Virchows Arch (2015) 467:417–425
DOI 10.1007/s00428-015-1819-3

ORIGINAL ARTICLE

In recurrent primary biliary cirrhosis after liver transplantation, biliary epithelial cells show increased expression of mitochondrial proteins

Motoko Sasaki[1] · **Maylee Hsu**[2] · **Matthew M. Yeh**[2] · **Yasuni Nakanuma**[1,3]

Received: 18 May 2015 / Revised: 6 July 2015 / Accepted: 28 July 2015 / Published online: 11 August 2015

Abstract In biliary epithelial lesions in primary biliary cirrhosis (PBC), mitochondrial proteins associated with deregulated autophagy are abnormally expressed. We examined whether this could be used as a diagnostic marker for end-stage PBC and recurrent PBC after liver transplantation. We examined the expression of the mitochondrial protein pyruvate dehydrogenase complex-E2 component and cytochrome c oxidase, subunit I (CCO), the autophagy-related marker microtubule-associated protein-light chain 3 (LC3), and p62/sequestosome-1 and the senescence markers $p16^{Ink4a}$ and $p21^{WAF1/Cip1}$ in small bile ducts and bile ductules in explanted livers from patients with PBC (n=20) in comparison with liver tissue from control patients (n=21) and post-transplant samples including recurrent PBC and cellular rejection (n=28). Intense granular expression of mitochondrial proteins was significantly more frequent in small bile ducts in explanted livers with PBC than in control livers (p<0.05). Post-transplant samples comprised of three groups: group A (positive for mitochondrial proteins, n=7), group B (positive for either autophagy-related or senescence markers but negative for mitochondrial proteins, n=7), and group C (all negative, n=14). All but one case of group A were clinically and histologically diagnosed as recurrent PBC. In contrast, all cases of group B were diagnosed as cellular rejection. This study suggests that the expression of mitochondrial proteins in small bile ducts may be a useful diagnostic marker for end-stage PBC and recurrent PBC after liver transplantation.

Keywords Primary biliary cirrhosis · Post-liver transplant rejection · Pyruvate dehydrogenase complex-E2 component · Biliary epithelial cell · Autophagy and cellular senescence

✉ Motoko Sasaki
m8sasaki@med.kanazawa-u.ac.jp

[1] Department of Human Pathology, Kanazawa University Graduate School of Medicine, Kanazawa 920-8640, Japan

[2] Department of Pathology, University of Washington, Seattle, WA 98195, USA

[3] Division of Pathology, Shizuoka Cancer Center, Shizuoka, Japan

Introduction

Primary biliary cirrhosis (PBC) is an organ-specific autoimmune disease that presents with chronic, progressive cholestasis, and liver failure [1–3]. PBC is characterized histologically as cholangitis of the small bile ducts (chronic non-suppurative destructive cholangitis; CNSDC), eventually followed by extensive loss of small bile ducts [1, 4]. Serum anti-mitochondrial antibodies are characteristic of PBC. Orthotopic liver transplantation (OLT) is widely performed for PBC and PBC may sometimes recur in the transplanted liver. Histological diagnosis of recurrent PBC can occasionally be difficult [5], because portal inflammation and bile duct damage similar to PBC rejection may also be found in other conditions. In addition, end-stage PBC can be challenging to distinguish from other chronic liver diseases, notably when histological hallmarks such as florid duct lesions are not readily present. This justifies the search for potential markers of PBC recurrence and end-stage PBC.

We have previously reported that biliary epithelial cells (BECs) in bile duct lesions in PBC show unique expression of mitochondrial antigens, autophagy and deregulated autophagy markers, and markers of senescence [6–9]. We reported that in PBC biliary epithelial cells (BECs) involved in CNSDC show cellular senescence, characterized by increased expression of senescence-associated β-galactosidase (SA-β-gal), $p16^{INK4a}$ and $p21^{WAF1/Cip1}$, and telomere shortening [6,

10, 7, 11]. We also reported that deregulated macroautophagy (hereafter referred to as autophagy) might be involved in the pathogenesis of bile duct lesions in PBC [8, 12]. Furthermore, our studies revealed that BECs in biliary epithelial lesions show abnormal expression of mitochondrial antigens associated with deregulated autophagy, which may play a role in the pathogenesis of PBC [9].

Given the specific pattern of expression of mitochondrial antigens in biliary epithelial lesions in PBC, we hypothesized that this might be helpful in the diagnosis of recurrent and end-stage PBC. In this study, we examined expression of mitochondrial antigens, autophagy markers, and markers of senescence in end-stage and post-transplant recurrent PBC cases. We show that expression of mitochondrial antigens might be useful as diagnostic marker for recurrent PBC after OLT and also for end-stage PBC.

Materials and methods

Classification of intrahepatic biliary tree We classified the intrahepatic biliary tree into intrahepatic large and small bile ducts (septal and interlobular bile ducts) by their size and distribution in the portal tracts [13]. Bile ductules, which are characterized by tubular or glandular structures with a poorly defined lumen and location at the periphery of the portal tracts [13, 14], were not considered small bile ducts.

Liver tissue preparation We collected 74 liver tissue specimens (biopsies or explants) from the liver disease files of our laboratory and affiliated hospitals. The Ethics Committee of Kanazawa University approved this study. The liver specimens in this study consisted of: (1) end-stage cirrhotic specimens: 20 cases of PBC (all explants), 7 of primary sclerosing cholangitis (PSC, 4 biopsies and 3 explants), 7 of autoimmune hepatitis (AIH, all explants), and 7 cases of nonalcoholic steatohepatitis (NASH, 1 biopsy and 6 explants); (2) 28 post-orthotopic liver transplant (OLT) samples (all biopsies, including 11 cases of recurrent PBC and 13 cases of acute cellular rejection). All PBC cases were from patients fulfilling clinical, serological, and histological characteristics consistent with a diagnosis of PBC [1]. PBC livers were staged histologically [1]; 3 cases were stage 3 and 17 stage 4. All explants of PSC, NASH, and AIH were stage 4.

