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Original Article

The pathological and biochemical identification of possible seed-lesions of transmitted transthyretin amyloidosis after domino liver transplantation

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Abstract

The most serious issue in domino liver transplantation (DLT) using liver grafts from patients with transthyretin (TTR)-related familial amyloid polyneuropathy (FAP) is the development of iatrogenic transmitted amyloidosis (*de novo* amyloidosis) in DLT-recipients. However, little is known regarding the mechanisms of the initial stage of amyloid formation in these recipients. We detected initial lesions (possible seed-lesions) of this iatrogenic amyloidosis in two recipients following liver grafting from FAP patients. Patient 1 underwent DLT at age 65 from an FAP patient with a Val30Met TTR variant and patient 2 received DLT from an FAP patient with a Val30Leu TTR variant at age 32. Patient 2 was started on diflunisal administration from 4 years after DLT. While neither patient had symptoms of FAP, small amyloid deposits were detected on the gastroduodenal mucosae 14 months and 12 years after DLT in patient 1 and patient 2, respectively. The amyloid was analyzed using a laser microdissection system and tandem mass spectrometry. Biochemical analysis indicated that the amyloid was composed mostly of variant TTR produced from the transplanted liver in both patients. In patient 1, wild-type TTR amyloid was detectable in the duodenal mucosa obtained 2 years after DLT. This is the first study to successfully capture the pathological and biochemical features of initial-stage amyloid lesions in DLT recipients. The findings clearly indicate that amyloid deposition can start by deposition of variant TTR followed by deposition of wild-type TTR, and blocking of amyloid seed formation from variant TTR may be a key to prevent or delay the development of DLT-associated amyloidosis.

Keywords: domino liver transplantation; familial amyloid polyneuropathy; transmitted amyloidosis; transthyretin; seed-lesions; laser microdissection system

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The authors have no conflicts of interest to declare.

Introduction

Transthyretin (TTR)-related familial amyloid polyneuropathy (FAP) is an inherited systemic amyloidosis caused by *TTR* gene mutations, and is characterized by peripheral and autonomic neuropathy in association with the involvement of many visceral organs [1]. As the liver is the main source of circulating TTR, including a variant form of TTR with one amino acid substitution [2], large numbers of FAP patients have received liver transplantation (LT) worldwide [3]. The liver of an FAP patient is

anatomically and functionally normal apart from the production of variant TTR, and the FAP liver has therefore been used as a graft donor in domino liver transplantation (DLT) to overcome organ shortages. DLT was first carried out in 1995 [4], and more than 1000 patients have since undergone DLT using grafts from FAP patients [3].

One of the most serious complications in domino recipients is the development of *de novo* TTR amyloidosis, ie iatrogenic transmission of amyloidosis [5]. Clinical symptoms associated with *de novo* TTR amyloidosis usually begin 6–10 years after the operation [5–7]. To prevent the development of *de novo* amyloidosis, it is important to gain an understanding of the pathophysiology of initial amyloid formation. However, the pathomechanism of initial amyloidogenesis in domino recipients remains unclear, as the biochemical characteristics of the amyloid fibril protein have not yet been investigated in preclinical-stage domino recipients. In addition, so far, it has been nearly impossible to extract amyloid fibril proteins from extremely small amyloid deposits in asymptomatic patients, including domino recipients as well as FAP patients, with conventional amyloid extraction methods by tissue-homogenization [8]. In this study, we successfully isolated and biochemically analyzed very tiny amyloid lesions on the gastroduodenal mucosae from two asymptomatic domino recipients using a laser microdissection (LMD) system. Here, we report the biochemical features of initial-stage amyloid lesions, possible seed-lesions, of transmitted amyloidosis. In addition, serial changes in biochemical composition of initial amyloid lesions were identified in one recipient, in whom amyloid deposition was first detected only 14 months after DLT. This is the first demonstration of an initial FAP lesion in a human subject in addition to the unexpectedly rapid transmission of amyloidosis in a domino recipient after receiving an FAP liver graft.

Patients and methods

Two DLT recipients were examined (patient 1: a 67-year-old woman; patient 2: a 44-year-old man). Both received DLT from FAP patients with a Val30Met TTR variant (patient 1) and a Val30Leu TTR variant (patient 2), respectively. The clinical picture by DLT of patient 2 was reported previously [9]. DNA analysis of the *TTR* gene [10] confirmed no mutations in either patient, although mass spectrometric analysis of serum TTR [11] showed a mutant TTR peak in addition to the wild-type TTR peak (data not shown). Tacrolimus was used as immunosuppressant therapy in both patients. Medical evaluation, including gastroduodenal mucosal biopsy, was carried out almost every year since DLT to monitor the development of *de novo* amyloidosis.

