contrast, in Sweden, the penetrance is very low and symptoms usually develop after the age of 50 [1]. Patients with TTR-related FAP commonly have ocular manifestations, especially vitreous opacity and glaucoma, which are troublesome and can restrict the daily lives of patients; these ocular involvements occur more frequently during the course of the illness [2–4].

We previously reported on the short-term outcomes of surgical treatment of patients with secondary glaucoma associated with TTR-related FAP and the clinical features of these patients [5]. Glaucoma occurred in 12 (24%) of 49 patients; amyloid deposition at the pupillary border, a pupillary border with irregularity, and vitreous opacity were strongly related to the occurrence of glaucoma; and intraocular pressure (IOP) was well controlled during the short follow-up period (mean, 1.2 years; range, 0.2–6.5 years). Also, we suggested that trabeculectomy with mitomycin C (MMC) may be the most promising treatment modality. However, during a longer follow-up period, certain patients required additional operations, and several specific complications developed after surgery.

The study described here therefore continued our evaluation of the efficacy of trabeculectomy with MMC in patients with glaucoma secondary to TTR-related FAP.

Methods

Subjects and Data Collection

We retrospectively reviewed the medical records of patients with secondary glaucoma associated with FAP who underwent trabeculectomy with MMC at the Department of Ophthalmology, Kumamoto University Hospital, between 1987 and 2011. Eyes

that had had a postoperative follow-up of less than 2 years were excluded. All records were evaluated for clinical signs of FAP, such as polyneuropathy, autonomic dysfunction, and visual disturbance, and later had a definitive diagnosis of FAP on the basis of amyloid deposits found in biopsy samples and genetic investigations. The age at onset of FAP was defined as the time at which the patient first complained of the typical symptoms just mentioned. Visual acuity was measured via standard Landolt C charts; values were converted to letter scores by using the Early Treatment Diabetic Retinopathy Study chart. The Institutional Review Board of Kumamoto University approved this retrospective review and analysis of patient data. Patient records/information was anonymized and de-identified prior to analysis.

Outcome Measures

The main outcome measure was success or failure of trabeculectomy with MMC as determined according to Kaplan-Meier analysis. Surgical success was defined as an IOP value of $\geq$6 mm Hg and $\leq$21 mm Hg with or without antiglaucoma medication. Failure was defined as an IOP value, on two consecutive visits, of $\leq$5 mm Hg or $\geq$22 mm Hg with or without antiglaucoma medication or as a need for additional glaucoma operations (excluding laser suture lysis) to reduce the IOP. Secondary outcome measures included complications, bleb characteristics, and postoperative interventions required. IOP data obtained within 2 months after trabeculectomy were excluded because of IOP fluctuations.

Surgical Procedures

We performed trabeculectomy with MMC according to a modified technique of Cairns: a limbus-based conjunctival flap was used until May 31, 2005, whereas a fornix-based conjunctival flap was used after June 1, 2005, followed by a half-layer scleral flap. We changed to conjunctival flap incisions as this made the surgery easier; the outcomes were similar in our general practice. Small pieces of MMC-soaked sponge (0.4 mg/ml) were applied to exposed tissues, including the Tenon capsule and the posterior surface of the conjunctiva, adjacent episcleral tissue, and scleral flap, for 3–4 min. After removal of all sponges, wounds were irrigated with 200 ml of balanced salt solution. A trabecular block was excised, and peripheral iridectomy was then performed. Closure of the scleral flap and conjunctiva was achieved with 10-0 monofilament nylon sutures. Postoperatively, all patients received the topical regimen of 0.1% betamethasone, 1% atropine sulfate, and 0.5% levofloxacin for 6–12 weeks [6]. Laser suture lysis was performed if the IOP and condition of bleb formation necessitated it. For eyes that required operations to reduce the IOP after the initial trabeculectomy with MMC, we used alternatives such as subconjunctival needle revision [7], repeated trabeculectomy with MMC, and reopening of the scleral flap with MMC [8–9].

Data Analysis

Kaplan-Meier survival analysis was used to determine the cumulative probability of success. We avoided comparing the results for both types of FAP because of low numbers of each type.

Results

Clinical Characteristics

Table 1 provides demographic and clinical characteristics of glaucoma patients due to FAP. Of 43 glaucomatous eyes, 27 (63%) had glaucoma surgery. The main indication for primary or additional glaucoma operations was inadequate IOP control despite use of maximal dosages of antiglaucoma medications; the surgical alternatives were selected at the clinician's discretion. Gonioscopic analysis revealed open angles with mild or heavy pigmentation in all eyes except two, which showed neovascularisation before surgery. These operations included trabeculectomy with MMC for 25 eyes; trabeculectomy without MMC for 1 eye; cyclodestructive procedures (performed at another hospital) for 1 eye; and trabeculotomy with sinusotomy for 1 eye, with a follow-up of only 4 months because of a poor systemic condition. Four of 25 eyes that had had trabeculectomy with MMC were excluded because the follow-up period was less than 2 years; therefore, 21 eyes were enrolled in a subsequent analysis. The mean follow-up period after trabeculectomy with MMC was 5.7 years (range, 2.2–12.7 years). Mean preoperative IOP and mean visual acuity values were 35.2 mm Hg and 78.2 letters, respectively, which changed to 20.4 mm Hg and 44.8 letters, respectively, at the last follow-up visit.

Outcomes

Figure 1 presents results of Kaplan-Meier survival analysis for 21 eyes that underwent trabeculectomy with MMC. According to treatment success criteria, cumulative survival values at 1 year were 0.76 for both groups, 0.88 for patients with FAP ATTR Val30Met, and 0.40 for patients with FAP ATTR Tyr114Cys. These respective values were 0.67, 0.81, and 0.20 at 2 years; 0.53, 0.63, and 0.20 at 3 years; 0.11, 0.17, and 0 at 4 years. Eight eyes were censored because regular follow-up ended. Eight of 16 eyes (50%) of patients with FAP ATTR Val30Met and all eyes (100%) of patients with FAP ATTR Tyr114Cys were classified as surgical failures.