Liver tissue samples were fixed in 10 % neutral-buffered formalin and embedded in paraffin. For each case, at least 20 serial sections, 4 μm thick, were cut from each block. Several slides were then stained routinely with hematoxylin and eosin, reticulin, and orcein for histology studies. The remaining sections were processed for immunohistochemistry.

Immunohistochemistry We examined immunohistochemically, as described previously [10], the expression of the following mitochondrial antigens: pyruvate dehydrogenase complex-E2 component (PDC-E2) and cytochrome c oxidase subunit I (CCO), the autophagy marker microtubule-associated protein-light chain 3β (LC3), the deregulated autophagy marker p62/sequestosome-1 (p62), and the senescence markers $p16^{INK4a}$ and $p21^{WAF1/Cip1}$. Details of the used primary antibodies are listed in Table 1. In brief, after pretreatment for antigen retrieval (Table 1) and blocking of endogenous peroxidase, the sections were incubated with the primary antibody at 4 °C overnight. The Envision+solution (Dako) was then applied for 30 min at room temperature. The reaction products were visualized using 3-3′-diaminobenizidine tetra hydrochloride (Sigma Chemical, Co., St. Louis, MO) and H_2O_2. The sections were then lightly counterstained with methyl green or hematoxylin. A similar dilution of control mouse or rabbit Immunoglobulin G (Dako) was applied instead of the primary antibody as negative

Table 1 Primary antibodies used in this study

Primary antibody	Type (clone)	Pre-treatment	Dilution	Source
Mitochondrial proteins				
PDC-E2	Rabbit poly	Proteinase K (RT, 10 min)	1:100	Santa-Cruz, Santa Cruz, CA
CCO	Mouse mono (1D6E1A8)	eARI–BA (121 °C, 5 min)	1:200	Invitrogen, Camarillo, CA
Autophagy-related markers				
LC3	Goat poly	eARI–BA (121 °C, 5 min)	1:50	Santa-Cruz, Santa Cruz, CA
p62	Rabbit poly	eARI–BA (121 °C, 5 min)	1:1,000	MBL, Nagoya, Japan
Senescent markers				
$p16^{INK4a}$	Mouse mono (JC8)	eARI–BA (121 °C, 5 min)	1:100	Neomarkers, Fremont, CA
$p21^{WAF1/Cip1}$	Mouse mono (70)	eARI–BA (121 °C, 5 min)	1:100	BD transduction, San Jose, CA

PDC-E2 pyruvate dehydrogenase complex-E2 component, *CCO* cytochrome c oxidase, subunit I, *p62* p62/sequestosome-1, *LC3* microtubule-associated proteins-light chain 3β, *RT* room temperature, *MW* microwave treatment, *CB* 0.05 M citric buffer (pH 6), *eARI*, electronic antigen retrieval instrument (Pascal, Dako), *BA* 0.05 M boric acid buffer (pH 8)

control. Positive and negative controls were routinely included. Histological analysis was performed in a blinded manner.

Extent of expression of immunohistochemical markers in small bile ducts was scored as follows: 0 (negative), 1 (focal, positive cells detected in three or fewer small bile ducts), and 2 (extensive, positive cells detected in more than three small bile ducts). Extent of expression of immunohistochemical markers in bile ductules was scored as follows: 0 (negative), score 1 (focal, positive cells detected in one third or fewer portal tracts), and 2 (extensive, positive cells detected in more than one third of portal tracts).

Laboratory data Laboratory data, including total bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (AP), and γ-glutamyltransferase (γ-GT), were available in 26 post-OLT patients. The correlation between laboratory data and the expression of PDC-E2, CCO, LC3, p62, $p16^{INK4a}$, and $p21^{WAF1/Cip1}$ in small bile ducts and bile ductules was assessed.

Statistical analysis Statistical analysis of the differences was performed using the Kruskal–Wallis test with Dunn's post-test. Correlation between two groups was assessed using Spearman's correlation test. When the p value was <0.05, the difference was considered significant.

Results

Marker expression in small bile ducts in end-stage livers

PDC-E2 and CCO Intense granular and vesicular expression of PDC-E2 (Fig. 1) and CCO was seen in biliary epithelial cells in small bile ducts in end-stage PBC livers (Figs. 1 and 2a). In our previous study, we also commonly observed PDC-E2 and CCO expression in damaged small bile ducts [9]. In contrast, the expression of PDC-E2 and CCO, if present, was focal in non-PBC end-stage control livers (Figs. 1 and 2a). PDC-E2 and CCO were significantly more frequently expressed in small bile ducts in end-stage PBC than in end-stage PSC ($p<0.05$; Fig. 2a).

LC3 and p62 Granular and vesicular expression of LC3 was observed in small bile ducts in end-stage PBC livers, but not in end-stage PSC, AIH, and NASH livers (Fig. 2a). Granular expression of p62 was observed in small bile ducts of end stage PBC livers, whereas the expression of p62 was absent or faint in end-stage PSC and NASH livers (Figs. 1 and 2a).

$p16^{INK4a}$ and $p21^{WAF1/Cip1}$ Nuclear and cytoplasmic expression of $p16^{INK4a}$ was observed, while expression of $p21^{WAF1/Cip1}$ was nuclear. Expression of $p16^{INK4a}$ and $p21^{WAF1/Cip1}$ was observed in small bile ducts in some end-stage PBC and end-stage PSC (Fig. 2a).