Histopathological examination and biochemical analysis of deposited amyloid to determine the composition ratio of wild-type and variant TTR in amyloid fibril protein

Amyloid deposits were histopathologically evaluated with Congo red staining and by immunohistochemistry using anti-TTR antibody [12]. For biochemical analysis, deposited amyloid was extracted using an LMD system (LMD 7000; Leica Microsystems Inc., Tokyo, Japan) after staining with Congo red. However, due to the extremely small size of the amyloid lesions, extraction of amyloid was impossible using this LMD system in patient 1. We, therefore, isolated the amyloid using an advanced LMD system, which was specially developed for isolation of intracellular inclusions by Hachiya and Kaneko [13]. Extracted amyloid was solubilized in 50 μl of 10 mM Tris/1 mM EDTA/0.002% Zwittergent buffer [14]. Microdissected amyloid proteins were digested with trypsin overnight, and the composition ratio of wild-type (Val-30) and variant (Met-30 or Leu-30) TTR in the amyloid was analyzed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) (Nano LC DiNa; KYA Technologies Co., Tokyo, Japan and QExactive; Thermo Fisher Scientific Inc., Waltham, MA) as reported previously [15]. The investigation was repeated twice and the mean composition ratio was calculated, except for the sample obtained at 14 months after DLT in patient 1 because the amount of the extracted amyloid was too small.

This study was performed with the approval of the Institute Review Board of Shinshu University (3088) and with written informed consent from both patients.

Results

Case reports

Patient 1. This domino recipient was a 67-year-old Japanese woman who had received DLT from a 35-year-old female FAP patient with Val30Met (p.Val50Met) at age 65 due to liver cirrhosis with hepatocellular carcinoma. Fourteen months after the operation, she was admitted to our hospital for the first check for amyloidosis. No abnormal findings

