Autophagy May Precede Cellular Senescence of Bile Ductular Cells in Ductular Reaction in Primary Biliary Cirrhosis

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Autophagy may precede cellular senescence of bile ductular cells in ductular reaction in primary biliary cirrhosis

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Abstract

Background and Aim. Recent studies disclosed that autophagy facilitates the process of senescence. Given cellular senescence is involved in the pathophysiology of ductular reaction (DR) in primary biliary cirrhosis (PBC), we examined an involvement of autophagy in DRs in PBC and control livers. **Methods.** We examined immunohistochemically the expression of microtubule-associated proteins-light chain 3β (LC3) as autophagy marker, p62/sequestosome-1 (p62) as autophagy-related marker in bile ductular cells in livers taken from the patients with PBC (n=42) and control livers (n=100). The expression of senescent markers ($p16^{INK4a}$ and $p21^{WAF1/Cip1}$) in bile ductular cells and their correlation with autophagy was also evaluated. **Results.** The expression of LC3 was seen in coarse vesicles in cytoplasm of bile ductular cells and significantly more frequent in PBC of both early and advanced stages, when compared with control livers ($p<0.01$). The expression of p62 was seen as intracytoplasmic aggregates and significantly more frequent in PBC, when compared with control livers ($p<0.05$). The expression of LC3 and p62 significantly correlated each other ($p<0.01$). The expression of LC3 and p62 significantly correlated with the expression of $p16^{INK4a}$, $p21^{WAF1/Cip1}$ ($p<0.05$). **Conclusion.** Autophagy is frequently seen in bile ductular cells in DRs in PBC. Since cellular senescence of bile ductular cells is rather frequent in the advanced stage of PBC, autophagy may precede cellular senescence of bile ductular cells in DRs in PBC.

Key words: autophagy, microtubule-associated proteins-light chain 3β (LC3), p62/sequestosome-1, ductular reaction, cellular senescence, primary biliary cirrhosis

Introduction

Ductular reaction (DR) is a reactive lesion at the portal tract interface comprising increased bile ductules with an accompanying complex of stromal and inflammatory cells (1). The involvement of DR has been implicated in the pathogenesis of progressive fibrosis, regeneration and hepatocarcinogenesis in chronic liver disease (1-3). We have recently reported that bile ductular cells undergoing cellular senescence, which are characterized by the augmented expression of senescence-associated β-galactosidase (SA-β-gal), $p16^{INK4a}$ and $p21^{WAF1/Cip}$ and telomere shortening, increase in PBC along with fibrous progression, especially in PBC (4-6). Cellular senescence is a state of stable cell arrest with active metabolism and a failsafe program against a variety of cellular insults. Cellular senescence is a delayed stress response involving multiple effector mechanisms such as the DNA damage response (7) and the senescence-associated secretion phenotype (SASP)(8-11). Such senescent bile ductular cells may be involved in the progression of fibrosis of these diseases, through the secretion of SASP (4-6, 11).

Autophagy (hereafter referred to as autophagy) in human diseases has been highlighted during the last decade (12-14) and recent data demonstrated that autophagy is significantly involved in the pathophysiology of liver diseases (15-21). Macroautophagy is a genetically regulated program responsible for the turnover of cellular proteins and damaged organelles. This evolutionarily conserved process is characterized by the formation of double membrane cytosolic vesicles, autophagosomes, which sequester cytoplasmic content and

deliver it to lysosomes (12, 13, 22, 23). Autophagy can enable adaptation to stress through the degradation of cellular proteins and organelles to suppress damage, maintain metabolism, and promote cellular viability and fitness (12-14, 23). An appropriate cellular stress response is critical for maintaining tissue integrity and function and for preventing diseases. Cells respond to stress with adaptation, repair, and recovery, or are diverted into irreversible cell cycle exit (senescence) or are eliminated through programmed cell death (apoptosis)(23). Dysfunctional autophagy appears to be associated with cellular senescence (23).

Autophagy and cellular senescence are two distinct cellular responses to stress. Recent studies disclosed that autophagy facilitates the process of senescence (22). We have also reported that autophagy may precedes biliary epithelial senescence in the damaged bile ducts in primary biliary cirrhosis (PBC)(20). Although cellular senescence is involved in the pathophysiology of DR along with fibrous progression in primary biliary cirrhosis (PBC) (4-6), there has been no study reporting the involvement of autophagy in ductular cells in PD. In the present study, we examined an involvement of autophagy and its association with cellular senescence in DRs in PBC and control livers.