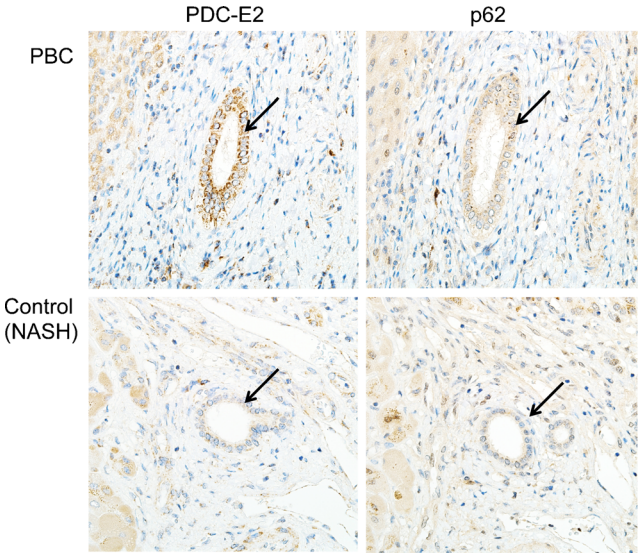


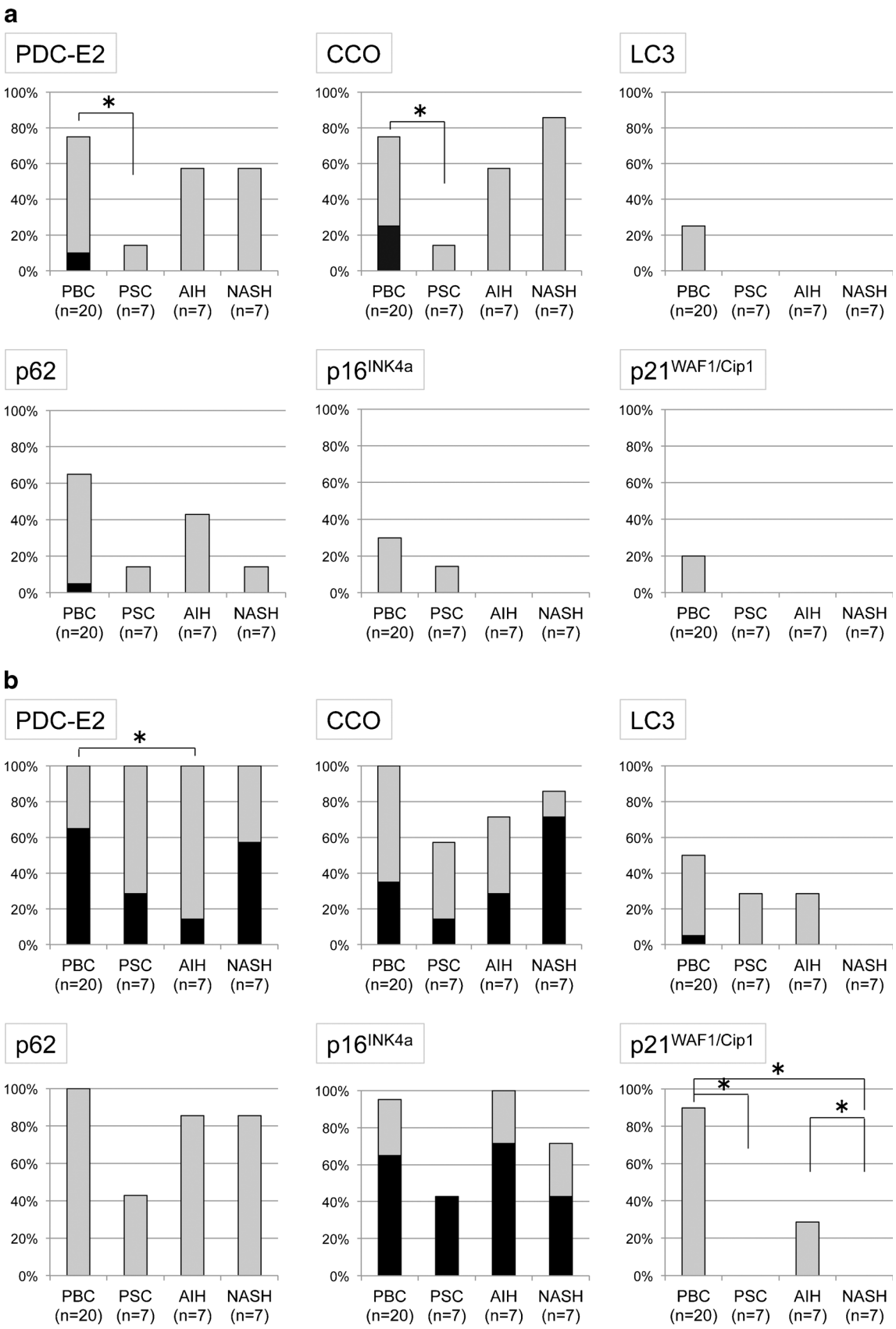
Fig. 1 Increased granular expression of pyruvate dehydrogenase, E2 component (PDC-E2) and p62 in small bile ducts in end-stage primary biliary cirrhosis (PBC). *Top*) Intense granular and vesicular expression of PDC-E2 was seen in biliary epithelial cells in small bile ducts (*arrow*) in PBC, stage 4. Granular expression of p62 was observed in small bile ducts (*arrow*) in PBC, stage 4. *Bottom*) The expression of PDC-E2 and p62/sequestosome-1 (p62) was absent in biliary epithelial cells in the small bile duct (arrow) in nonalcoholic steatohepatitis (NASH), stage 4. Immunostaining for PDC-E2 and p62. Original magnification, x400

Marker expression in bile ductules in end-stage livers

PDC-E2 and CCO Intense granular expression of PDC-E2 and CCO was seen in bile ductules in end-stage PBC and other diseases. PDC-E2 was significantly more frequently expressed in end-stage PBC than in end-stage AIH livers ($p<0.05$; Fig. 2b).