Interventions and Complications

Table 2 lists reasons for treatment failure, as well as complications, bleb characteristics, and additional operations. Three of five eyes (60%) of patients with FAP ATTR Tyr114Cys had no light perception because of subsequent neovascular glaucoma, which may have been caused by retinal ischemia induced by ocular amyloid angiopathy. Significant complications during the follow-up period included ocular decompression retinopathy (7 eyes, 33%) and bleb encapsulation (10 eyes, 48%).

Twelve eyes (57%) of 8 patients required additional procedures to reduce IOP. Four eyes required more than one additional treatment. Six eyes had the scleral flap reopened via MMC; five eyes had another trabeculectomy with MMC. The mean time from initial trabeculectomy to additional surgery was 1.9 years (range, 0.2–6.0 years).

Discussion

Before the utilization of liver transplantation, early-onset FAP (before the age of 50) was fatal, with an expected survival of about 10 years from disease onset [1]. Because the liver is the main site of synthesis of ATTR found in serum, liver transplantation has been thought to be a promising approach for halting progression of neurological complications in patients with TTR-related FAP [10]. However, ocular complications have continued and indeed worsened after liver transplantation [5], [11], because ocular tissues—the retinal and ciliary pigment epithelia—also synthesised ATTR [12], [13]. In continuing investigations of these ocular complications and their treatment, we previously evaluated 17 glaucomatous eyes of 10 patients and 15 surgically treated eyes of 9 patients, but these numbers increased to 43 eyes of 27 patients and 21 eyes of 13 patients, respectively, in the present study.

Glaucoma is the most serious ocular manifestation of FAP and can cause severe visual disturbances. Previously reported evidence led to the common belief that ocular amyloid deposition may

Table 1. Demographic and Clinical Characteristics of Patients with Secondary Glaucoma due to FAP.

Characteristic	Val30Met	Tyr114Cys	Total
No. of eyes (patients)	33 (21)	10 (6)	43 (27)
Sex (male/female), n	8/13	1/5	9/18
Age at onset of FAP, y (range)	40.1 (28–73)	37.5 (32–43)	39.5 (28–73)
Age at onset of glaucoma, y (range)	49.5 (44–77)	45.5 (39–54)	48.5 (39–77)
Time from onset of FAP to onset of glaucoma, y (range)	10.4 (4–12)	7.8 (4–10)	8.6 (4–12)
No. (%) of eyes with neovascular glaucoma during follow-up	0 (0%)	8 (80%)	8 (19%)
No. (%) of patients who had LT	14 (67%)	3 (50%)	17 (59%)
No. of eyes (patients) that had trabeculectomy with MMC and at least 2-year follow-up	16 (10)	5 (3)	21 (13)
Age at surgery, y (range)	55.3 (42–79)	48.0 (39–50)	52.6 (39–79)
Follow-up period after surgery, y (range)	5.4 (2.2–12.7)	6.3 (2.4–9.2)	5.7 (2.2–12.7)
Prior surgery, no. (%) of eyes			
Vitrectomy	8 (50%)	5 (100%)	13 (62%)
Cataract surgery	8 (50%)	5 (100%)	13 (62%)
Glaucoma surgery			
Trabeculotomy with sinusotomy	1 (6%)	0 (0%)	1 (5%)
NPT with MMC	1 (6%)	1 (20%)	2 (10%)
IOP, mmHg (range)			
Preoperative	34.8 (24–42)	36.5 (30–43)	35.2 (24–43)
Last follow-up	16.0 (8–29)	34.6 (20–52)	20.4 (12–52)
Visual acuity, letters (range)			
Preoperative	83.8 (58–94)	60.6 (15–89)	78.2 (15–94)
Last follow-up	57.8 (0–89)	3.0 (0–15)	44.8 (0–89)

FAP = familial amyloidotic polyneuropathy; IOP = intraocular pressure; LT = liver transplantation; MMC = mitomycin C; NPT = nonpenetrating trabeculectomy.
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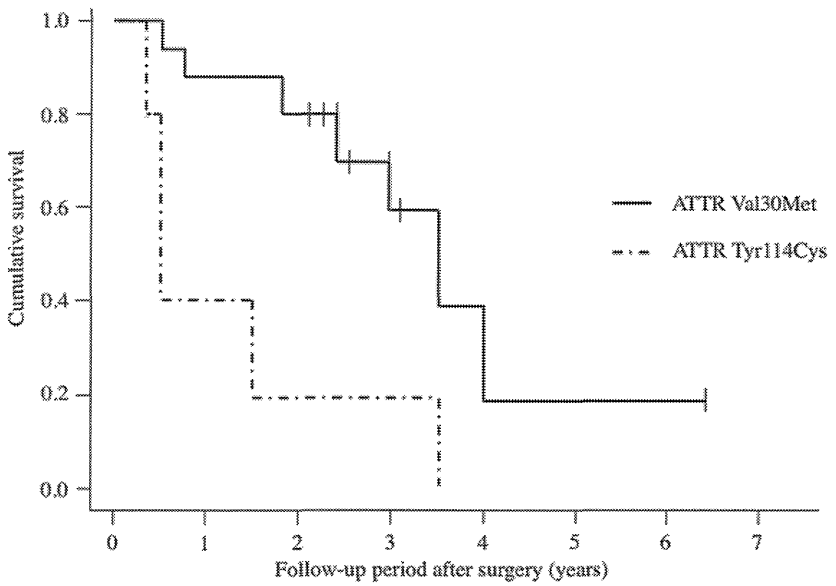

Figure 1. Kaplan-Meier survival curve for patients treated with trabeculectomy with mitomycin C. Treatment failure was defined as an intraocular pressure (IOP) value of ≤5 mm Hg or ≥22 mm Hg on at least two consecutive visits or as additional glaucoma operations (excluding laser suture lysis) required to reduce the IOP. The small vertical dashes along the curves represent times of censored observations.
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Table 2. Summary of Interventions and Complications.

