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Kumamoto University
Title: Effect of recipient-derived cells on the progression of familial amyloidotic polyneuropathy after liver transplantation: a retrospective study

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A short title: Microchimerism on FAP patients after LT
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DECLARATIONS

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Abstract

Background: Some familial amyloidotic polyneuropathy (FAP) patients show the post-transplant progression of the clinical symptoms. Although the presence of recipient-derived cells in transplanted livers has been reported, no studies investigating the functional significance of this post-transplant chimerism in transplanted FAP patients was performed. The aims of this study were to evaluate amyloidogenic transthyretin (ATTR) production of recipient-derived cells and the relationship between the protein from recipient-derived cells and the progression of FAP symptoms after liver transplantation (LT).

Methods: Seven FAP ATTR Val30Met patients who underwent LT were included in this
study. In one male patient with sex-mismatched donor, fluorescence in situ hybridization (FISH) method was performed on a liver biopsy sample using DNA probes for visualizing X and Y chromosomes to detect the recipient-derived cells. In three patients including the FISH-analyzed patient, ATTR mRNA expression in transplanted livers was evaluated by the polymerase chain reaction (PCR)-restriction fragment length polymorphism method and real-time quantitative reverse transcription-PCR. In five of the seven patients, ATTR in serum protein expression was measured by mass spectrometry.

Results: One FAP patient has 3.1% recipient-derived cells in the transplanted liver. The ATTR mRNA was not expressed in any of the three transplanted livers. The ATTR was not detected in any sera of measured patients.

Conclusion: Although the FAP patient had recipient-derived cells in the transplanted liver, the recipient-derived cells did not contribute to the production of ATTR in our specific case. Effect of recipient-derived cells on the post-transplant progression of FAP symptoms may be negligible.
Introduction

Familial amyloidotic polyneuropathy (FAP) is a disease, inherited in an autosomal dominant fashion, that is caused by mutant transthyretin (TTR).\(^1,2\) The disease is characterized by sensory-dominant peripheral neuropathy, as well as symptoms in the gastrointestinal tract, heart, eyes, kidneys, and autonomic nervous system.\(^3-6\) More than 90 amyloidogenic transthyretin (ATTR) mutations leading to FAP have been reported.\(^7\) Of those, Val30Met is the most common type mutation. TTR is produced by the liver, retinal pigment epithelium, choroid plexus, and alpha cells of the pancreas. Because the liver synthesizes more than 90% of serum TTR,\(^8,9\) liver transplantation (LT) has been performed to treat FAP since 1990. LT is now considered as a promising therapy to halt the progression of clinical symptoms in FAP.\(^9,10\) However, it was reported that some FAP patients showed progression of FAP symptoms even after LT,\(^11-14\) while the mechanism remain to be elucidated.

There is a possibility that the recipient-derived cells in transplanted liver have impact on the progression of FAP symptoms after LT. The presence of microchimerism was reported in transplanted livers and in other transplanted organs.\(^15-17\) Chimerism is
defined as cell migration from the host to the transplanted organ. The mechanism of microchimerism was reported to be transdifferentiation and/or cell fusion of bone marrow cells.\textsuperscript{18-20} Many reports have been published on the presence of microchimerism in transplanted liver.\textsuperscript{21,22} It was reported that transplanted livers have recipient-derived cells and these cells play a role in liver regeneration.\textsuperscript{21-23} In FAP cases, if transplanted liver has recipient-derived cells in FAP patients, these cells have a possibility to produce ATTR which may cause the progression of FAP symptoms. However, there have been no studies investigating recipient derived cells in FAP patients.

The aims of this study were to evaluate the protein production of recipient-derived cells and the impact of recipient-derived cells on the progression of FAP symptoms after LT. We evaluated recipient-derived cells in transplanted FAP patients by fluorescent in situ hybridization (FISH). We also evaluated the serum ATTR level and ATTR mRNA expression in transplanted liver in FAP patients.

\textbf{Patients and methods}

We performed a retrospective study of 7 Japanese FAP ATTR Val30Met patients in
Kumamoto University Hospital who underwent LT from 1994 to 2008 (Table 1). All FAP patients in this study had a definite diagnosis of FAP on the basis of genetic investigations and clinical manifestations of FAP. All patients had standard immunosuppression with tacrolimus or cyclosporine and corticosteroids with or without mycophenolate mofetil (Table 1). We evaluated preoperative factors, graft types, and the changes of clinical manifestations after LT by retrospective analysis of medical charts (Table 1). The patient who has sex-mismatched donor and shows the typical progression, would be an ideal and a suitable case to evaluate the effect of recipient-derived cells. Especially, Patient 4 was the ideal and suitable case. Therefore, we focused on Patient 4. Two frozen autopsy samples of Patient 1 and Patient 2 and one frozen biopsy sample of Patient 4 were used to evaluate ATTR mRNA expression in the transplanted livers. In three patients including the FISH-analyzed one, expression of ATTR Val30Met mRNA in transplanted livers was evaluated using the polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method and real-time quantitative reverse transcription-polymerase chain reaction (RT-PCR) method. In five of the seven patients, ATTR in sera was measured using matrix-assisted laser desorption
ionization/time-of-flight mass spectrometry (MALDI/TOF-MS).