LC3 and p62 Granular and vesicular expression of LC3 and p62 was seen in bile ductules in end-stage PBC and other diseases (Fig. 2b). Granular expression of p62 was also seen in bile ductules in end-stage PBC and other diseases (Fig. 2b). Expression of LC3 and p62 in bile ductules was not significantly different between PBC and other diseases.

$p16^{INK4a}$ and $p21^{WAF1/Cip1}$ $p16^{INK4a}$ was frequently expressed in bile ductules in end-stage PBC and other liver diseases (Fig. 2b). Expression of $p21^{WAF1/Cip1}$ was more frequent in bile ductules in end-stage PBC, compared to PSC and NASH ($p<0.05$; Fig. 2b). Expression of $p21^{WAF1/Cip1}$ was more frequent in bile ductules in end-stage AIH, compared to NASH ($p<0.05$; Fig. 2b)


a
PDC-E2
CCO
LC3
p62
p16INK4a
p21WAF1/Cip1
b
PDC-E2
CCO
LC3
p62
p16INK4a
p21WAF1/Cip1
100%
80%
60%
40%
20%
0%
PBC (n=20)
PSC (n=7)
AIH (n=7)
NASH (n=7)
*

◀ **Fig. 2** Semiquantitative evaluation of mitochondrial, autophagy-related and senescent markers expression in biliary epithelial cells in PBC and control livers. **a**) Expression in small bile ducts. *Grey column*, score 1 (mild) and *black*, score 2 (extensive). *, $p<0.05$. *PDC-E2* pyruvate dehydrogenase complex-E2 component, *CCO* cytochrome c oxidase, subunit I, *p62*, *p62/sequestosome-1*;*LC3* microtubule-associated proteins-light chain 3β, *PBC* primary biliary cirrhosis, *PSC* primary sclerosing cholangitis, *AIH* autoimmune hepatitis, *NASH* nonalcoholic steatohepatitis, *TL* post-liver transplantation. **b**) Expression in bile ductules. *Grey column*, score 1 (mild) and *black*, score 2 (extensive). *, $p<0.05$. *PDC-E2* pyruvate dehydrogenase complex-E2 component, *CCO* cytochrome c oxidase, subunit I, *p62* p62/sequestosome-1, *LC3* microtubule-associated proteins-light chain 3β, *PBC* primary biliary cirrhosis, *PSC* primary sclerosing cholangitis, *AIH* autoimmune hepatitis, *NASH* nonalcoholic steatohepatitis, *TL* post-liver transplantation

Marker expression in small bile ducts in post-OLT livers

PDC-E2 and CCO Intense granular and vesicular expression of PDC-E2 was seen in biliary epithelial cells in small bile ducts in recurrent PBC after liver transplantation (Fig. 3). In contrast, the expression of PDC-E2 was faint in small ductular biliary epithelial cells in acute cellular rejection after liver transplantation (Fig. 3). Expression of PDC-E2 was significantly more frequent in small bile ducts in recurrent PBC than in acute cellular rejection ($p<0.05$; Fig. 4a).

LC3 and p62 Granular expression of LC3 and p62 was observed in small bile ducts in recurrent PBC after liver transplantation (Fig. 3). Granular expression of LC3 and p62 was also seen in small bile ducts in acute cellular rejection after liver transplantation (Fig. 3). Expression of LC3 and p62 in small bile ducts was not significantly different between recurrent PBC, acute cellular rejection, and other diseases (Fig. 4a).

$p16^{INK4a}$ and $p21^{WAF1/Cip1}$ $p16^{INK4a}$ and $p21^{WAF1/Cip1}$ expression was present in small bile ducts in some recurrent PBC and in acute cellular rejection (Fig. 4a).

Table 2 and Fig. 5 summarize the expression pattern in all post-OLT livers examined and the rate of expression in each histological category (recurrent PBC, acute cellular rejection, or others). Post-OLT samples were divided into group A (positive for both PDC-E2 and CCO; $n=7$), group B (positive for either of LC3, p62, p21, or p16; negative for PDC-E2 and CCO; $n=7$), and group C (all negative; $n=14$; Fig. 5). All but one case of group A were clinically and histologically diagnosed as recurrent PBC. In contrast, all but one case of group B were diagnosed as rejection.

Marker expression in bile ductules in post-OLT livers

PDC-E2 and CCO Granular expression of PDC-E2 and CCO in bile ductules in post-OLT livers was not significantly different between recurrent PBC, acute cellular rejection and others (Fig. 4b).