Materials and Methods

Classification of intrahepatic biliary tree. The intrahepatic biliary tree is classified into the intrahepatic large and small bile ducts (septal and interlobular bile ducts) by their size and distributions in the portal tracts (24). In this study, septal and interlobular bile ducts are

termed small bile ducts. Bile ductules are not included in the small bile ducts. Bile ductules are characterized by tubular or glandular structures with poorly defined lumen and located at the periphery of the portal tracts and are not accompanied by parallel running hepatic arterial branches (1, 24). Ductular cells in ductular reaction (1) including intermediate hepatobiliary cells with heterogeneous phenotype in the diseased liver were also evaluated

Liver tissue preparation. A total of 142 liver tissue specimens (all were biopsied or surgically-resected) were collected from the liver disease file of our laboratory and affiliated hospitals. The liver specimens enrolled in this study were 42 PBC, 41 chronic viral hepatitis (CVH) livers, 27 nonalcoholic steatohepatitis (NASH), 10 extrahepatic biliary obstruction (EBO) livers and 22 “histologically normal” livers. All PBC were from the patients fulfilling the clinical, serological and histological characteristics consistent with the diagnosis of PBC (25). PBC livers were staged histologically (26) and 29 and 13 of PBC were of stages 1, 2 (early PBC) and of stages 3, 4 (advanced PBC), respectively. Twenty-seven CVH were regarded as F0-2 and 14 as F3, 4, respectively (27). Three and 38 of CVH cases were serologically positive for hepatitis B surface antigen (HBsAg) and anti-hepatitis C viral antibody (HCVAb), respectively. The grade of activity and stage in the patients with NASH were assessed by using the criteria proposed by Brunt et al. (28), and 14 and 13 NASH were regarded as stages 1, 2 and stages 3, 4, respectively. Causes of EBO were obstruction of the bile duct at the hepatic hilum or the extrahepatic bile ducts, because of carcinoma or stone, and the duration of jaundice was less than 1 month. “Histologically normal” livers were

obtained from surgically resected livers for traumatic hepatic rupture or metastatic liver tumor. The liver tissues used were taken from the part sufficiently away from the trauma and tumor.

Liver tissue samples were fixed in 10% neutral buffered formalin, and embedded in paraffin. More than twenty serial sections, 4 μm thick, were cut from each block. Several were processed routinely for histopathologic study, and the remainder was processed for the following immunohistochemistry. The Committee of Ethics in Kanazawa University approved this study.

Immunohistochemistry. We examined immunohistochemically the expression of microtubule-associated proteins-light chain 3β (LC3) as an autophagy marker, p62/sequestosome-1 (p62)(29, 30) as an autophagy-related marker in bile ductular cells. The expression of senescent markers ($p16^{INK4a}$ and $p21^{WAF1/Cip1}$) in bile ductular cells and their correlation with autophagy was also examined in same sample series. Immunostaining was performed using the antibodies shown in Table 1, as described previously (31). In brief, after pretreatment for antigen retrieval as described in Table 1, blocking endogenous peroxidase, the sections were incubated with the primary antibody at 4 degree 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 Chemica, Co., St. Louis, MO) and H_2O_2. The sections were then lightly counterstained with methyl green or hematoxylin. A similar dilution of the control mouse IgG (Dako) was applied instead of the

primary antibody as negative control. Positive and negative controls were routinely included.

Assessment of immunostaining. All fields of each liver specimen were observed under light microscope for the evaluation of immunohistochemical expression of LC3, p62, $p16^{INK4a}$ and $p21^{WAF1/Cip1}$. The extent of expression was semiquantitatively evaluated as 1+ (focal, positive cells are detected in one third or fewer portal tracts), and 2+ (extensive, positive cells are detected in more than one third of portal tracts).

Statistical analysis. Statistical analysis for the difference used the Wilcoxon rank sum test. The correlation coefficient of 2 factors was evaluated using Spearman's rank correlation test. When the *P* value was less than 0.05, the difference was regarded as significant.

Results

LC3. The expression of LC3 was seen in coarse vesicles in the cytoplasm of bile ductular cells, when detectable (Fig.1A). As shown in Figure 2A, the expression of LC3 was more frequent in ductular cells in the early stages of PBC (1+, 50%; 2+, 15%) and the advanced stages of PBC (1+, 23%; 2+, 31%), compared to other groups; CVH, F1/2 (1+, 6.7%; 2+, 0%), CVH, F3/4 (1+, 18%; 2+, 0%), NASH stages 1 and 2 (1+, 0%; 2+, 0%), NASH stages 3 and 4 (1+, 8.3%; 2+, 0%), EBO (1+, 10%; 2+, 0%) and normal livers (1+, 0%; 2+, 0%), respectively

($p<0.01$). The expression of LC3 in CVH, F3/4 was significantly more frequent, compared to normal livers ($p<0.05$).

p62. The expression of p62 was seen as intracytoplasmic aggregates in bile ductular cells, when detectable (Fig.1B). As shown in Figure 2B, the expression of p62 was more frequent in ductular cells in the early stages of PBC (1+, 45%; 2+, 17%) and the advanced stages of PBC (1+, 31%; 2+, 31v%), compared to NASH stages 1 and 2 (1+, 0%; 2+, 0%) and normal livers (1+, 9%; 2+, 0%), respectively ($p<0.01$). The expression of p62 was more frequent in the early and advanced stages of PBC, compared to CVH, F1/2 (1+, 33%; 2+, 0%), CVH, F3/4 (1+, 14%; 2+, 7.1%), respectively ($p<0.05$).