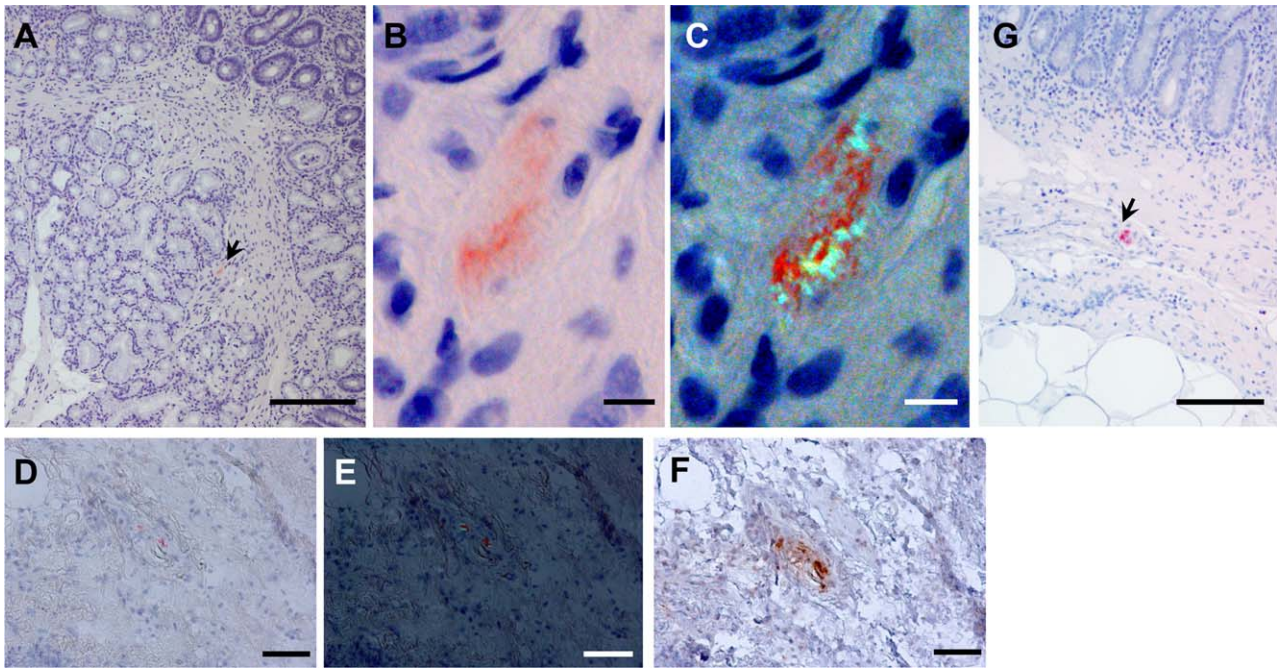

Figure 1. Histopathology of duodenal mucosa of patient 1. (A–F) Biopsy samples at 14 months after DLT. (G) Biopsy sample at 24 months after DLT. (A–E, G) Congo red staining. (F) Dual staining with Congo red (D, E) followed by immunohistochemistry (F) on frozen duodenal mucosa. (A–C) At lower magnification, a small Congo red-positive spot was seen on the lamina muscularis mucosae (arrow) (A, bar = 100 μm). At higher magnification, the amyloid was clearly positive for Congo red (B), showing strong apple-green birefringence under polarized light (C) (B, C, bars = 5 μm). Formalin-fixed and paraffin-embedded tissue sections. (D–F) Frozen tissue sections were stained with Congo red. After confirming the presence of amyloid (D, E), the cover glass was removed in Hemo-De solution (FALMA Co., Ltd., Tokyo, Japan), and the tissue sections were rehydrated. After treatment with formic acid for 1 minute, immunohistochemical staining using anti-TTR115–124 peptide antibody (1:4000 dilution) [12] was performed using the standard immunoperoxidase method (Vectastain ABC kit; Vector Laboratories Inc., Burlingame, CA). (E) Congo red staining under polarized light. The deposited amyloid was positive for anti-TTR antibody (F) (D–F, bars = 50 μm). (G) A small Congo red-positive spot was also present on the lamina muscularis mucosae at 24 months after DLT (arrow, bar = 100 μm).

were seen in nerve conduction studies or echocardiography. Abdominal fat pad aspiration biopsy showed no amyloid deposition. Biopsy of the gastroduodenal mucosa was performed under endoscopic control, and one tiny Congo red-positive deposit, showing strong apple-green birefringence under polarized light, was detected on the lamina muscularis mucosa of the duodenum in only one tissue section among five different biopsy samples (Figure 1A–C). For immunohistochemical analysis using anti-TTR antibody [12], we prepared 235 consecutive tissue sections from the same paraffin-embedded tissue block; no amyloid deposition was detected and we failed to detect TTR-deposited lesions. After obtaining informed consent, biopsies were taken from an additional five different portions of the duodenal mucosa. Of the five samples, two were fixed in 10% formalin, embedded in paraffin, and cut into sections, while the remaining three were stored at −80°C and used to prepare frozen sections. We prepared 258 consecutive tissue sections from the paraffin-embedded tissue block, and no amyloid was detected. However, we found small amyloid deposits in the duodenal mucosa in 3 of 465 consecutive tissue sections prepared from the three frozen samples (Figure 1D, E). We carried out dual staining with Congo red and immunohistochemical staining with anti-TTR antibody [12] on the same tissue sections, and positive staining of the amyloid deposits was observed (Figure 1F). The area positive with anti-TTR antibody was somewhat widely expanded compared to the Congo red-positive area. We isolated amyloid fibril protein using the advanced LMD system from the two tissue sections dual-stained with Congo red followed by immunostaining with anti-TTR antibody. In LC-MS/MS analysis of TTR 22–34 tryptic peptides, only peptide derived from variant TTR (Met-30) was found (Figure 2A).

Oral administration of tafamidis, a stabilizer of tetrameric TTR structure [16], was started 20 months after DLT despite the preclinical stage of amyloidosis. Twenty-four months after DLT, she was again