Outcome	Val30Met	Tyr114Cys	Total
	(n = 16 eyes)	(n = 5 eyes)	(n = 21 eyes)
High IOP	8 (50%)	5 (100%)	13 (62%)
Loss of light perception	1 (6%)	3 (60%)	3 (14%)
Complications			
Bleb leakage	1 (6%)	0 (0%)	1 (5%)
Choroidal detachment	2 (13%)	0 (0%)	2 (10%)
Ocular decompression retinopathy	3 (19%)	4 (80%)	7 (33%)
Bleb characteristics			
Avascularity	2 (13%)	1 (20%)	3 (14%)
Delayed bleb leaks (Seidel's test)	2 (13%)	0 (0%)	2 (10%)
Encapsulation	7 (44%)	3 (60%)	10 (48%)
Additional surgical procedures*			
Bleb needling	2 (13%)	0 (0%)	2 (10%)
Bleb revision for leakage	1 (6%)	0 (0%)	1 (5%)
Bleb revision (Reopening of the scleral flap with MMC)	4 (25%)	2 (40%)	6 (29%)
Trabeculectomy with MMC	5 (31%)	0 (0%)	5 (24%)
Cyclodestructive procedure	0 (0%)	1 (20%)	1 (5%)

IOP = intraocular pressure; MMC = mitomycin C.
*Some eyes had more than 1 procedure.
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result in obstruction of the aqueous outflow route and/or perivascular amyloid deposition in conjunctival and episcleral tissues, which may contribute to elevated episcleral venous pressure and thereby cause glaucoma [5], [14], [15]. Our previous study demonstrated that the findings of amyloid deposition at the pupillary border, a pupillary border with irregularity, and vitreous opacity seemed to be reliable for predicting glaucoma onset [5]. Also, trabeculectomy with MMC appeared to be the most promising treatment modality available to us on the basis of our previous short-term results [5]. However, our present study providing longer term follow-up results revealed that the effect of trabeculectomy with MMC seemed to be limited on FAP-associated glaucoma. The cumulative probability of treatment success in our study was 0.76 at 1 year, 0.67 at 2 years, 0.53 at 3 years and 0.11 at 4 years. Approximately half of the trabeculectomized eyes needed additional surgical procedures within 2 years after surgery. We recently reported on the outcomes for 101 patients who underwent trabeculectomy with MMC for neovascular glaucoma, which is particularly difficult to manage; the probability of treatment success at 1, 2, and 5 years after surgery was 0.63, 0.58, and 0.52, respectively [6]. Thus, glaucoma associated with FAP appears to present serious management problems.

Bleb encapsulation, whose features include a dome-shaped, tense, opalescent, thick-walled bleb with vascular engorgement of overlying conjunctiva, is a relatively uncommon complication of filtering surgery. The occurrence of bleb encapsulation in different types of glaucoma is reportedly between 2.5% and 29%, depending on the surgical technique used [16–18]. Various risk factors, including male sex, prior use of topical medications, argon laser trabeculoplasty, glaucoma diagnosis, and surgery involving the conjunctiva, have been associated with development of this complication [16], [17]. In our study, we found 10 eyes (48%) with bleb encapsulation, accompanied by an elevated IOP. Because 7 of these eyes had had vitrectomy for vitreous opacity before the trabeculectomy, vitrectomy may be related to the development of bleb encapsulation. Ophir proposed that comparatively mild triggers of fibroblast activation, such as prolonged preoperative use of antiglaucoma medication and ocular trauma, may promote bleb encapsulation, whereas stronger stimuli such as previous intraocular surgery promote bleb scarring [18]. Other researchers reported that, in patients with TTR-related FAP, TTR aggregates that induce abnormal, sustained activation of extracellular signal-regulated kinases 1/2 may result in activation of nuclear transcription factor-κB, up-regulation of proinflammatory cytokines, oxidative stress, and ultimately neurodegeneration [19], [20]. Amyloid was also often deposited in the subepithelial layer and vascular wall of the conjunctiva [14], [21]. Therefore, TTR aggregates in the conjunctiva induce mild fibroblast activation, which may result in bleb encapsulation. Further study is needed to elucidate whether amyloid deposition affects development of bleb encapsulation.

In the present study, almost half of trabeculectomized eyes needed additional surgical interventions because trabeculectomy failed within 2 years after the operation. For the most recent cases, we utilized MMC to reopen the scleral flap, because most patients were relatively young and because of possible advantages of reopening the failed filter: reopening ensures superior filtration and preserves the superior conjunctiva for possible future surgery. However, this procedure, as well as other methods such as repeated trabeculectomy and needle revision, had limited efficacy. Although glaucoma drainage implants are now regarded as promising therapeutic options in types of glaucoma that are difficult to manage such as neovascular glaucoma, drainage implant procedures had not been performed at our institution yet, because these devices had not been approved for clinical use in Japan until 2012. After their approval, further study will be needed to determine the effects of drainage implants on FAP-associated glaucoma. We recently reported results of a pilot trial in which panretinal laser photocoagulation prevented progression of amy-

loid deposition in the vitreous and on the retinal surface [22]. However, the effect of laser photocoagulation on glaucoma progression is still unknown, and additional study is warranted.