The correlation between expressions of LC3, p62, $p16^{INK4a}$ and $p21^{WAF1/Cip1}$.

The expression of LC3 and p62 ($p<0.01$, rs=0.38703) were significantly correlated in bile ductular cells (Fig.2C). The expression of $p16^{INK4a}$ and $p21^{WAF1/Cip1}$ were significantly more frequent in bile ductular cells in PBC, stage 3, 4, when compared with control livers ($p<0.05$), as previously reported (5, 6). There were significant correlation between the expression of LC3 and $p16^{INK4a}$ ($p<0.01$, rs=0.574), the expression of LC3 and $p21^{WAF1/Cip1}$ ($p<0.01$, rs=0.462), the expression of p62 and $p16^{INK4a}$ ($p<0.01$, rs=0.336), the expression of p62 and $p21^{WAF1/Cip1}$ ($p<0.05$, rs=0.285) (Figs 3A and 3B).

Discussion

The data obtained in this study are summarized as follows; 1) LC3, an autophagy marker, and p62, an autophagy-related marker, were frequently expressed in ductular cells in DR in PBC, compared to those in CVH, NASH, EBO and normal livers. 2) The expression of LC3 and p62 was significantly correlated each other in ductular cells in DR. 3) The expression of LC3 and p62 was significantly correlated with the expression of senescent markers: $p16^{INK4a}$ and $p21^{WAF1/Cip1}$ in ductular cells in DR.

The present study firstly disclosed that ductular cells in DR frequently show autophagy detected by immunostaining for LC3 in PBC, whereas autophagy is infrequently detected in DR in control livers. This finding clearly indicates that autophagy is involved in the pathogenesis of DR in PBC and DR in PBC may be different from other liver disease. We have shown that autophagy is upregulated in the damage bile ducts in PBC (20). Therefore, the autophagy appears to be a common feature of biliary epithelial cells in small bile ducts and DR in PBC.

Furthermore, it is of interest that the accumulation of p62 is frequently seen in DR in PBC, similarly to LC3. p62 is an adaptor protein involved in the delivery of ubiquitin-bound cargo to the autophagosome and regulates the formation of protein aggregates (29, 32-34). An accumulation of p62 is seen in autphagy-deficient condition, so, the accumulation of p62 may be a marker of dysfunctional autophagy in which the capacity of autophagy is not much enough to process the damaged proteins bound to p62(19, 21, 35).

Therefore, the accumulation of p62 may reflect dysfunctional autophagy in ductular cells in DR in PBC. In addition, the expression of LC3 and p62 was significantly correlated each other in ductular cells in DR. Taken together, autophagy, especially dysfunctional autophagy may be involved in the pathophysiology of ductular cells in PBC.

Recent studies have disclosed that autophagy preceded and accelerated cellular senescence (22) and we have also reported that autophagy mediates biliary epithelial senescence (20). Our previous study shows that some of ductular cells in DR in chronic liver diseases were at G1- arrest and undergoing cellular senescence and that such senescent cells may be involved in the progression of fibrosis of these diseases, particularly in PBC (6). The present study revealed that the expression of autophagy markers: LC3 and p62 was significantly correlated with the expression of senescent markers: $p16^{INK4a}$ and $p21^{WAF1/Cip1}$ in ductular cells in DR. This finding suggests that autophagy is involved in the biliary epithelial senescence in DR. Dysfunctional autphagy may induce cellular senescence. It is of interest that autophagy is upregulated in DR in both early and advanced PBC, whereas cellular senescence is more frequent in DR in the advanced PBC in our previous study. This may also support hypothesis that biliary epithelial senescence may be induced via process of autophagy. Taken together, the regulation of autophagy may be a therapeutic target to prevent the progression of fibrosis along with biliary epithelial senescence in PBC.

In conclusion, autophagy is frequently seen and correlated with cellular senescene in bile ductular cells in DRs in PBC, both in the early and advanced stage. These findings

suggest that autophagy may be involved in the pathophysiology of DRs in PBC and may precede cellular senescence of bile ductular cells in DRs in PBC. Since cellular senescence of bile ductular cells is rather frequent in the adanced stage of PBC, autophagy may precede cellular senescence of bile ductular cells in DRs in PBC.

References

1. Roskams TA, Theise ND, Balabaud C, et al. Nomenclature of the finer branches of the biliary tree: canals, ductules, and ductular reactions in human livers. *Hepatology*. 2004;39(6):1739-45.