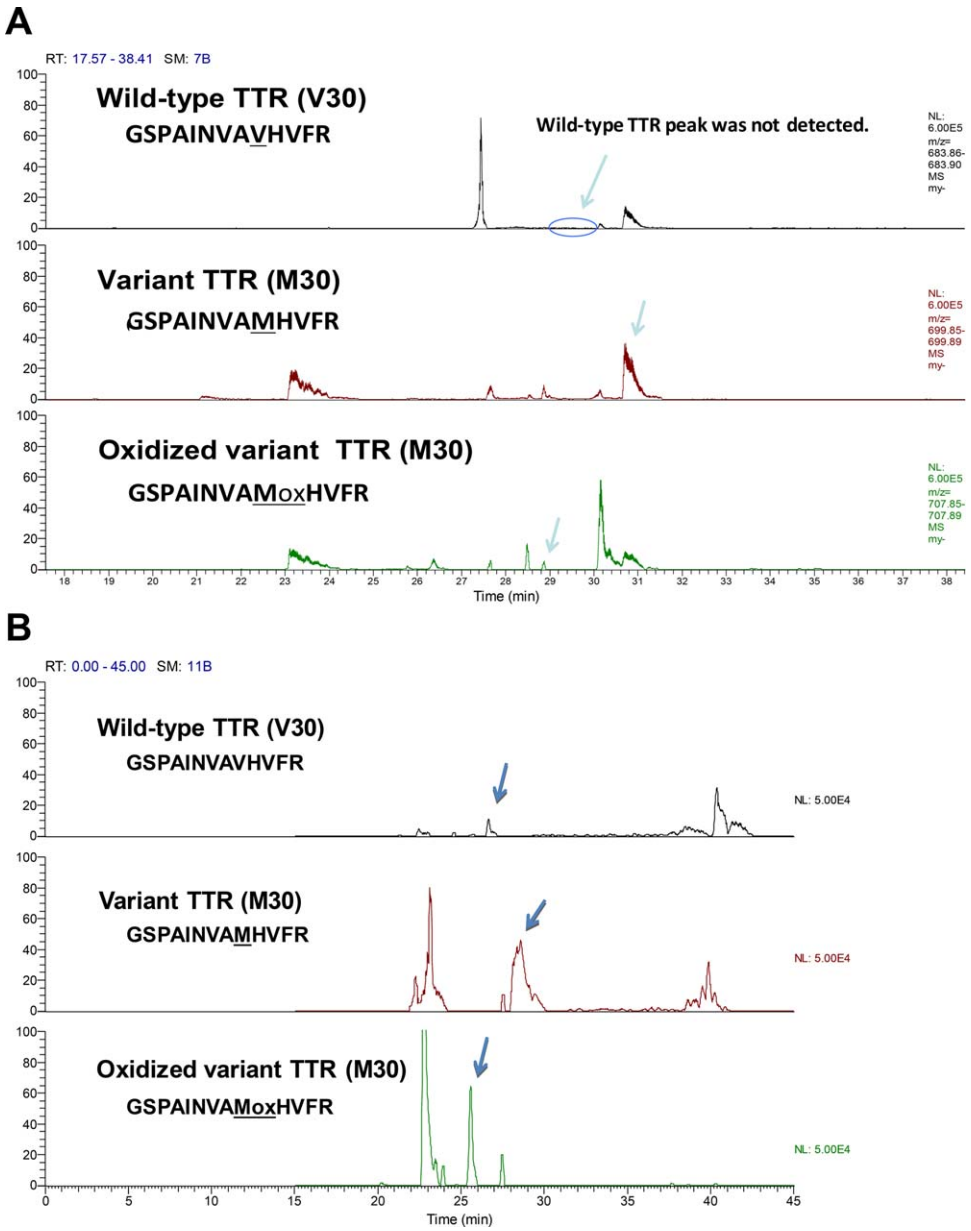

Figure 2. Serial changes in relative quantification of TTR 22–34 tryptic peptide in LC-MS/MS analysis of amyloid isolated at 14 months (A) and 24 months (B) after DLT in patient 1. Proteomics analysis by LMD-LC-MS/MS was performed as follows. The area positive with both Congo red and immunohistochemical staining of frozen biopsied samples (Figure 1D–F) was isolated using an advanced LMD system [13]. Selected mass range profiles obtained from the ion chromatograms of LC-MS/MS analysis are shown (A, B). The chromatograms were filtered with mass ranges, *m/z* 683.86–683.90, *m/z* 699.85–699.89 and *m/z* 707.85–707.89. These ranges correspond to TTR 22–34 wild-type (GSPAINVAVHVFR, *m/z* 683.88), variant peptide (GSPAINVAMHVFR, *m/z* 699.87) and variant peptide oxidized at Met-30 (GSPAINVAM(ox)HVFR, *m/z* 707.87), respectively. Only TTR 22–34 peptide peaks derived from variant TTR and oxidized variant TTR were detected at 14 months after DLT, and no clear peak derived from wild-type TTR was seen in the extracted amyloid (sequence data of both variant and oxidized variant TTR on LC-MS/MS analysis are not shown). We also investigated synthetic wild-type TTR peptide 22–34 by mass spectrometry, and confirmed a peak derived from synthetic peptide at 29.97 minutes (data not shown). Conversely, in amyloid isolated at 24 months after DLT, the mass peak of wild-type TTR became apparent and the composition ratio of wild-type versus variant TTR was 6:94%.