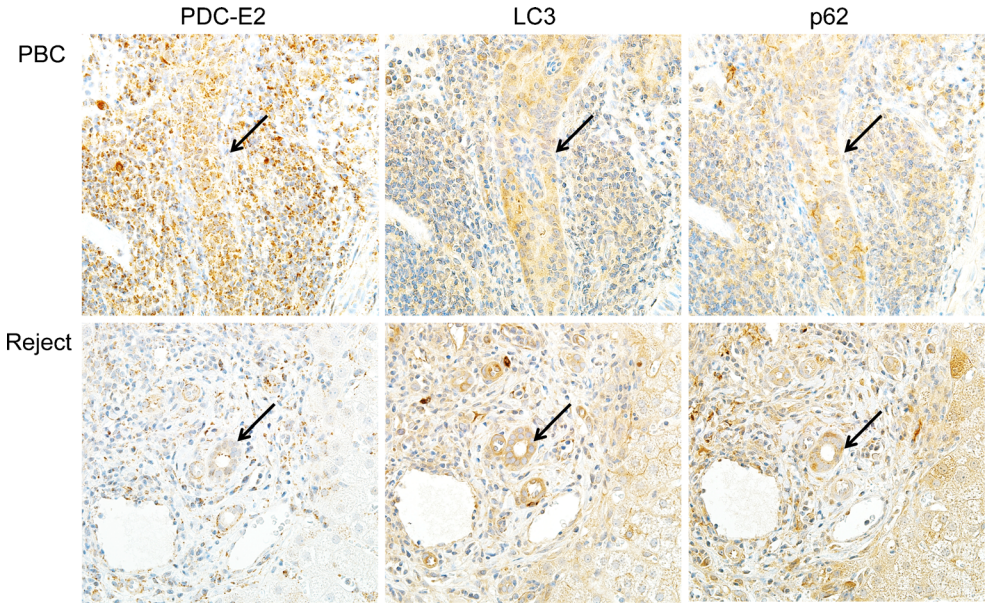
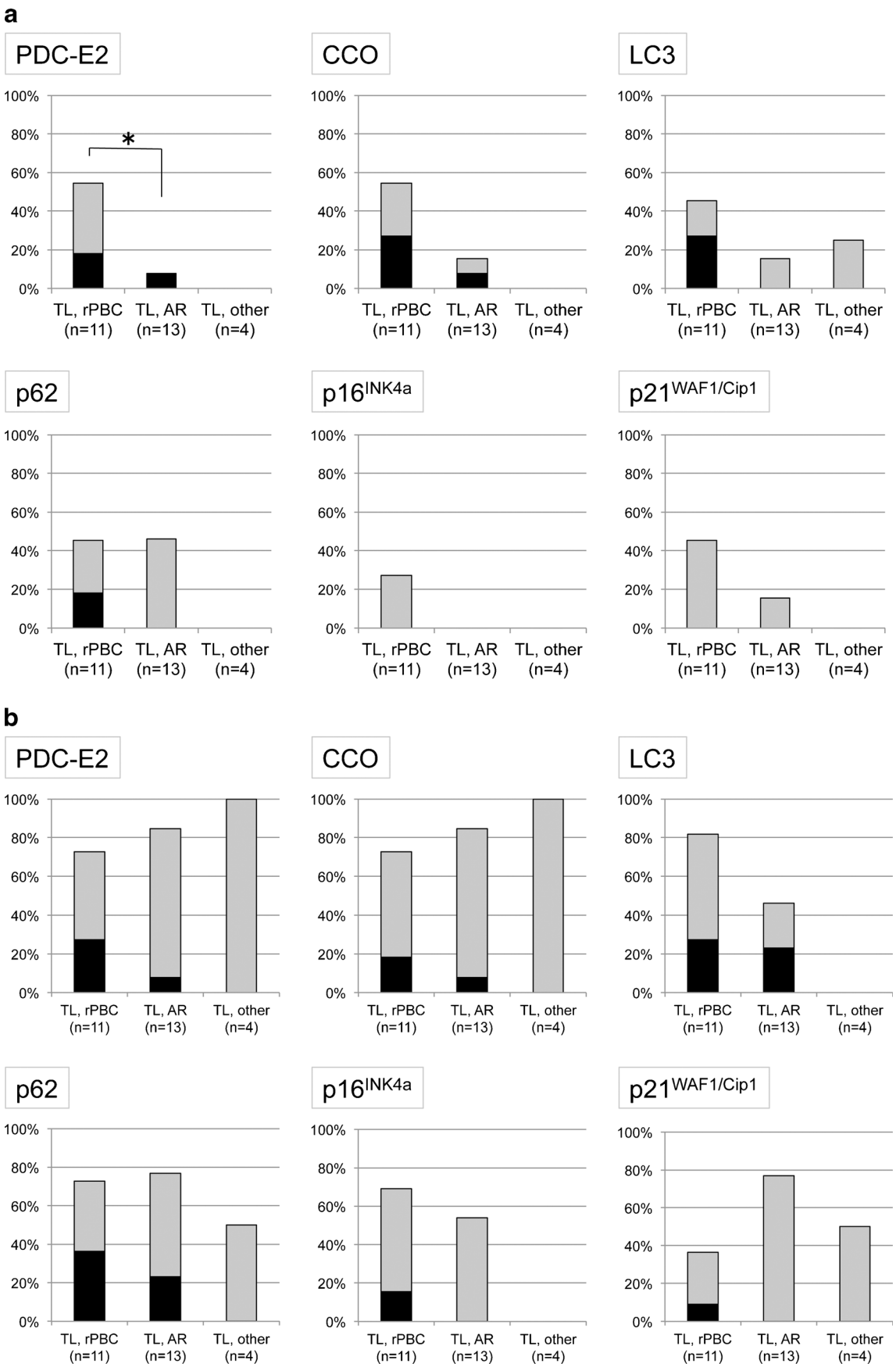