In our study described here, we detected 7 eyes (33%) with ocular decompression retinopathy, which is a rare complication characterized by scattered retinal hemorrhages occurring immediately after uncomplicated filtering surgery [23], [24]. Fechtner *et al* hypothesized that one possible cause of this complication is a loss of retinal vessel autoregulation, which overwhelms the ability of the vessels to respond to IOP changes and results in retinal hemorrhages [23]. Our present FAP patients manifested autonomic dysfunction as one of their systemic symptoms, which increased their susceptibility to such a phenomenon. The hemorrhages resolved spontaneously in all patients, and only one patient showed decreased visual acuity. In addition, 8 eyes (80%) of 5 patients with FAP ATTR Tyr114Cys developed neovascular glaucoma later, possibly caused by retinal ischemia. We previously reported the occurrence of ocular amyloid angiopathy in patients with FAP ATTR Tyr114Cys, in whom vascular amyloid deposition caused a steno-occlusive vascular disorder that resulted in ischemic changes [25]. Patients with FAP ATTR Tyr114Cys have a susceptibility to retinal ischemic changes, so they have a poor prognosis compared with patients with FAP ATTR Val30Met. Thus, these complications—ocular decompression retinopathy and neovascular glaucoma caused by amyloid angiopathy—appear to be related to the FAP disease process rather than to surgical procedure.

Our study has several limitations. This is a retrospective review of cases performed consecutively. There is no control group for comparison of our results. All patients are Japanese, and the number of patients is small. Another limitation is the use of both eyes in a single patient. However, given the rarity of this disease, we do not feel that it was inappropriate to do so.

In summary, as a consequence of the significantly improved survival of patients with FAP that is achieved by liver transplantation, glaucoma may become a more common serious complication. Our results suggest that trabeculectomy with MMC may not have a sufficient effect on secondary glaucoma in patients with FAP and that this method has several significant complications. Besides larger and long-term follow-up studies, glaucoma drainage implant studies are needed to determine effective therapeutic strategies.

Author Contributions

Conceived and designed the experiments: TK TI YA HT. Analyzed the data: TK RH DE. Wrote the paper: TK.

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Amyloid deposits derived from transthyretin in the ligamentum flavum as related to lumbar spinal canal stenosis

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Amyloidosis is a protein conformational disorder with the distinctive feature of extracellular accumulation of amyloid fibrils that come from different proteins. In the ligamentum flavum of the lumbar spine, amyloid deposits were frequently found in elderly patients with lumbar spinal canal stenosis and were at least partially formed by wild-type transthyretin. However, how amyloid deposits in the ligamentum flavum affect lumbar spinal canal stenosis has remained unclear. In this study, we analyzed clinical, pathologic, and radiologic findings of patients with lumbar spinal canal stenosis who had amyloid deposits in the ligamentum flavum. We studied 95 ligamentum flavum specimens obtained from 56 patients with lumbar spinal canal stenosis and 21 ligamentum flavum specimens obtained from 19 patients with lumbar disk herniation. We evaluated histopathologic findings and clinicoradiologic manifestations, such as thickness of the ligamentum flavum and lumbar spinal segmental instability. We found that all 95 ligamentum flavum specimens resected from patients with lumbar spinal canal stenosis had amyloid deposits, which we classified into two types, transthyretin-positive and transthyretin-negative, and that transthyretin amyloid formation in the ligamentum flavum of patients with lumbar spinal canal stenosis was an age-associated phenomenon. The amount of amyloid in the ligamentum flavum was related to clinical manifestations of lumbar spinal canal stenosis, such as thickness of the ligamentum flavum and lumbar spinal segmental instability, in the patients with lumbar spinal canal stenosis with transthyretin-positive amyloid deposits. To our knowledge, this report is the first to show clinicopathologic correlations in transthyretin amyloid deposits of the ligamentum flavum. In conclusion, transthyretin amyloid deposits in the ligamentum flavum may be related to the pathogenesis of lumbar spinal canal stenosis in elderly patients.
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Amyloidosis is a disorder of protein conformation in which extracellular accumulation of amyloid fibrils that derive from different proteins occurs.[1,2] Thus far, 30 distinct protein precursors of amyloid fibrils were identified as causing different kinds of amyloid-associated diseases.[3] In each kind of amyloid deposits, specific features, including aging, mutation, inflammation, and tumors, can effect disease-causing polymerization of soluble proteins that function in the body.[1,2] Transthyretin, a serum protein synthesized mainly in the liver, causes two types of systemic amyloidosis. One type is a hereditary systemic amyloidosis (familial amyloid polyneuropathy), which is induced by mutant transthyretin.[4–6] The other type is senile systemic amyloidosis, which is an age-related sporadic systemic amyloidosis that is induced by wild-type

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transthyretin.[7–9] Besides full-length wild-type transthyretin, C-terminal wild-type transthyretin fragments beginning at positions 46–52 usually occur in amyloid deposits obtained from patients with senile systemic amyloidosis as well as many cases with hereditary transthyretin amyloidosis.[8,10]

Lumbar spinal canal stenosis is the most common spinal disorder in elderly people. Although hypertrophy of the ligamentum flavum is one of the major factors associated with lumbar spinal canal stenosis, the mechanism of ligamentum flavum hypertrophy is not well understood. Spinal ligaments of elderly patients reportedly often contained amyloid deposits, but precursor proteins of those amyloid fibrils were not clarified.[11,12] We recently reported that amyloid deposits in the ligamentum flavum were at least partially derived from wild-type transthyretin in elderly lumbar spinal canal stenosis patients.[13] Another recent paper suggested that transthyretin amyloid formation may be a common cause of lumbar spinal canal stenosis.[14] We also reported that a senile systemic amyloidosis patient with multifocal spinal transthyretin amyloid deposits developed severe destructive spondylosis and radiculomyelopathy.[15] However, how amyloid deposits in the ligamentum flavum affect lumbar spinal canal stenosis in which the ligamentum flavum shows degeneration and hypertrophy remains unclear. In this study, we analyzed clinical, pathologic, and radiologic findings of lumbar spinal canal stenosis patients with amyloid deposits. To our knowledge, this report is the first to show clinical–radiological–pathological correlations in transthyretin amyloid deposits in lumbar spinal canal stenosis.