admitted to our hospital for the second check for amyloidosis. Duodenal mucosal biopsy was performed and very small amyloid deposits were seen in 5 of 300 consecutive paraffin-embedded tissue sections (Figure 1G). We also prepared 200 consecutive frozen tissue sections, but no amyloid deposits were

detected. In LC-MS/MS analysis of the isolated amyloid from paraffin-embedded tissue sections, a small wild-type TTR peak became detectable and the composition ratio of wild-type TTR versus variant TTR was 6:94% (Figure 2B). The patient still had no clinical symptoms 24 months after DLT.

Patient 2. This recipient was a 45-year-old Japanese man with adult-onset type II citrullinemia, which developed at the age of 32. One month after onset, he received DLT from a 52-year-old female FAP patient with ATTR Val30Leu (p.Val50Leu) [9]. However, his donor died of rapidly progressive amyloid cardiomyopathy 1 year after the operation due to paradoxical ongoing wild-type TTR-derived amyloid deposition [17]. Therefore, the recipient has been carefully checked for the presence of *de novo* amyloid deposits almost every year, paying special attention to the development of amyloid cardiomyopathy as well as neuropathy. In addition, he was started on oral intake of diflunisal (500 mg a day), a stabilizer of TTR structure [18,19], from 4 years after DLT as a preventive step against *de novo* amyloidosis. To date, he has shown no clinical symptoms, including congestive heart failure or peripheral neuropathy, and no abnormal findings have been found on echocardiography. However, in nerve conduction studies, motor nerve compound muscle action potential of the tibial nerve and sensory nerve action potential of the sural nerve gradually decreased over the last 2 years (from 9.9 to 1.1 mV, normal value >6.2 mV; from 14.7 to 4.4 μV, normal value >5 μV, respectively), although the nerve conduction velocities were within the normal limits. The presence of amyloid deposits has been checked almost every year with gastroduodenal mucosal biopsy, and amyloid deposits were first detected on both gastric and duodenal mucosae at 45 years old (12 years after DLT) (Figure 3A–C). We investigated the gastroduodenal mucosal biopsy samples obtained at 11 years after DLT again, with preparation of 500 consecutive tissue sections, but no amyloid deposits were seen (data not shown). In LC-MS/MS analysis of the amyloid microdissected from formalin-fixed paraffin-embedded tissue sections, the composition ratio of wild-type TTR versus variant TTR was 5:95% (Figure 3D).

Discussion

De novo amyloidosis associated with DLT was first reported in 2005 as transmission of amyloidosis in a 55-year-old man who developed neuropathic symptoms 8 years after DLT [5]. Since then, similar cases of *de novo* amyloidosis have been described in a number of domino recipients [5–7]. To date, the shortest periods from DLT to identification of amyloid deposits have been reported as 3 years in skin [20] and 4 years in gastroduodenal mucosa [21], and there have been no reports of amyloid deposition identified within 3 years after DLT [22].

Of particular interest in this study was that very small and primitive amyloid lesions were first analyzed biochemically as well as histopathologically at such an early stage that we are not usually able to observe in human FAP patients. In addition, the serial changes in biochemical composition of the amyloid lesions could clearly be captured. In patient 1, as the amyloid deposits were identified at an unexpectedly early postoperative stage, these deposits were initially thought to be preexisting wild-type TTR-related amyloid produced preoperatively from the patient's native liver as senile systemic amyloidosis. However, the amyloid was purely composed of variant TTR, all of which was produced by the transplanted FAP liver during the 14-month post-operative period. Wild-type TTR is known to be inherently amyloidogenic, as shown in senile systemic amyloidosis [23]. In FAP patients, the contribution of wild-type TTR to amyloid formation is significant in all visceral organs [17,24,25] and our previous study showed that large amounts of wild-type TTR were already present even in biopsy samples of FAP patients in their 20s obtained soon after onset of the disease [25]. However, the biochemical characteristics of amyloid fibrils of preclinical FAP patients have not been investigated, and it remains unknown whether wild-type TTR can be deposited from the initial stage of amyloid formation. Our biochemical results showed that variant TTR alone can be strongly associated with initial amyloid formation, and wild-type TTR seems to be deposited later in the early stage amyloidogenesis of domino recipients. In the histopathological dual staining with Congo red and anti-TTR antibody in this study, Congo red-positive lesions seemed to be located at the centre of Congo red-negative/anti-TTR-positive lesions (Figure 1D–F), which looked like an amyloid lesion in the process of initial fibril formation from non-fibrillar TTR aggregates. Thus, the amyloid lesions of patient 1 may be very primitive 'amyloid seeds' before the involvement of wild-type TTR in amyloid fibrils.