2. Richardson MM, Jonsson JR, Powell EE, et al. Progressive fibrosis in nonalcoholic steatohepatitis: association with altered regeneration and a ductular reaction. *Gastroenterology*. 2007;133(1):80-90.

3. Clouston AD, Powell EE, Walsh MJ, Richardson MM, Demetris AJ, Jonsson JR. Fibrosis correlates with a ductular reaction in hepatitis C: roles of impaired replication, progenitor cells and steatosis. *Hepatology*. 2005;41(4):809-18.

4. Sasaki M, Ikeda H, Haga H, Manabe T, Nakanuma Y. Frequent cellular senescence in small bile ducts in primary biliary cirrhosis: a possible role in bile duct loss. *J Pathol*. 2005;205(4):451-9.

5. Sasaki M, Ikeda H, Yamaguchi J, Nakada S, Nakanuma Y. Telomere shortening in the damaged small bile ducts in primary biliary cirrhosis reflects ongoing cellular senescence. *Hepatology*. 2008;48(1):186-95.

6. Sasaki M, Ikeda H, Yamaguchi J, Miyakoshi M, Sato Y, Nakanuma Y. Bile ductular cells undergoing cellular senescence increase in chronic liver diseases along with fibrous progression. *Am J Clin Pathol*. 2010;133(2):212-23. doi:133/2/212 [pii]
10.1309/AJCPWMX47TREYWZG

7. Bartkova J, Rezaei N, Liontos M, et al. Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. *Nature*. 2006;444(7119):633-7. doi:nature05268 [pii]
10.1038/nature05268

8. Acosta JC, O'Loghlen A, Banito A, et al. Chemokine signaling via the CXCR2 receptor reinforces senescence. *Cell*. 2008;133(6):1006-18.

9. Kuilman T, Michaloglou C, Vredeveld LC, et al. Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. *Cell*. 2008;133(6):1019-31.

10. Wajapeyee N, Serra RW, Zhu X, Mahalingam M, Green MR. Oncogenic BRAF induces senescence and apoptosis through pathways mediated by the secreted protein

IGFBP7. *Cell*. 2008;132(3):363-74.

11. Sasaki M, Miyakoshi M, Sato Y, Nakanuma Y. Modulation of the microenvironment by senescent biliary epithelial cells may be involved in the pathogenesis of primary biliary cirrhosis. *J Hepatol*. 2010;53(2):318-25. doi:S0168-8278(10)00324-7 [pii]
10.1016/j.jhep.2010.03.008

12. Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. *Nature*. 2008;451(7182):1069-75. doi:nature06639 [pii]
10.1038/nature06639

13. Ohsumi Y. Molecular dissection of autophagy: two ubiquitin-like systems. *Nat Rev Mol Cell Biol*. 2001;2(3):211-6. doi:10.1038/35056522

14. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. *Cell*. 2008;132(1):27-42. doi:S0092-8674(07)01685-6 [pii]
10.1016/j.cell.2007.12.018

15. Yin XM, Ding WX, Gao W. Autophagy in the liver. *Hepatology*. 2008;47(5):1773-85. doi:10.1002/hep.22146

16. Teckman JH, An JK, Blomenkamp K, Schmidt B, Perlmutter D. Mitochondrial autophagy and injury in the liver in alpha 1-antitrypsin deficiency. *Am J Physiol Gastrointest Liver Physiol*. 2004;286(5):G851-62. doi:10.1152/ajpgi.00175.2003
00175.2003 [pii]

17. Wang Y, Singh R, Xiang Y, Czaja MJ. Macroautophagy and chaperone-mediated autophagy are required for hepatocyte resistance to oxidant stress. *Hepatology*. 2010;52(1):266-77. doi:10.1002/hep.23645

18. Singh R, Kaushik S, Wang Y, et al. Autophagy regulates lipid metabolism. *Nature*. 2009;458(7242):1131-5. doi:nature07976 [pii]
10.1038/nature07976

19. Komatsu M, Kurokawa H, Waguri S, et al. The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. *Nat Cell Biol*. 2010;12(3):213-23. doi:ncb2021 [pii]
10.1038/ncb2021

20. Sasaki M, Miyakoshi M, Sato Y, Nakanuma Y. Autophagy mediates the process

of cellular senescence characterizing bile duct damages in primary biliary cirrhosis. *Lab Invest*. 2010;90(6):835-43. doi:labinvest201056 [pii]
10.1038/labinvest.2010.56

21. Mathew R, Karp CM, Beaudoin B, et al. Autophagy suppresses tumorigenesis through elimination of p62. *Cell*. 2009;137(6):1062-75. doi:S0092-8674(09)00391-2 [pii]
10.1016/j.cell.2009.03.048

22. Young AR, Narita M, Ferreira M, et al. Autophagy mediates the mitotic senescence transition. *Genes Dev*. 2009;23(7):798-803. doi:gad.519709 [pii]
10.1101/gad.519709

23. White E, Lowe SW. Eating to exit: autophagy-enabled senescence revealed. *Genes Dev*. 2009;23(7):784-7. doi:23/7/784 [pii]
10.1101/gad.1795309

24. Nakanuma Y, Sasaki M. Expression of blood-group-related antigens in the intrahepatic biliary tree and hepatocytes in normal livers and various hepatobiliary diseases. *Hepatology*. 1989;10:174-8.