The ethical committee at Kumamoto University School of Medicine approved this study and informed consent was obtained from patients after explanation about this study.

**FISH**

The biopsied liver at 7 years after LT of Patient 4 was analyzed. FISH was performed in Nihon Gene Research Laboratories. For malin-fixed paraffin-embedded sections were sectioned at 6 µm. The sections were prepared for FISH by the NaSCN incubation, denaturation, and proteinase K digestion steps, one after the other. After the last formamide denaturation, the slides were dehydrated in graded ethyl alcohols and air dried. FISH was performed on pretreated slides using spectrum green labeled DXZ1 probe (Vysis, Downers Grove, IL) and spectrum red labeled DYZ1 probe (Vysis) for labeling X and Y chromosomes. Probe-applied, covered, and sealed slides were denatured on hot plate and hybridized over night at 37°C using a hybrid incubation chamber (Vysis). DAPI was performed for nuclear counter-staining.
**Determination of TTR using MALDI/TOF-MS**

We examined the presence of ATTR Val30Met in sera of five post-transplanted FAP patients. Fifty microliter of test serum was mixed with 10 µl of 2.7 mM dithiothreitol and 20 µl of an anti-TTR polyclonal antibody (DAKO, Glostrup, Denmark). TTR in the serum was isolated as described by Ando *et al.* All experiments were performed using a Bruker Reflex mass spectrometer (Bruker Franzen Analytik GmbH, Bremen, Germany) operated at a wavelength of 337 nm. The isolated TTR was assayed as described previously.

**Isolation of RNA and reverse transcription**

Total RNA was extracted from each frozen samples using the RNeasy Mini Kit Isolation System (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA was quantified by spectrophotometric analysis using NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, DE). The RNA was reverse-transcribed into complementary DNA (cDNA) by one cycle at 37 °C 15 min followed by one cycle 85 °C for 5 s using
PrimeScript RT reagent Kit (Takara Bio, Shiga, Japan).

**PCR-RFLP**

The generated cDNA was used as the template in real-time PCR reactions. PCR primers were designed to amplify the Val30Met mutation in exon 2 of the TTR gene. Primers were: forward 5’-CA TTCTTGGCAGGATGGCTTC-3’ and reverse 5’-CT CCCAGGTGTC ATCAGCAG-3’. The PCR conditions were 5 min at 98 °C; 35 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min; followed by 7 min 72 °C. The PCR product of 199 bp was digested with 5 U NsiI restriction enzyme (New England Biolabs, UK) that recognized mutation site at 37 °C for 2 h. PCR products were then separated on 3.5% NuSieve GTG agarose gel (BMA products, Rockland, ME) by electrophoresis in 1× TBE buffer, approximately 2 h at 50 V. The expected PCR products are visualized using SYBR SafeTM DNA Gel Stain (Invitrogen, Carlsbad, CA). PCR products were evaluated using microchip electrophoresis system (Cosmo-I SV1210; Hitachi Electronics Engineering, Tokyo, Japan), too. Measurement was according to manufacture’s manual.
**Real-time quantitative RT-PCR**

The generated cDNA was used as the template in real-time PCR reactions. Real-time PCR primers were designed to amplify the Val30Met mutation of the TTR gene. Primers were: forward 5’-CTCTGA TGGTCAAAGTTCTAGATGCT-3’ and reverse 5’-GTGTCATCAGCAGCCTTTCTG-3’. We used FAM-labeled TaqMan probe and VIC-labeled TaqMan probe to detect the Val30Met TTR gene and wild-type TTR gene, respectively. Val30Met TTR specific FAM-labeled TaqMan probe was 5’-FAM-AATGTGGCCATGCAT-3’. Wild-type TTR specific VIC-labeled TaqMan probe was 5’-VIC-CAATGTGGCCGTGCAT-3’. The real-time PCR conditions were 2 min at 50 °C, 10 min at 95 °C; 40 cycles of 95 °C for 15 s, 65 °C for 1 min. Real-time RT-PCR was performed with TaqMan Genotyping Master Mix (Applied Biosystems, Foster City, CA), according to the manufacturer’s protocol. Real-time PCR for wild-type and Val30Met TTR mRNA was validated on the Applied Biosystems 7500 Real-Time PCR system according to the manufacturer’s instructions. 50:50, 90:10, 98:2, 99.6:0, 4, 99.92:0.08, and 100:0 of wild-type TTR genes and ATTR Val30Met genes served as
standards for analyses of TTR mRNA expression ratio in transplanted liver.