Fig. 3 Increased granular expression of pyruvate dehydrogenase, E2 component (PDC-E2) in small bile ducts in recurrent primary biliary cirrhosis (rPBC). *Top*) Intense granular and vesicular expression of PDC-E2 was seen in biliary epithelial cells in small bile ducts (*arrow*) in recurrent PBC after liver transplantation. Granular expression of microtubule-associated proteins-light chain 3β (LC3) and p62/sequestosome-1 (p62) was also observed in small bile ducts (arrows) in recurrent PBC after liver transplantation. *Bottom*) The expression of PDC-E2 was faint in biliary epithelial cells in the small bile duct (*arrow*) in acute cellular rejection after liver transplantation. Granular expression of LC3 and p62 was seen in small bile ducts (*arrows*) in acute cellular rejection after liver transplantation (Reject). Immunostaining for PDC-E2, LC3 and p62. Original magnification, x400


a
PDC-E2
CCO
LC3
p62
p16INK4a
p21WAF1/Cip1
100%
80%
60%
40%
20%
0%
*
TL, rPBC (n=11)
TL, AR (n=13)
TL, other (n=4)
b
PDC-E2
CCO
LC3
p62
p16INK4a
p21WAF1/Cip1
TL, rPBC (n=11)
TL, AR (n=13)
TL, other (n=4)

◀ **Fig. 4** Semiquantitative evaluation of mitochondrial, autophagy-related and senescent markers expression in biliary epithelial cells in post-orthotopic liver transplantation **a**) Expression in small bile ducts. *Grey column*, score 1 (mild) and *black*, score 2 (extensive). *, $p<0.05$. PDC-E2, pyruvate dehydrogenase complex-E2 component; CCO, cytochrome c oxidase, subunit I; p62, p62/sequestosome-1; LC3, microtubule-associated proteins-light chain 3β; TL, post-liver transplantation; rPBC, recurrent primary biliary cirrhosis; AR, acute cellular rejection. **b**) Expression in bile ductules. Grey column, score 1 (mild) and black, score 2 (extensive). *, $p<0.05$; **, $p<0.01$. *PDC-E2* pyruvate dehydrogenase complex-E2 component, *CCO* cytochrome c oxidase, subunit I, *p62* p62/sequestosome-1, *LC3* microtubule-associated proteins-light chain 3β, *TL* post-liver transplantation, *rPBC* recurrent primary biliary cirrhosis, *AR* acute cellular rejection

Table 2 Expression of mitochondrial, autophagy-related, and senescent markers in small bile ducts in post-transplantation samples

Case no.	Clin-diag	Mitochondrial		Autophagy-related		Senescent	
		PDC-E2	CCO	LC3	p62	p16	p21
1	rPBC	1	1	2	1	1	1
2	rPBC	1	1	2	2	1	1
3	rPBC	1	1	2	2	1	1
4	rPBC	2	2	1	1	0	1
5	rPBC	2	2	1	1	0	1
6	rPBC	1	2	0	0	0	0
7	rPBC	0	0	0	0	0	0
8	rPBC	0	0	0	0	0	0
9	rPBC	0	0	0	0	0	0
10	rPBC	0	0	0	0	0	0
11	rPBC	0	0	0	0	0	0
12	ACR	2	2	1	1	0	0
13	ACR	0	1	0	1	0	0
14	ACR	0	0	1	0	0	0
15	ACR	0	0	0	1	0	1
16	ACR	0	0	0	1	0	0
17	ACR	0	0	0	1	0	0
18	ACR	0	0	0	1	0	0
19	ACR	0	0	0	0	0	1
20	ACR	0	0	0	0	0	0
21	ACR	0	0	0	0	0	0
22	ACR	0	0	0	0	0	0
23	ACR	0	0	0	0	0	0
24	ACR	0	0	0	0	0	0
25		0	0	0	0	0	0
26		0	0	0	0	0	0
27		0	0	0	0	0	0
28		0	0	0	0	0	0

PDC-E2 pyruvate dehydrogenase complex-E2 component, *CCO* cytochrome c oxidase, subunit I, *p62* p62/sequestosome-1, *LC3* microtubule-associated proteins-light chain 3β, *N* needle biopsy, *rPBC* recurrent primary biliary cirrhosis, *ACR* acute cellular rejection

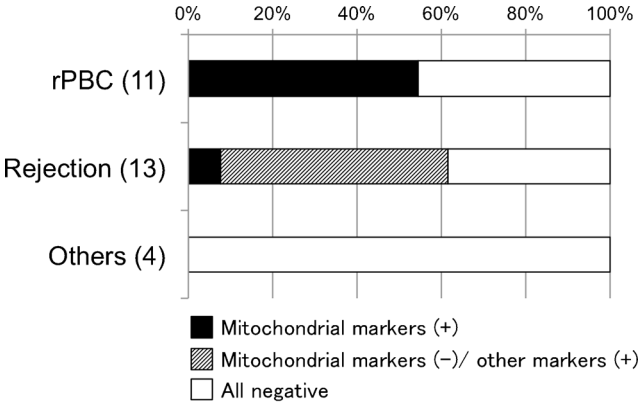


Fig. 5 Histologic diagnosis and expression pattern of mitochondrial, autophagy-related and senescent markers in small bile ducts in post-transplantation samples. *Black column*, mitochondrial markers (pyruvate dehydrogenase complex-E2component [PDC-E2] and cytochrome c oxidase, subunit I [CCO])-positive; *stripe column*, mitochondrial markers-negative and at least one of microtubule-associated proteins-light chain 3β (LC3), p62/sequestosome-1 (p62), $p16^{INK4a}$, $p21^{WAF1/Cip1}$-positive; *white column*, all negative. rPBC, Cases histologically diagnosed as recurrent primary biliary cirrhosis; Rejection; acute cellular rejection

LC3 and p62 Granular and vesicular expression of LC3 and p62 in bile ductules in some cases of recurrent PBC and acute cellular rejection (Fig. 4b) was not significantly different between recurrent PBC, acute cellular rejection, and others (Fig. 4b).