In patient 2, there have been no clinical symptoms of amyloidosis even 12 years after DLT, although abnormal findings on nerve conductions studies suggestive of amyloid neuropathy were observed. In addition, amyloid deposition had not been detected until 1 year ago. Thus, the extracted amyloid in this

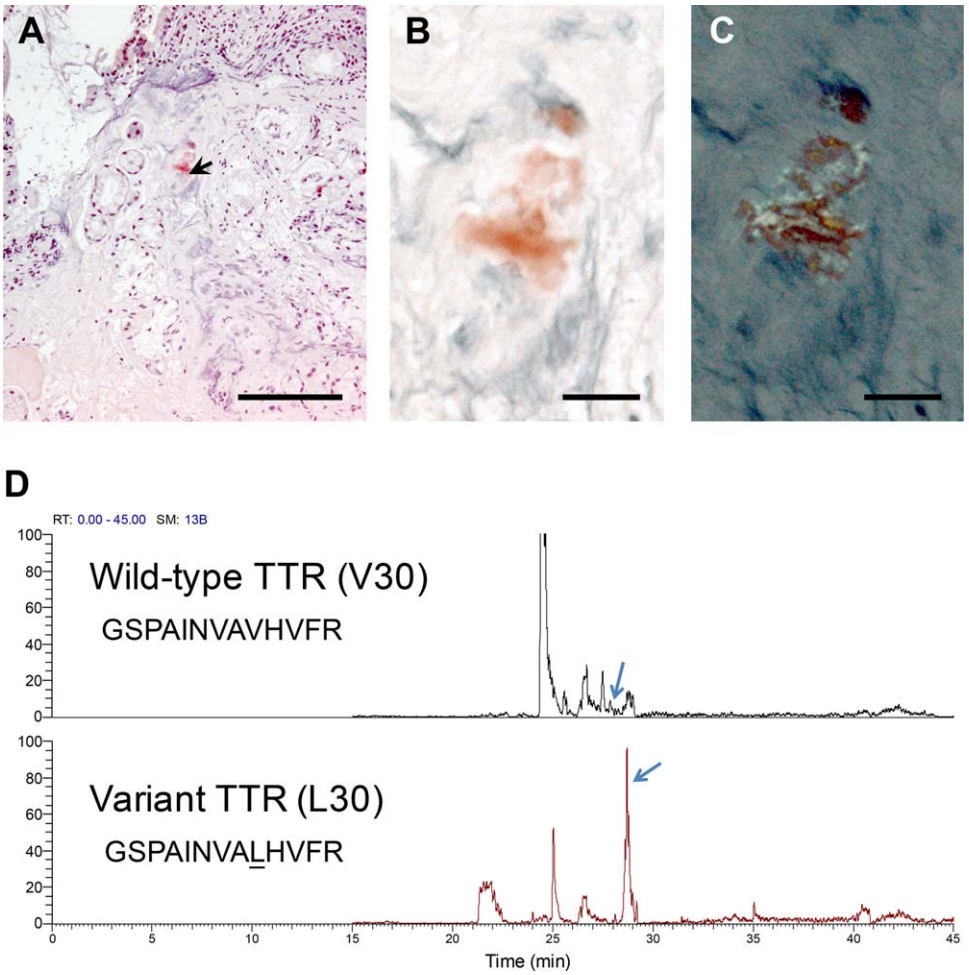

Figure 3. Congo red-stained duodenal mucosa (A–C) and relative quantification of TTR 22–34 tryptic peptide in LC-MS/MS analysis of amyloid isolated (D) at 12 years after DLT in patient 2. (A–C) A small Congo red-positive spot was seen on the lamina muscularis mucosae (arrow) (A, bar = 100 μm). At higher magnification, the amyloid was positive for Congo red (B), showing strong apple-green birefringence under polarized light (C) (B, C, bars = 10 μm). Formalin-fixed and paraffin-embedded tissue sections. Deposited amyloid was isolated with an LMD system (LMD7000; Leica Microsystems Inc., Tokyo, Japan). In LC-MS/MS analysis, the chromatograms were filtered with mass ranges, *m/z* 683.86–683.90 and *m/z* 690.86–690.90. These ranges correspond to TTR 22–34 wild-type (GSPAINVAVHVFR, *m/z* 683.88) and variant peptide (GSPAINVALHVFR, *m/z* 690.87), respectively. The composition ratio of wild-type versus variant TTR was 5:95%.

study also represented early-stage amyloid lesions, and these results support the importance of variant TTR in the initial stage of TTR amyloid formation, as in patient 1.

In addition, our data may provide valuable information for understanding the pathogenesis of TTR amyloid formation. In current dogma of TTR-related amyloidogenesis, the dissociation into monomeric TTR due to instability of the TTR tetramer structure is thought to be important, as confirmed by *in vitro* studies [26]. However, this has not been fully confirmed *in vivo* and it is still