Patients and methods

Patients

We used 95 ligamentum flavum specimens obtained from 56 patients with lumbar spinal canal stenosis and 21 ligamentum flavum specimens obtained from 19 patients with lumbar disk herniation; all patients underwent lumbar spinal surgery for in Kumamoto Orthopaedic Hospital from 2012 to 2013.

Histologic Examination

Tissue samples were fixed in 10% formalin, embedded in paraffin, serially sectioned at a thickness of 4 μm, and placed onto microscope slides. Sections were stained with hematoxylin–eosin and alkaline Congo red, after which they were viewed under polarized light to check for green birefringence. The extent of amyloid deposition, determined by measuring the degree of Congo red-positive areas, was ascertained with a computer and the public domain ImageJ program developed by the US National Institutes of Health and available at http://rsb.info.nih.gov/ij/index.html.

Immunohistochemistry

Deparaffinized sections were incubated in 3% hydrogen peroxide for 15 min to block endogenous peroxidase. Goat serum (Dako, Glostrup, Denmark) was used to block nonspecific background staining. A rabbit polyclonal anti-transthyretin antibody (Dako) and a horseradish peroxidase-conjugated goat anti-rabbit immunoglobulin antibody (Dako) were used at 1:100 dilution as the first and second antibodies, respectively. Reactivity was visualized by using the DAB Liquid System (Dako). Sections were counterstained with hematoxylin.

Magnetic Resonance Imaging of the Ligamentum Flavum

The thickness of the ligamentum flavum at the facet joint of the affected spinal level was measured on axial T1-weighted magnetic resonance images.[16] We measured the thickness of the ligamentum flavum three times and used the average of the three repeated measurements as the final value.

Radiography of the Lumbar Spine

Lateral functional flexion–extension radiographs were used to evaluate lumbar spinal segmental instability.[17] The adjacent vertebral end-plate angle (total degrees) was measured by using the lumbar flexion and extension radiographs at the operative level.[18]

Nephelometry to Measure Serum Transthyretin Concentrations and Mass Spectrometric Analysis of Serum Transthyretin

Serum transthyretin concentrations were determined via nephelometry in seven serum samples from lumbar spinal canal stenosis patients with transthyretin-positive amyloid deposits and eight serum samples from lumbar spinal canal stenosis patients with transthyretin-negative amyloid deposits. We excluded patients with low albumin concentrations (<4.1 g/dl) and/or high C-reactive protein concentrations (>0.3 mg/dl) in serum. To detect wild-type and mutant transthyretins, serum samples were analyzed by using surface-enhanced laser desorption/ionization time-of-flight mass spectrometry with the Q10 ProteinChip (Bio-Rad, Hercules, CA, USA), a strong anion exchange array with quaternary amine functionality, as described previously.[19]

Detection of Fragmented Transthyretin in Amyloid Deposits

To extract amyloid-forming proteins from formalin-fixed, paraffin-embedded specimens, we used biochemical methods of Layfield *et al.*[20] In brief, sodium dodecyl sulfate-polyacrylamide gel electrophoresis was applied to process isolated tissue pellets that

were incubated in Laemmli sample buffer (Bio-Rad) containing 8 M urea at 90 °C for 15 min, after which samples were transferred to a nitrocellulose membrane (Bio-Rad) for immunoblotting. A polyclonal antiserum against truncated transthyretin 50-127 (transthyretin 50-127), produced in rabbits, was diluted 1:2000 and used as the primary antibody.[21] A goat anti-rabbit antibody conjugated with horseradish peroxidase (Dako), diluted 1:5000, was used as the secondary antibody. The reaction was visualized by using an enhanced chemiluminescence system (GE Healthcare, Buckinghamshire, UK).

Statistical Analysis

Data were evaluated with Student's *t*-test and Fisher's exact test. All analyses were performed with JMP Version 5.1 (SAS Institute Japan, Tokyo, Japan). *P*-values of <0.05 were regarded as statistically significant.

Ethics

The study protocol was approved by Human Ethics Review Committee of Kumamoto University and Kumamoto Orthopaedic Hospital. A signed consent form was obtained from all patients or a family member.

Results

Amyloid Deposits in the Ligamentum Flavum

All 95 ligamentum flavum specimens resected from lumbar spinal canal stenosis patients contained amyloid deposits. Immunohistochemistry revealed positive transthyretin staining in amyloid deposits in 43 of 95 specimens (Table 1). The anti-transthyretin antibody did not react with amyloid deposits in the other specimens. The amount of amyloid deposited in transthyretin-positive amyloid cases was higher than that in transthyretin-negative amyloid cases. As Figure 1 shows, transthyretin-positive amyloid occurred in massive deposits, whereas transthyretin-negative amyloid formed small spotty deposits. Lumbar spinal canal stenosis patients with transthyretin-positive amyloid deposits had no symptoms of cardiac amyloidosis, which is major symptom of senile systemic amyloidosis. We found no amyloid deposition in blood vessels in 40 (95.6%) of 41 specimens containing transthyretin-positive amyloid deposits in the ligamentum flavum (Supplementary Figure S1 and Supplementary Table S1). One case having transthyretin-positive amyloid deposits in blood vessels of the ligamentum flavum did not show any clinical symptoms suggesting senile systemic amyloidosis such as cardiac failure (Supplementary Figure S1 and Supplementary Table S1). We also investigated the presence of amyloid deposits in the ligamentum flavum of lumbar disk herniation patients. Transthyretin-negative amyloid deposits were found 9 (42.9%) of 21 specimens (Table 2). We found no transthyretin-positive amyloid deposits in the ligamentum flavum of lumbar disk herniation patients.