25. Portmann B, Nakanuma Y. Diseases of the bile ducts. In: MacSween R, Burt A, BC P, Ishak K, Scheuer P, Anthony P, eds. *Pathology of the Liver*. 4th Eds ed. London: Churchill Livingstone; 2001. p. 435-506.

26. Ludwig J. Small-duct primary sclerosing cholangitis. *Semin Liver Dis*. 1991;11(1):11-7.

27. Desmet V, Gerber M, Hoofnagle J, Manns M, Scheuer P. Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology*. 1994;19:1513-20.

28. Brunt EM, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. *Am J Gastroenterol*. 1999;94(9):2467-74.

29. Bjorkoy G, Lamark T, Brech A, et al. p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *J Cell Biol*. 2005;171(4):603-14. doi:jcb.200507002 [pii]
10.1083/jcb.200507002

30. Komatsu M, Waguri S, Koike M, et al. Homeostatic levels of p62 control cytoplasmic inclusion body formation in autophagy-deficient mice. *Cell*.

2007;131(6):1149-63. doi:S0092-8674(07)01354-2 [pii]
10.1016/j.cell.2007.10.035

31. Sasaki M, Ikeda H, Sato Y, Nakanuma Y. Decreased expression of Bmi1 is closely associated with cellular senescence in small bile ducts in primary biliary cirrhosis. *Am J Pathol*. 2006;169(3):831-45.

32. Lamark T, Kirkin V, Dikic I, Johansen T. NBR1 and p62 as cargo receptors for selective autophagy of ubiquitinated targets. *Cell Cycle*. 2009;8(13):1986-90. doi:8892 [pii]

33. Ichimura Y, Kumanomidou T, Sou YS, et al. Structural basis for sorting mechanism of p62 in selective autophagy. *J Biol Chem*. 2008;283(33):22847-57. doi:M802182200 [pii]
10.1074/jbc.M802182200

34. Pankiv S, Clausen TH, Lamark T, et al. p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *J Biol Chem*. 2007;282(33):24131-45. doi:M702824200 [pii]
10.1074/jbc.M702824200

35. Monick MM, Powers LS, Walters K, et al. Identification of an autophagy defect in smokers' alveolar macrophages. *J Immunol*. 2010;185(9):5425-35. doi:jimmunol.1001603 [pii]
10.4049/jimmunol.1001603

Figure legends

1. Expression of LC3 and p62 in ductular reaction (DR) in PBC and control liver.

A) Coarse vesicular expression of LC3 in DRs in PBC. Coarse vesicular expression of LC3 is seen in the cytoplasm of ductular cells in PBC (left, arrows), whereas no expression of LC3 is found in ductular cells in CVH, C (right, arrows). Immunostaining for LC3. Original magnification, x400. B) Coarse vesicular expression of p62 is seen in the cytoplasm of ductular cells in PBC (left, arrows), whereas no or faint expression of p62 is found in ductular cells in CVH, C (right, arrows). Immunostaining for p62. Original magnification, x400.

2. Increased expression of LC3 and p62 in ductular reaction (DR) in PBC.

A) The frequency and extent of LC3 expression. White column, focal expression (1+), gray columns, extensive expression (2+). a, $p<0.01$ vs other groups, b, $p<0.05$ vs normal liver (NL). B) The frequency and extent of p62 expression. White column, focal expression (1+), gray columns, extensive expression (2+). a, $p<0.01$ vs normal liver (NL) and nonalcoholic steatohepatitis (NASH), stage (st)1 and 2, b, $p<0.05$ vs chronic viral hepatitis (CVH), F1 and F2 and F3 and F4. C) Correlation between expression of LC3 and p62 in ductular reaction (DR) in primary biliary cirrhosis (PBC) and control diseases. In PBC, both molecules are expressed frequently and extensively, while expression of these two molecules are infrequent or focal in control livers (chronic viral hepatitis (CVH) and extrahepatic biliary obstruction (EBO)). There is a statistical correlation in the

distribution of these two molecules ($p<0.01$, $rs=0.558$).

3. Correlation between autophagy markers and senescent markers.

A) Correlation between expression of LC3 and p16 in ductular reaction (DR) in primary biliary cirrhosis (PBC) and control diseases. In PBC, both molecules are expressed frequently and extensively, while expression of these two molecules are infrequent or focal in control livers (chronic viral hepatitis (CVH) and extrahepatic biliary obstruction (EBO)). There is a statistical correlation in the distribution of these two molecules ($p<0.01$, $rs=0.574$).