controversial whether dissociated monomeric TTR is really a precursor or dissociated dimer with one or two variant monomers may be the real precursor because amyloid fibrils commonly contain large amounts of wild-type TTR [27]. In this study, the contribution of wild-type TTR to early-stage amyloid formation was not significant, and therefore, our results indicate, as the first *in vivo* data, the importance of aggregation from monomeric variant TTR, not from dimeric forms, in amyloid formation.

The precise pathomechanism underlying the difference in time from DLT to identification of amyloid deposits between patients 1 and 2 remains unclear. Differences in age or type of TTR mutation may be

important factors. However, our data strongly suggest that blocking of amyloid formation of variant TTR in the early postoperative stage may be a key to preventing the development of this transmitted amyloidosis. In patient 2, administration of diflunisal [18,19] was begun in the preclinical stage of amyloidosis, and it is, therefore, possible that diflunisal delayed the onset of transmission of amyloidosis associated with DLT.

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Author Contributions

TY: Data collection, amyloid analysis, data interpretation, writing the manuscript; MY: study design, data collection, amyloid analysis, immunohistochemistry, writing the manuscript; YS: amyloid analysis, data interpretation; FK: amyloid analysis, making the figures; KM: amyloid analysis; NH: amyloid analysis, data interpretation; TT: data collection, patient's treatment; NK: data collection, patient's treatment; KH: amyloid analysis, data interpretation; SI: amyloid analysis, data collection, data interpretation, correction of the draft. All authors were involved in writing the paper and approved the submitted version.