Relationship Between Transthyretin Amyloid Deposits and Age

Lumbar spinal canal stenosis patients with transthyretin-positive amyloid deposits were older than patients with transthyretin-negative findings (Table 1). The occurrence of transthyretin-positive amyloid deposits increased with age (Figure 2a), but no relationship existed between the amount of transthyretin amyloid and age (Figure 2b).

Table 1 Characteristics of lumbar spinal canal stenosis patients with transthyretin-positive or transthyretin-negative amyloid deposits in the ligamentum flavum

Characteristic	*Transthyretin +*	*Transthyretin −*	P-*value*
Frequency of occurrence, *n* (%)	43 (45.3%)	52 (54.7%)	
Males/females, *n*	26/17	42/10	
Age (years; mean ± s.d.)	74 ± 7.6	68 ± 6.4	<0.0001
Amount of amyloid deposited (%)	4.34 ± 3.89	0.26 ± 0.33	<0.0001
Mean ligamentum flavum thickness (mm)	4.36 ± 0.79	3.39 ± 0.75	<0.0001
Lumbar spinal segmental instability (deg)	8.1 ± 3.3	5.6 ± 2.6	<0.0005
Destructive spondylosis, *n*	0	0	
Cardiac failure, *n*	0	0	
Level of harvested ligamentum flavum, n			
L1/2	2	3	
L2/3	6	13	
L3/4	13	15	
L4/5	18	17	
L5/S	4	4	

Abbreviations: Transthyretin +, lumbar spinal canal stenosis patients with transthyretin-positive amyloid deposits; transthyretin −, lumbar spinal canal stenosis patients with transthyretin-negative amyloid deposits.

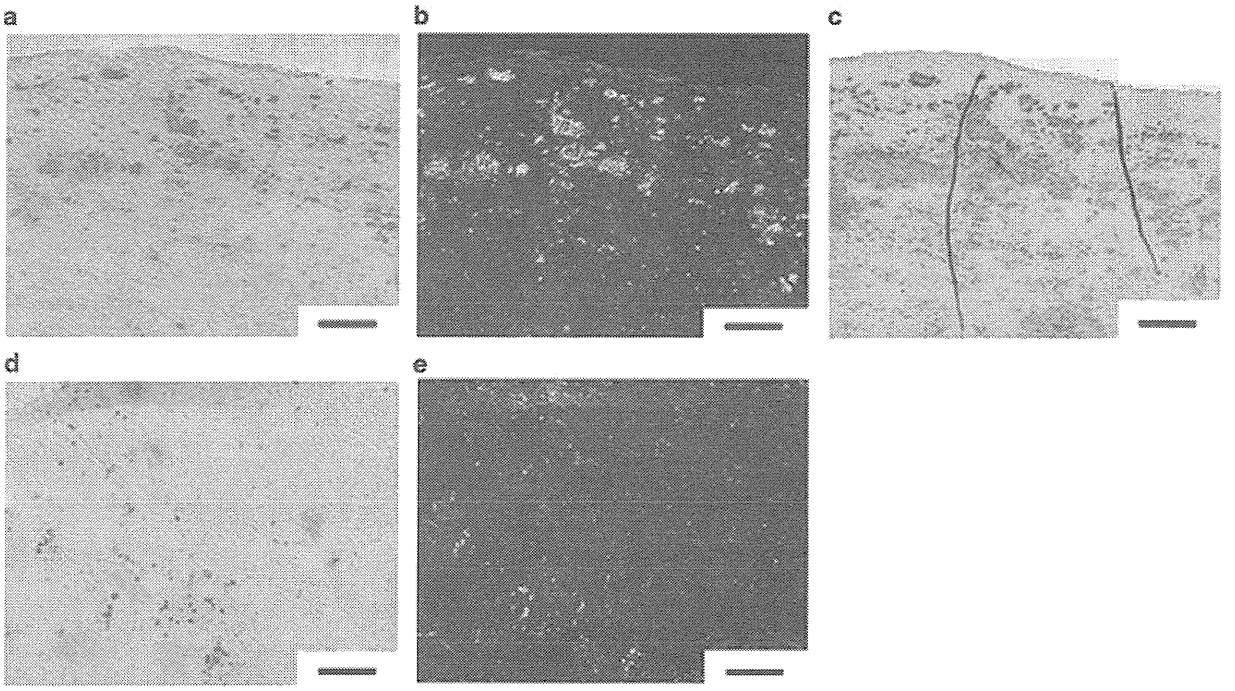

Figure 1 Amyloid deposits in the ligamentum flavum of lumbar spinal canal stenosis patients. (**a–c**) Transthyretin-positive amyloid deposits in the ligamentum flavum. (**d** and **e**) Transthyretin-negative amyloid deposits in the ligamentum flavum. (**a** and **d**) Congo red staining. (**b** and **e**) The same sections viewed with polarized light. (**c**) Immunohistochemical staining with an anti-human transthyretin polyclonal antibody (Dako). Scar bars, 200 μm.

Table 2 Characteristics of lumbar disk herniation patients with or without amyloid deposits in the ligamentum flavum

Characteristic	*Without amyloid deposits*	*With amyloid deposits*	P-*value*
Frequency of occurrence, *n* (%)	12 (57.1%)	9 (42.9%)	
Transthyretin-positive amyloid	Not applicable	0 (0%)	
Males/females, *n*	10/2	7/2	
Age (years, mean ± s.d.)	41.1 ± 10.6	57.6 ± 10.1	<0.005
Mean ligamentum flavum thickness (mm)	2.54 ± 0.38	2.98 ± 0.27	0.051
Level of harvested ligamentum flavum, n			
L1/2	0	0	
L2/3	0	1	
L3/4	1	2	
L4/5	7	4	
L5/S	4	2	

Relationship Between the Amount of Transthyretin Amyloid in the Ligamentum Flavum and Ligamentum Flavum Thickness

Magnetic resonance imaging revealed that the ligamentum flavum of lumbar spinal canal stenosis patients with transthyretin-positive amyloid deposits was thicker than the ligamentum flavum of lumbar spinal canal stenosis patients with transthyretin-negative amyloid deposits (Table 1). The amount of amyloid in the ligamentum flavum was related to ligamentum flavum thickness in lumbar spinal canal stenosis patients with transthyretin amyloid deposits (Figure 3a).