Results

Clinical outcome

Table 1 presents preoperative factors, graft types, and the changes of clinical manifestations after LT. Patient 1 and Patient 2 received whole liver grafts from deceased donors. Patient 1 showed the progression of cardiac symptoms even after LT. He underwent pacemaker implantation 8 years after LT due to complete atrioventricular block. In addition, although the ocular manifestation was not observed before LT, he developed mild degree of vitreous opacity in his right eye 12 years after LT. He did not show the progression of neuropathy after LT and died of hypokalemia-induced pulseless ventricular tachycardia caused by alcoholism 13.8 years after LT. Patient 2 showed the progression of cardiac symptom too. She underwent pacemaker implantation 5 years after LT due to heart failure. Because the progression of vitreous opacity and glaucoma was observed, she underwent trabeculectomies 7 and 8 years after LT and vitrectomy 9 years after LT. She did not show the progression of neuropathy after LT and died of...
severe congestive heart failure with cardiac liver cirrhosis 9.2 years after LT. Patient 3, 5, 6 and 7 did not show the progression of FAP symptoms. Patient 4 received the right lobe graft from the living donor and underwent liver biopsy 7 years after LT. The donor was his daughter without pregnancy history. He showed the granular sparkling echo and circumferential ventricular wall thickening with echocardiography 4 years after LT and developed symptoms of heart failure. He underwent pacemaker implantation 5 years after LT due to trifascicular block. He underwent liver biopsy to check his liver, because he had ascites and pleural effusion 7 years after LT. The transplanted liver was almost normal according to the results of biopsy. These results showed that the cardiac amyloidosis caused the ascites and pleural effusion and cardiac symptoms progressed after LT. He showed the progression of neuropathy and deterioration in nerve conduction velocity gradually after LT. In addition, because he also exhibited the progression of vitreous opacity, he underwent anterior vitrectomy of his right eye 3 years after LT and vitrectomy of his left eye 4 years after LT.

**Recipient-derived cells in liver allograft**
FISH analysis was performed in Patient 4 with sex-mismatched donor to evaluate the ratio of recipient-derived cells in transplanted liver. There were 31 cells that had XY signals out of 1000 cells in his transplanted liver, indicating that his transplanted liver had 3.1% recipient-derived cells 7 years after LT (Figure 1).

**Serum ATTR in FAP patients after LT**

TTR in sera of five transplanted FAP patients was measured by MALDI/TOF-MS to evaluate the serum ATTR after LT. The results of a FAP patient before LT as a positive control and Patient 4 were shown in Figure 2. While the peak of approximately 13,761 kDa of wild-type TTR was detected, the peak of ATTR Val30Met was not detected in Patient 4. Other transplanted FAP patients did not exhibit the peak of ATTR Val30Met.

**ATTR Val30Met mRNA expression in transplanted livers**

ATTR mRNA levels in transplanted livers were evaluated by using PCR-RFLP and real time quantitative R T-PCR to elucidate the ATTR expression of recipient-derived hepatocytes. In three transplanted livers, PCR-RFLP analysis revealed wild-type TTR
mRNA expression, but not ATTR Val30Met mRNA expression in transplanted livers.

ATTR mRNA expression in transplanted livers was evaluated by using real time quantitative RT-PCR to elucidate the ATTR production of recipient-derived hepatocytes more sensitively. Delta threshold cycle (Ct) value was calculated by taking Ct of wild-type TTR from that of Val30Met ATTR in real time quantitative RT-PCR. The chart of standard curve that has delta Ct scale and logarithmic scale of mixing ratio was made. The approximation of standard curve was expressed as follows: \[ Y = -15.35\ln(X) + 33.218 \]. Using purified wild-type and ATTR Val30Met DNAs, we verified approximately 0.1% ATTR Val30Met mRNA expression at the lowest level. However, in three transplanted livers, ratio of ATTR mRNA expression was calculated by the approximation (Table 2). Ratio of ATTR expression in three transplanted livers was below the detectable level. In this analysis, ATTR mRNA was not expressed in transplanted livers.

Discussion

Results of this study indicate that the transplanted liver had recipient-derived cells
which did not produce A TTR. Although it has been reported the progression of FAP symptoms after LT,\textsuperscript{11-14} effect of recipient-derived cells on the progression of FAP have not been reported until now. If recipient-derived cells have some effects on the progression, we need to consider the way of inhibition for the recipient-derived cells. Therefore, we enrolled FAP patients with or without progression of symptoms in our study.