References

1. Ikeda S, Nakazato M, Ando Y, *et al*. Familial transthyretin-type amyloid polyneuropathy in Japan: clinical and genetic heterogeneity. *Neurology* 2002;**58**:1001–1007.
2. Connors LH, Lim A, Prokaeva T, *et al*. Tabulation of human transthyretin (TTR) variants, 2003. *Amyloid* 2003;**10**:160–184.
3. Online domino liver transplant registry [Internet]. Familial Amyloidotic Polyneuropathy World Transplant Registry and Domino Liver Transplant Registry; [Accessed November 1, 2015]: Available from: http://www.fapwtr.org/.
4. Azoulay D, Samuel D, Castaing D, *et al*. Domino liver transplantation for metabolic disorders: experience with familial amyloidotic polyneuropathy. *J Am Coll Surg* 1999;**189**:584–593.
5. Stangou AJ, Heaton ND, Hawkins PN. Transmission of systemic transthyretin amyloidosis by means of domino liver transplantation. *N Engl J Med* 2005;**352**:2356.
6. Goto T, Yamashita T, Ueda M, *et al*. Iatrogenic amyloid neuropathy in a Japanese patient after sequential liver transplantation. *Am J Trasnplant* 2006;**6**:2512–2515.
7. Barreiros AP, Geber C, Birlklein F, *et al*. Clinical symptomatic de novo systemic transthyretin amyloidosis 9 years after domino liver transplantation. *Liver Transpl* 2010;**16**:109.
8. Pras M, Schubert M, Zucker-Franklin D, *et al*. The characterization of soluble amyloid prepared in water. *J Clin Invest* 1968;**47**:924–933.
9. Yazaki M, Hashikura Y, Takei Y, *et al*. Feasibility of auxiliary partial orthotopic liver transplantation from living donors for patients with adult-onset type II citrullinemia. *Liver Transpl* 2004;**10**:550–554.
10. Ikeda S, Nakano T, Yanagisawa N, *et al*. Asymptomatic homozygous gene carrier in a family with type I familial amyloid polyneuropathy. *Eur Neurol* 1992;**32**:308–313.
11. Tachibana N, Tokuda T, Yoshida K, *et al*. Usefulness of MALDI/TOF mass spectrometry of immunoprecipitated serum variant transthyretin in the diagnosis of familial amyloid polyneuropathy. *Amyloid* 1999;**6**:282–288.
12. Gustavsson A, Engström U, Westermark P. Mechanisms of transthyretin amyloidogenesis. Antigenic mapping of transthyretin purified from plasma and amyloid fibrils and within in situ tissue localizations. *Am J Pathol* 1994;**144**:1301–1311.
13. Hachiya NS, Kaneko K. Investigation of laser-microdissected inclusion bodies. *Methods Cell Biol* 2007;**82**:355–375.
14. Sethi S, Theis JD, Vrana JA, *et al*. Laser microdissection and proteomic analysis of amyloidosis, cryoglobulinemic GN, fibrillary GN, and immunotoctoid glomerulopathy. *Clin J Am Soc Nephrol* 2013;**8**:915–921.
15. Kametani F, Haga S. Accumulation of carboxy-terminal fragments of APP increases phosphodiesterase 8B. *Neurobiol Aging* 2015;**36**:634–637.
16. Bulawa CE, Connelly S, Devit M, *et al*. Tafamidis, a potent and selective transthyretin kinetic stabilizer that inhibits the amyloid cascade. *Proc Natl Acad Sci USA* 2012;**109**:9629–9634.
17. Yazaki M, Mitsuhashi S, Tokuda T, *et al*. Progressive wild-type transthyretin deposition after liver transplantation preferentially occurs onto myocardium in FAP patients. *Am J Transplant* 2007;**7**:235–242.
18. Berk JL, Suhr OB, Obici L, *et al*. Repurposing diflunisal for familial amyloid polyneuropathy: a randomized clinical trial. *JAMA* 2013;**310**:2658–2667.
19. Sekijima Y, Tojo K, Morita H, *et al*. Safety and efficacy of long-term diflunisal administration in hereditary transthyretin (ATTR) amyloidosis. *Amyloid* 2015;**22**:79–83.
20. Sousa MM, Ferrão J, Fernandes R, *et al*. Deposition and passage of transthyretin through the blood-nerve barrier in recipients of familial amyloid polyneuropathy livers. *Lab Invest* 2004;**84**:865–873.
21. Takei Y, Gono T, Yazaki M, *et al*. Transthyretin-derived amyloid deposition on the gastric mucosa in domino recipients of familial amyloid polyneuropathy liver. *Liver Transpl* 2007;**13**:215–218.
22. Bittencourt PL, Couto CA, Leitão RM, *et al*. No evidence of de novo amyloidosis in recipients of domino liver transplantation: 12 to 40 (mean 24) month follow-up. *Amyloid* 2002;**9**:194–196.
23. Gustavsson A, Engström U, Westermark P. Normal transthyretin and synthetic transthyretin fragments from amyloid-like fibrils in vitro. *Biochem Biophys Res Commun* 1991;**175**:1159–1164.

24. Yazaki M, Tokuda T, Nakamura A, *et al*. Cardiac amyloid in patients with familial amyloid polyneuropathy consists of abundant wild-type transthyretin. *Biochem Biophys Res Commun* 2000;**274**:702–706.
25. Tsuchiya-Suzuki A, Yazaki M, Kametani F, *et al*. Wild-type transthyretin significantly contributes to the formation of amyloid fibrils in familial amyloid polyneuropathy patients with amyloidogenic transthyretin Val30Met. *Hum Pathol* 2011;**42**: 236–243.
26. Kelly JW, Lansbury PT, Jr. A chemical approach to elucidate the mechanism of transthyretin and b-protein amyloid fibril formation. *Amyloid* 1994;**1**:186–205.
27. Benson MD. Pathogenesis of transthyretin amyloidosis. *Amyloid* 2012;**19**:14–15.