Relationship Between the Amount of Transthyretin Amyloid in the Ligamentum Flavum and Lumbar Spinal Segmental Instability

Lateral functional flexion–extension radiography revealed that the lumbar spinal segmental instability of lumbar spinal canal stenosis patients with transthyretin-positive amyloid deposits was greater

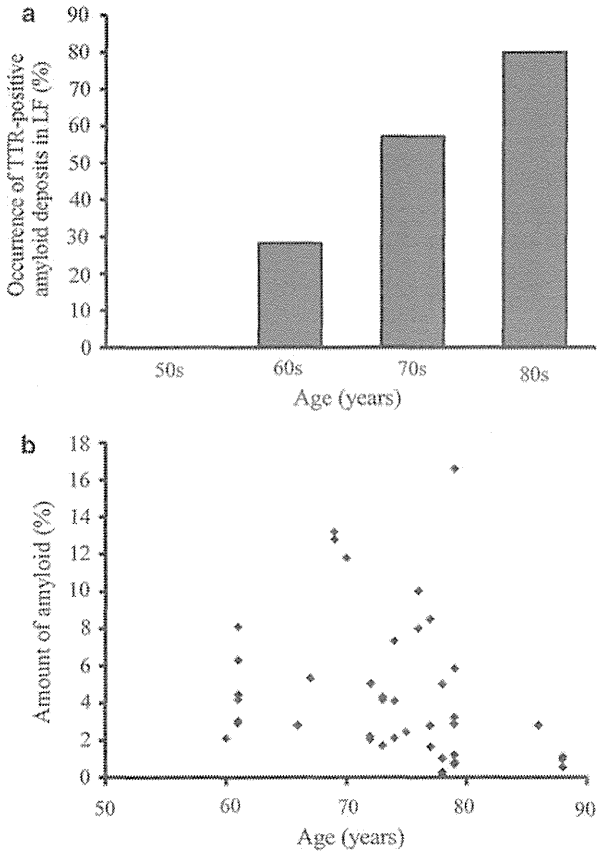

Figure 2 Occurrence and amount of transthyretin amyloid in the ligamentum flavum of lumbar spinal canal stenosis patients. (**a**) Occurrence of transthyretin-positive amyloid deposits according to age. (**b**) The amount of transthyretin-positive amyloid according to age ($r=0.25$, $P=0.1$).

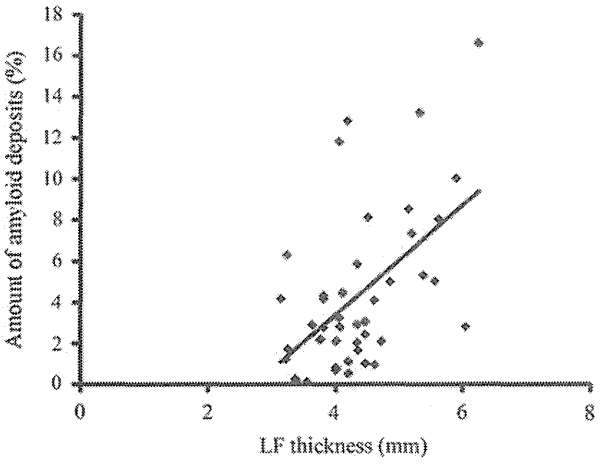

Figure 3 Relationship between the amount of transthyretin amyloid in the ligamentum flavum and ligamentum flavum thickness ($r=0.54$, $P<0.0005$).

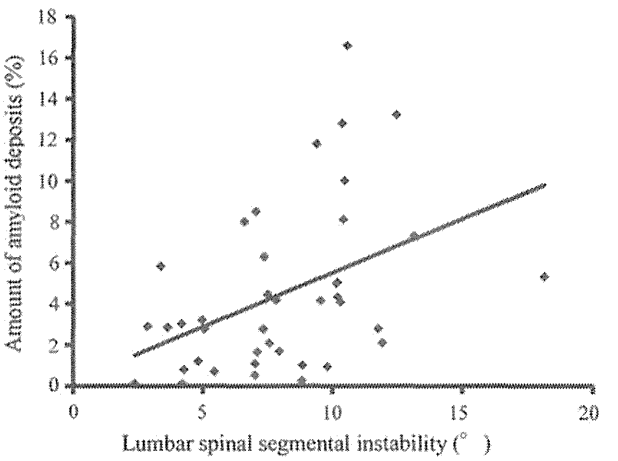

Figure 4 Relationship between the amount of transthyretin amyloid in the ligamentum flavum and lumbar spinal segmental instability ($r=0.43$, $P<0.01$).

than that of lumbar spinal canal stenosis patients with transthyretin-negative amyloid deposits (Table 1). The amount of amyloid in the ligamentum flavum was related to the lumbar spinal segmental instability of lumbar spinal canal stenosis patients with transthyretin-positive amyloid deposits (Figure 4).

Serum Transthyretin Concentrations and Mass Spectrometric Analysis of Serum Transthyretin

We analyzed serum transthyretin concentrations in lumbar spinal canal stenosis patients with transthyretin-positive amyloid deposits and lumbar spinal canal stenosis patients with transthyretin-negative amyloid deposits. No significant difference existed in serum transthyretin concentrations of lumbar spinal canal stenosis patients with transthyretin-positive amyloid deposits and those with transthyretin-negative amyloid deposits ($22.9\pm5.9$and 23.9 ± 5.6 mg/dl, respectively; $P=0.74$). In addition, mass spectrometric analysis, which can detect wild-type and mutant forms of transthyretin in serum samples, indicated that those lumbar spinal canal stenosis patients had wild-type transthyretin but no mutant transthyretin in the bloodstream.