In this study, the presence of recipient-derived cells in transplanted liver was first confirmed in the FAP patient by using FISH analysis, indicating that microchimerism occurred in the FAP patient after LT. Although FISH analysis was performed in only one FAP patient, the presence of recipient-derived cells were also confirmed with almost the equal frequency of previous reports in other diseases.\textsuperscript{21,22} However, despite the presence of recipient-derived cells, our results showed that ATTR mRNA was not expressed in the transplanted liver and serum ATTR could not be detected. Previous studies reported that hepatocyte transdifferentiation from recipient bone marrow stem cells was rare,\textsuperscript{27-30} suggesting that the recipient-derived cells in the transplanted liver may be too immature to synthesize TTR. In addition, previous studies have also shown
that the recipient-derived cells transdifferentiated into cholangiocytes or vein endothelium cells in transplanted livers,\textsuperscript{21,30-33} implying that the recipient-derived cells may transdifferentiate into non-hepatocytes. Because the sample was limited in this study, further investigation will be needed to identify the type of recipient-derived cells by multiple methods such as immunohistological staining for hepatocyte marker.

Taken together, in the view of microchimerism, values of LT for FAP patients were revalidated by showing that recipient-derived cells did not produce ATTR in our cases. However, because transplanted liver actually had recipient-derived cells in FAP patients, it still remains possible that these recipient-derived cells may have impact on the progression of FAP symptom in some FAP patients. Furthermore, the transplanted FAP patient who showed the progression and had sex-mismatched donor was very rare. We need to analyze in more number of FAP patients hereafter.

Although it has been reported that some FAP patients showed progression of FAP symptoms even after LT, the mechanism remains to be elucidated. In this study, our data did not confirm the effect of recipient-derived cells on the progression of FAP. It is well documented that TTR was produced by different organs, such as retinal pigment...
epithelium, choroid plexus, and alpha cells of the pancreas. Even though A TTR levels were not detectable in our study, the trace amount of ATTR from extrahepatic organs may contribute to the progression of FAP symptoms after LT. In addition, because the part of amyloid deposits in some organs have been proven to be derived from the wild-type TTR, the TTR may have some impact on the progression of FAP symptoms after LT. Further investigations will be needed to clarify the detailed mechanism of the progression of FAP symptoms after LT.

Another interesting finding in this study is that LT in FAP has the possibility to be a unique and natural human model to evaluate the recipient-derived transdifferentiated hepatocytes by detecting A TTR gene or protein functionally, because only recipient-derived hepatocytes can express ATTR gene. Previously, the presence of recipient-derived hepatocytes was usually shown by sex-mismatch analysis. FISH and immunohistochemical staining methods on transplanted tissues in sex-mismatched individuals cannot fully evaluate the function of recipient-derived cells. It is well documented that not only albumin but also TTR is produced by differentiated
hepatocytes. If recipient-derived cells transdifferentiate to hepatocytes in heterozygous FAP patients, these recipient-derived hepatocytes should produce both wild-type TTR and ATTR. We could not detect the expression of ATTR Val30Met in this study. This result may imply that the recipient-derived cells did not transdifferentiate to hepatocytes in our cases. Although we need to study in more transplanted FAP patients, we might provide a new tool to evaluate functional chimerism in transplanted livers. However, because our data do not exclude the possibility that ATTR mRNA expression and serum ATTR protein levels were too low to be detected, we need to evaluate ATTR expression by more sensitive method such as radioimmunoassay procedure.

In conclusion, although it was confirmed that the FAP patient had recipient-derived cells in the transplanted liver in our series, the recipient-derived cells did not contribute to the production of ATTR. Although the case volume was small, our study suggests that microchimerism do not have effect on the progression of FAP symptoms after LT in our cases. Our results provide one milestone into LT in FAP patients.
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Figure legends

Figure 1. Presence of recipient-derived cells is confirmed in the liver biopsy sample of male FAP recipient with sex-mismatched donor. Nuclear red spots were interpreted as Y chromosomal signal, green spots as X chromosomal signals. Recipient-derived cells (arrow) were detected in the transplanted liver.

Figure 2. Analysis of TTR forms by MALDI/TOF-MS in the serum of FAP patients. (A) A FAP patient before LT as a positive control. The peaks of 13,761 kDa and 13,792 were free forms of wild-type TTR and ATTR Val30Met, respectively. (B) Patient 4 after LT. The peak of 13,761 kDa was free form of wild-type TTR. The peak of A TTR was not detected.