B) Correlation between expression of LC3 and p21 in ductular reaction (DR) in primary biliary cirrhosis (PBC) and control diseases. In PBC, both molecules are expressed frequently and extensively, while expression of these two molecules are infrequent or focal in control livers (CVH and EBO). There is a statistical correlation in the distribution of these two molecules ($p<0.01$, $rs=0.462$).

C) Correlation between expression of p62 and p16 in ductular reaction (DR) in primary biliary cirrhosis (PBC) and control diseases. In PBC, both molecules are expressed frequently and extensively, while expression of these two molecules are infrequent or focal in control livers (CVH and EBO). There is a statistical correlation in the distribution of these two molecules ($p<0.01$, $rs=0.335$).

D) Correlation between expression of p62 and p21 in ductular reaction (DR) in primary

biliary cirrhosis (PBC) and control diseases. In PBC, both molecules are expressed frequently and extensively, while expression of these two molecules are infrequent or focal in control livers (CVH and EBO). There is a statistical correlation in the distribution of these two molecules ($p<0.05$, $rs=0.285$).

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Abstract

Background and Aim. Recent studies disclosed that autophagy facilitates the process of senescence. Given cellular senescence is involved in the pathophysiology of ductular reaction (DR) in primary biliary cirrhosis (PBC), we examined an involvement of autophagy in DRs in PBC and control livers. **Methods.** We examined immunohistochemically the expression of microtubule-associated proteins-light chain 3β (LC3) as autophagy marker, p62/sequestosome-1 (p62) as autophagy-related marker in bile ductular cells in livers taken from the patients with PBC (n=42) and control livers (n=100). The expression of senescent markers ($p16^{INK4a}$ and $p21^{WAF1/Cip1}$) in bile ductular cells and their correlation with autophagy was also evaluated. **Results.** The expression of LC3 was seen in coarse vesicles in cytoplasm of bile ductular cells and significantly more frequent in PBC of both early and advanced stages, when compared with control livers ($p<0.01$). The expression of p62 was seen as intracytoplasmic aggregates and significantly more frequent in PBC, when compared with control livers ($p<0.05$). The expression of LC3 and p62 significantly correlated each other ($p<0.01$). The expression of LC3 and p62 significantly correlated with the expression of $p16^{INK4a}$, $p21^{WAF1/Cip1}$ ($p<0.05$). **Conclusion.** Autophagy is frequently seen in bile ductular cells in DRs in PBC. Since cellular senescence of bile ductular cells is rather frequent in the advanced stage of PBC, autophagy may precede cellular senescence of bile ductular cells in DRs in PBC.

Key words: autophagy, microtubule-associated proteins-light chain 3β (LC3), p62/sequestosome-1, ductular reaction, cellular senescence, primary biliary cirrhosis

Introduction

Ductular reaction (DR) is a reactive lesion at the portal tract interface comprising increased bile ductules with an accompanying complex of stromal and inflammatory cells (1). The involvement of DR has been implicated in the pathogenesis of progressive fibrosis, regeneration and hepatocarcinogenesis in chronic liver disease (1-3). We have recently reported that bile ductular cells undergoing cellular senescence, which are characterized by the augmented expression of senescence-associated β-galactosidase (SA-β-gal), $p16^{INK4a}$ and $p21^{WAF1/Cip}$ and telomere shortening, increase in PBC along with fibrous progression, especially in PBC (4-6). Cellular senescence is a state of stable cell arrest with active metabolism and a failsafe program against a variety of cellular insults. Cellular senescence is a delayed stress response involving multiple effector mechanisms such as the DNA damage response (7) and the senescence-associated secretion phenotype (SASP)(8-11). Such senescent bile ductular cells may be involved in the progression of fibrosis of these diseases, through the secretion of SASP (4-6, 11).

Autophagy (hereafter referred to as autophagy) in human diseases has been highlighted during the last decade (12-14) and recent data demonstrated that autophagy is significantly involved in the pathophysiology of liver diseases (15-21). Macroautophagy is a genetically regulated program responsible for the turnover of cellular proteins and damaged organelles. This evolutionarily conserved process is characterized by the formation of double membrane cytosolic vesicles, autophagosomes, which sequester cytoplasmic content and

deliver it to lysosomes (12, 13, 22, 23). Autophagy can enable adaptation to stress through the degradation of cellular proteins and organelles to suppress damage, maintain metabolism, and promote cellular viability and fitness (12-14, 23). An appropriate cellular stress response is critical for maintaining tissue integrity and function and for preventing diseases. Cells respond to stress with adaptation, repair, and recovery, or are diverted into irreversible cell cycle exit (senescence) or are eliminated through programmed cell death (apoptosis)(23). Dysfunctional autophagy appears to be associated with cellular senescence (23).