$p16^{INK4a}$ and $p21^{WAF1/Cip1}$ $p16^{INK4a}$ expression was present in bile ductules in some cases of recurrent PBC and acute cellular rejection (Fig. 4b). $p21^{WAF1/Cip1}$ expression was present in bile ductules in some post-OLT livers (Fig. 4b).

Correlation between laboratory data and marker expression

Serum AST and ALT levels correlated with the expression of LC3 in small bile ducts (AST, $p=0.028$, $r=0.479$; ALT, $p=0.035$, $r=0.462$). Serum AST and ALT levels correlated with the expression of LC3 in bile ductules (AST, $p=0.0045$, $r=0.594$; ALT, $p=0.016$, $r=0.519$) and of p21 in bile ductules (AST, $p=0.0055$, $r=0.584$; ALT, $p=0.032$, $r=0.468$). Serum AST level correlated with the expression of p16 in bile ductules ($p=0.046$, $r=0.440$).

Discussion

This study confirms that expression of mitochondrial proteins (PDC-E2 and CCO) is significantly more frequent in small bile ducts in end stage PBC, suggesting that this might be used as a diagnostic marker for end-stage PBC. We have previously demonstrated that in BECs in damaged small bile ducts in

PBC, especially in early stages, expression of mitochondrial proteins is increased due to deregulated autophagy [9]. Since all our PBC cases were end stage, the difference in frequency of staining as compared to what we reported previously is likely secondary to the lack of early stages in the present case series [9]. When present, extensive expression of mitochondrial proteins (PDC-E2 and CCO) in small bile ducts suggests PBC, even in the end stage.

The present study also clearly shows that the expression of PDC-E2 and CCO in small bile ducts may be useful as a diagnostic marker for recurrent PBC. Interestingly, most cases showing expression of mitochondrial proteins in small bile ducts were clinically and histologically diagnosed as recurrent PBC. In contrast, most cases showing in small bile ducts either LC3, p62, $p16^{INK4a}$, or $p21^{WAF1/Cip1}$ expression and no expression of PDC-E2 and CCO were diagnosed as acute cellular rejection. To distinguish recurrent PBC from rejection can be problematic because of overlapping histologic features of bile duct injury [5]. Although the presence of florid (granulomatous) bile duct lesions is regarded as presumptive evidence of PBC recurrence, florid duct lesions are only seen in around 30 % of liver biopsies from patients with PBC [5]. A few reports have considered diagnostic markers for recurrent PBC. One study suggests that epithelial–mesenchymal transition may be involved in the pathogenesis of bile duct loss during early stages of recurrent PBC [15]. Immunohistochemical studies of PDC-E2 expression in liver allografts have produced conflicting results in previous studies [16, 17]. Our study provides further evidence that extensive expression of mitochondrial proteins (PDC-E2 and CCO) in small bile ducts is a characteristic of recurrent as well as native PBC. Although one case (case no. 19 in Table 2) showed positive PDC-E2 and CCO staining, it was not histologically and clinically definitive for recurrent PBC and further follow-up is needed to clarify this issue.

We found in most cases diagnosed as acute cellular rejection in small bile ducts expression of LC3, p62, $p16^{INK4a}$, or $p21^{WAF1/Cip1}$, but not of PDC-E2 and CCO. Several studies have focused on expression of $p16^{INK4a}$ and $p21^{WAF1/Cip1}$ in small bile ducts in liver allograft rejection. In chronic liver allograft rejection as well as in PBC, both characterized by progressive loss of intrahepatic bile ducts, cellular senescence of biliary epithelial cells is reportedly critical to the mechanism of bile duct loss [18]. We also reported increased expression of $p16^{INK4a}$ and $p21^{WAF1/Cip1}$ in small bile ducts in chronic rejection [6]. Biliary epithelial senescence with increased expression of $p21^{WAF1/Cip1}$ has also been reported in acute cellular rejection [19]. The present study presents further evidence of the occurrence of biliary epithelial senescence in small bile ducts in acute and chronic rejection.

We found that small bile ducts in acute cellular rejection frequently show expression of LC3 and/or p62. Expression of LC3, p62, $p16^{INK4a}$, or $p21^{WAF1/Cip1}$ is frequently observed in damaged small bile ducts in PBC [6, 10, 7, 11, 8]. We previously reported that autophagy precedes cellular senescence in BECs in PBC [8]. In vitro studies showed that oxidative stress, starvation, and hydrophobic bile acid (GCDC) induce autophagy and deregulated autophagy in BECs [12, 8]. Taken together, these data suggest that in the liver allograft bile duct injury by acute cellular and chronic rejection deregulates autophagy and induces subsequent cellular senescence. Although bile duct injury in terms of deregulated autophagy and cellular senescence resembles that in PBC, our study shows that in acute cellular and chronic rejection expression of mitochondrial proteins is different from that in PBC. We hypothesize that different mechanisms may be involved in the pathogenesis of bile duct injury in acute and chronic rejection as compared to those operative in PBC.

We noted that in post-OLT livers AST and ALT levels significantly correlated with expression of LC3 in small bile ducts and bile ductules and with the expression of $p21^{WAF1/Cip1}$ in bile ductules, but the responsible mechanisms remain unclear. One possibility is that severe liver injury might lead to hepatocellular damage as well as biliary epithelial autophagy/deregulated autophagy in post-OLT livers.

In conclusion, our data show that expression of mitochondrial proteins in small bile ducts is a potentially useful diagnostic marker for end-stage PBC and recurrent PBC following liver transplantation.