Detection of Fragmented Transthyretin in Amyloid Deposits

To investigate the proteolytic cleavage of transthyretin in amyloid deposits, we performed immunoblotting with anti-transthyretin 50-127 antiserum. As seen in Figure 5, amyloid fibrils consisted of both full-length transthyretin and C-terminal fragmented transthyretin in lumbar spinal canal stenosis patients with transthyretin-positive amyloid deposits.

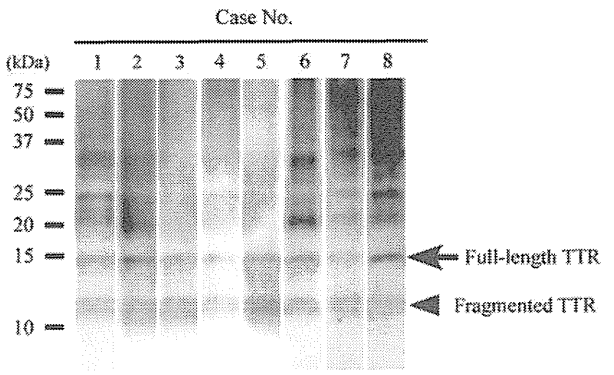

Figure 5 Immunoblotting with an antiserum against transthyretin 50-127, which can detect C-terminal transthyretin fragments in addition to full-length transthyretin. Cases 1–8 from among lumbar spinal canal stenosis patients with transthyretin-positive amyloid deposits in the ligamentum flavum were randomly selected for analysis.

Discussion

In this study, we first demonstrated that lumbar spinal canal stenosis patients often had amyloid deposits in the ligamentum flavum, which we classified into two types, transthyretin-positive and transthyretin-negative, and that transthyretin amyloid formation in the ligamentum flavum of lumbar spinal canal stenosis patients was an age-associated phenomenon. We next established that in lumbar spinal canal stenosis patients with transthyretin-positive amyloid deposits the amount of amyloid in the ligamentum flavum was related to clinical manifestations of lumbar spinal canal stenosis, such as thickness of the ligamentum flavum and lumbar spinal segmental instability. A surprising result from this study was that all lumbar spinal canal stenosis patients had amyloid deposits in the ligamentum flavum, but the frequency of occurrence of amyloid deposits in the ligamentum flavum of lumbar disk herniation patients was limited to about 40%. Amyloid deposits in the ligamentum flavum can be classified on the basis of their precursor proteins. In this study, we mainly focused on transthyretin amyloid formation in the ligamentum flavum. Transthyretin-positive amyloid deposits in the ligamentum flavum were massive and increased with the age of the lumbar spinal canal stenosis patients. Because those patients had only wild-type transthyretin in the serum, transthyretin-positive amyloid deposits in the ligamentum flavum of lumbar spinal canal stenosis patients were thought to be derived from wild-type transthyretin. These features of transthyretin amyloid deposits in the ligamentum flavum were consistent with a few previous reports that evaluated smaller numbers of lumbar spinal canal stenosis patients.[13,14,22] In addition, because we discovered no relationship between the amount of transthyretin amyloid and age, we speculate that aging mainly affects the initiation of transthyretin amyloid formation in the ligamentum flavum. Because we found no symptoms that would indicate cardiac amyloidosis, which is major symptom of senile systemic amyloidosis, an age-related systemic form of amyloidosis, transthyretin-positive amyloid deposition in the ligamentum flavum of lumbar spinal canal stenosis patients may be a localized form of amyloidosis. Biochemical analysis revealed that amyloid deposits in the ligamentum flavum of lumbar spinal canal stenosis patients consisted of C-terminal transthyretin fragments, which constantly occur in amyloid deposits in senile systemic amyloidosis, in addition to full-length transthyretin.[21,23] Although the roles of transthyretin fragmentation in amyloid formation remain to be clarified, there may be a common pathologic process for transthyretin amyloid formation in the ligamentum flavum of lumbar spinal canal stenosis patients and that in senile systemic amyloidosis.

The small spotty transthyretin-negative amyloid deposits were more frequently seen in younger patients, but precursor proteins of those amyloid deposits remain to be determined. To clarify features of those amyloid deposits, detailed biochemical analyses such as mass spectrometry of extracts of the deposits should be performed.

The most important finding of this study was that the amount of transthyretin amyloid in the ligamentum flavum was related to clinical manifestations of lumbar spinal canal stenosis such as the thickness of the ligamentum flavum and lumbar spinal segmental instability, because whether amyloid deposits in the ligamentum flavum would affect clinical manifestations of lumbar spinal canal stenosis had not been previously determined. This finding suggests that transthyretin amyloid deposits in the lumbar spine are related to degenerative changes in lumbar spinal canal stenosis. Dialysis-related amyloidosis, which is a complication of chronic dialysis in patients with chronic renal failure and is derived from β_2-microglobulin, has been well documented as causing orthopedic disorders such as destructive osteoarthropathy, carpal tunnel syndrome, and spinal involvement.[24] Although we previously reported that a patient suspected of having senile systemic amyloidosis developed multifocal spinal transthyretin amyloidosis with destructive spondylosis of the lumbar spine,[15] in our study here no lumbar spinal canal stenosis patients with ligamentum flavum transthyretin amyloid deposits also had destructive spondylosis. Our data here thus suggest that transthyretin amyloid deposits in the lumbar spine are related to degenerative changes and instability of the lumbar spine but rarely cause destructive changes in the lumbar spine. To clarify detailed mechanisms of transthyretin amyloid formation in the lumbar spine including causes of lumbar spinal canal stenosis or other results in lumbar spinal canal stenosis, additional studies with cell cultures and animal models are required.