Autophagy and cellular senescence are two distinct cellular responses to stress. Recent studies disclosed that autophagy facilitates the process of senescence (22). We have also reported that autophagy may precedes biliary epithelial senescence in the damaged bile ducts in primary biliary cirrhosis (PBC)(20). Although cellular senescence is involved in the pathophysiology of DR along with fibrous progression in primary biliary cirrhosis (PBC) (4-6), there has been no study reporting the involvement of autophagy in ductular cells in PD. In the present study, we examined an involvement of autophagy and its association with cellular senescence in DRs in PBC and control livers.

Materials and Methods

Classification of intrahepatic biliary tree. The intrahepatic biliary tree is classified into the intrahepatic large and small bile ducts (septal and interlobular bile ducts) by their size and distributions in the portal tracts (24). In this study, septal and interlobular bile ducts are

termed small bile ducts. Bile ductules are not included in the small bile ducts. Bile ductules are characterized by tubular or glandular structures with poorly defined lumen and located at the periphery of the portal tracts and are not accompanied by parallel running hepatic arterial branches (1, 24). Ductular cells in ductular reaction (1) including intermediate hepatobiliary cells with heterogeneous phenotype in the diseased liver were also evaluated

Liver tissue preparation. A total of 142 liver tissue specimens (all were biopsied or surgically-resected) were collected from the liver disease file of our laboratory and affiliated hospitals. The liver specimens enrolled in this study were 42 PBC, 41 chronic viral hepatitis (CVH) livers, 27 nonalcoholic steatohepatitis (NASH), 10 extrahepatic biliary obstruction (EBO) livers and 22 “histologically normal” livers. All PBC were from the patients fulfilling the clinical, serological and histological characteristics consistent with the diagnosis of PBC (25). PBC livers were staged histologically (26) and 29 and 13 of PBC were of stages 1, 2 (early PBC) and of stages 3, 4 (advanced PBC), respectively. Twenty-seven CVH were regarded as F0-2 and 14 as F3, 4, respectively (27). Three and 38 of CVH cases were serologically positive for hepatitis B surface antigen (HBsAg) and anti-hepatitis C viral antibody (HCVAb), respectively. The grade of activity and stage in the patients with NASH were assessed by using the criteria proposed by Brunt et al. (28), and 14 and 13 NASH were regarded as stages 1, 2 and stages 3, 4, respectively. Causes of EBO were obstruction of the bile duct at the hepatic hilum or the extrahepatic bile ducts, because of carcinoma or stone, and the duration of jaundice was less than 1 month. “Histologically normal” livers were

obtained from surgically resected livers for traumatic hepatic rupture or metastatic liver tumor. The liver tissues used were taken from the part sufficiently away from the trauma and tumor.

Liver tissue samples were fixed in 10% neutral buffered formalin, and embedded in paraffin. More than twenty serial sections, 4 μm thick, were cut from each block. Several were processed routinely for histopathologic study, and the remainder was processed for the following immunohistochemistry. The Committee of Ethics in Kanazawa University approved this study.

Immunohistochemistry. We examined immunohistochemically the expression of microtubule-associated proteins-light chain 3β (LC3) as an autophagy marker, p62/sequestosome-1 (p62)(29, 30) as an autophagy-related marker in bile ductular cells. The expression of senescent markers ($p16^{INK4a}$ and $p21^{WAF1/Cip1}$) in bile ductular cells and their correlation with autophagy was also examined in same sample series. Immunostaining was performed using the antibodies shown in Table 1, as described previously (31). In brief, after pretreatment for antigen retrieval as described in Table 1, blocking endogenous peroxidase, the sections were incubated with the primary antibody at 4 degree 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 Chemica, Co., St. Louis, MO) and H_2O_2. The sections were then lightly counterstained with methyl green or hematoxylin. A similar dilution of the control mouse IgG (Dako) was applied instead of the

primary antibody as negative control. Positive and negative controls were routinely included.

Assessment of immunostaining. All fields of each liver specimen were observed under light microscope for the evaluation of immunohistochemical expression of LC3, p62, $p16^{INK4a}$ and $p21^{WAF1/Cip1}$. The extent of expression was semiquantitatively evaluated as 1+ (focal, positive cells are detected in one third or fewer portal tracts), and 2+ (extensive, positive cells are detected in more than one third of portal tracts).

Statistical analysis. Statistical analysis for the difference used the Wilcoxon rank sum test. The correlation coefficient of 2 factors was evaluated using Spearman's rank correlation test. When the *P* value was less than 0.05, the difference was regarded as significant.