Acknowledgments This study was supported in part by a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Culture, Sports and Science and Technology of Japan (24590409 and 15K08341).

Conflict of interest The authors declare that they have no conflict of interest.

References

1. Portmann B, Nakanuma Y (2007) Diseases of the bile ducts. In: Burt A, BC P, LD F (eds) Pathology of the liver, 5th edn. Churchill Livingstone, London, pp 517–581
2. Kaplan MM, Gershwin ME (2005) Primary biliary cirrhosis. N Engl J Med 353(12):1261–1273. doi:10.1056/NEJMra043898
3. Lindor KD, Gershwin ME, Poupon R, Kaplan M, Bergasa NV, Heathcote EJ (2009) Primary biliary cirrhosis. Hepatology 50(1): 291–308. doi:10.1002/hep.22906
4. Nakanuma Y, Ohta G (1979) Histometric and serial section observations of the intrahepatic bile ducts in primary biliary cirrhosis. Gastroenterology 76(6):1326–1332
5. Hubscher S, Clousen A (2012) Transplantation pathology. In: Burt A, Portmann B, Ferrell L (eds) Pathology of the liver, 6th edn. Churchill Livingstone, London, pp 853–934
6. Sasaki M, Ikeda H, Haga H, Manabe T, Nakanuma Y (2005) Frequent cellular senescence in small bile ducts in primary biliary cirrhosis: a possible role in bile duct loss. J Pathol 205(4):451–459
7. Sasaki M, Ikeda H, Yamaguchi J, Nakada S, Nakanuma Y (2008) Telomere shortening in the damaged small bile ducts in primary

biliary cirrhosis reflects ongoing cellular senescence. Hepatology 48(1):186–195
8. Sasaki M, Miyakoshi M, Sato Y, Nakanuma Y (2010) Autophagy mediates the process of cellular senescence characterizing bile duct damages in primary biliary cirrhosis. Lab Invest 90(6):835–843. doi:10.1038/labinvest.2010.56
9. Sasaki M, Miyakoshi M, Sato Y, Nakanuma Y (2013) Increased expression of mitochondrial proteins associated with autophagy in biliary epithelial lesions in primary biliary cirrhosis. Liver Int : Off J Int Assoc Stud Liver 33(2):312–320. doi:10.1111/liv.12049
10. Sasaki M, Ikeda H, Sato Y, Nakanuma Y (2006) Decreased expression of Bmi1 is closely associated with cellular senescence in small bile ducts in primary biliary cirrhosis. Am J Pathol 169(3):831–845
11. Sasaki M, Ikeda H, Nakanuma Y (2008) Activation of ATM signaling pathway is involved in oxidative stress-induced expression of mito-inhibitory p21(WAF1/Cip1) in chronic non-suppurative destructive cholangitis in primary biliary cirrhosis: an immunohistochemical study. J Autoimmun 31(1):73–78
12. Sasaki M, Miyakoshi M, Sato Y, Nakanuma Y (2012) A possible involvement of p62/sequestosome-1 in the process of biliary epithelial autophagy and senescence in primary biliary cirrhosis. Liv Int 32(3):487–499. doi:10.1111/j.1478-3231.2011.02656.x
13. Nakanuma Y, Sasaki M (1989) Expression of blood-group-related antigens in the intrahepatic biliary tree and hepatocytes in normal livers and various hepatobiliary diseases. Hepatology 10:174–178
14. Roskams TA, Theise ND, Balabaud C, Bhagat G, Bhathal PS, Bioulac-Sage P, Brunt EM, Crawford JM, Crosby HA, Desmet V, Finegold MJ, Geller SA, Gouw AS, Hytiroglou P, Knisely AS, Kojiro M, Lefkowitch JH, Nakanuma Y, Olynyk JK, Park YN, Portmann B, Saxena R, Scheuer PJ, Strain AJ, Thung SN, Wanless IR, West AB (2004) Nomenclature of the finer branches of the biliary tree: canals, ductules, and ductular reactions in human livers. Hepatology 39(6):1739–1745
15. Robertson H, Kirby JA, Yip WW, Jones DE, Burt AD (2007) Biliary epithelial-mesenchymal transition in posttransplantation recurrence of primary biliary cirrhosis. Hepatology 45(4):977–981. doi:10.1002/hep.21624
16. Neuberger J, Wallace L, Joplin R, Hubscher S (1995) Hepatic distribution of E2 component of pyruvate dehydrogenase complex after transplantation. Hepatology 22(3):798–801
17. Van de Water J, Gerson LB, Ferrell LD, Lake JR, Coppel RL, Batts KP, Wiesner RH, Gershwin ME (1996) Immunohistochemical evidence of disease recurrence after liver transplantation for primary biliary cirrhosis. Hepatology 24(5):1079–1084. doi:10.1002/hep.510240517
18. Lunz JG 3rd, Contrucci S, Ruppert K, Murase N, Fung JJ, Starzl TE, Demetris AJ (2001) Replicative senescence of biliary epithelial cells precedes bile duct loss in chronic liver allograft rejection: increased expression of p21(WAF1/Cip1) as a disease marker and the influence of immunosuppressive drugs. Am J Pathol 158(4):1379–1390
19. Brain JG, Robertson H, Thompson E, Humphreys EH, Gardner A, Booth TA, Jones DE, Afford SC, von Zglinicki T, Burt AD, Kirby JA (2013) Biliary epithelial senescence and plasticity in acute cellular rejection. Am J Transplant : Off J Am Soc Transplant Am Soc Transplant Surg 13(7):1688–1702. doi:10.1111/ajt.12271