Results

LC3. The expression of LC3 was seen in coarse vesicles in the cytoplasm of bile ductular cells, when detectable (Fig.1A). As shown in Figure 2A, the expression of LC3 was more frequent in ductular cells in the early stages of PBC (1+, 50%; 2+, 15%) and the advanced stages of PBC (1+, 23%; 2+, 31%), compared to other groups; CVH, F1/2 (1+, 6.7%; 2+, 0%), CVH, F3/4 (1+, 18%; 2+, 0%), NASH stages 1 and 2 (1+, 0%; 2+, 0%), NASH stages 3 and 4 (1+, 8.3%; 2+, 0%), EBO (1+, 10%; 2+, 0%) and normal livers (1+, 0%; 2+, 0%), respectively

($p<0.01$). The expression of LC3 in CVH, F3/4 was significantly more frequent, compared to normal livers ($p<0.05$).

p62. The expression of p62 was seen as intracytoplasmic aggregates in bile ductular cells, when detectable (Fig.1B). As shown in Figure 2B, the expression of p62 was more frequent in ductular cells in the early stages of PBC (1+, 45%; 2+, 17%) and the advanced stages of PBC (1+, 31%; 2+, 31v%), compared to NASH stages 1 and 2 (1+, 0%; 2+, 0%) and normal livers (1+, 9%; 2+, 0%), respectively ($p<0.01$). The expression of p62 was more frequent in the early and advanced stages of PBC, compared to CVH, F1/2 (1+, 33%; 2+, 0%), CVH, F3/4 (1+, 14%; 2+, 7.1%), respectively ($p<0.05$).

The correlation between expressions of LC3, p62, $p16^{INK4a}$ and $p21^{WAF1/Cip1}$.

The expression of LC3 and p62 ($p<0.01$, rs=0.38703) were significantly correlated in bile ductular cells (Fig.2C). The expression of $p16^{INK4a}$ and $p21^{WAF1/Cip1}$ were significantly more frequent in bile ductular cells in PBC, stage 3, 4, when compared with control livers ($p<0.05$), as previously reported (5, 6). There were significant correlation between the expression of LC3 and $p16^{INK4a}$ ($p<0.01$, rs=0.574), the expression of LC3 and $p21^{WAF1/Cip1}$ ($p<0.01$, rs=0.462), the expression of p62 and $p16^{INK4a}$ ($p<0.01$, rs=0.336), the expression of p62 and $p21^{WAF1/Cip1}$ ($p<0.05$, rs=0.285) (Figs 3A and 3B).

Discussion

The data obtained in this study are summarized as follows; 1) LC3, an autophagy marker, and p62, an autophagy-related marker, were frequently expressed in ductular cells in DR in PBC, compared to those in CVH, NASH, EBO and normal livers. 2) The expression of LC3 and p62 was significantly correlated each other in ductular cells in DR. 3) The expression of LC3 and p62 was significantly correlated with the expression of senescent markers: $p16^{INK4a}$ and $p21^{WAF1/Cip1}$ in ductular cells in DR.

The present study firstly disclosed that ductular cells in DR frequently show autophagy detected by immunostaining for LC3 in PBC, whereas autophagy is infrequently detected in DR in control livers. This finding clearly indicates that autophagy is involved in the pathogenesis of DR in PBC and DR in PBC may be different from other liver disease. We have shown that autophagy is upregulated in the damage bile ducts in PBC (20). Therefore, the autophagy appears to be a common feature of biliary epithelial cells in small bile ducts and DR in PBC.

Furthermore, it is of interest that the accumulation of p62 is frequently seen in DR in PBC, similarly to LC3. p62 is an adaptor protein involved in the delivery of ubiquitin-bound cargo to the autophagosome and regulates the formation of protein aggregates (29, 32-34). An accumulation of p62 is seen in autphagy-deficient condition, so, the accumulation of p62 may be a marker of dysfunctional autophagy in which the capacity of autophagy is not much enough to process the damaged proteins bound to p62(19, 21, 35).

Therefore, the accumulation of p62 may reflect dysfunctional autophagy in ductular cells in DR in PBC. In addition, the expression of LC3 and p62 was significantly correlated each other in ductular cells in DR. Taken together, autophagy, especially dysfunctional autophagy may be involved in the pathophysiology of ductular cells in PBC.

Recent studies have disclosed that autophagy preceded and accelerated cellular senescence (22) and we have also reported that autophagy mediates biliary epithelial senescence (20). Our previous study shows that some of ductular cells in DR in chronic liver diseases were at G1- arrest and undergoing cellular senescence and that such senescent cells may be involved in the progression of fibrosis of these diseases, particularly in PBC (6). The present study revealed that the expression of autophagy markers: LC3 and p62 was significantly correlated with the expression of senescent markers: $p16^{INK4a}$ and $p21^{WAF1/Cip1}$ in ductular cells in DR. This finding suggests that autophagy is involved in the biliary epithelial senescence in DR. Dysfunctional autphagy may induce cellular senescence. It is of interest that autophagy is upregulated in DR in both early and advanced PBC, whereas cellular senescence is more frequent in DR in the advanced PBC in our previous study. This may also support hypothesis that biliary epithelial senescence may be induced via process of autophagy. Taken together, the regulation of autophagy may be a therapeutic target to prevent the progression of fibrosis along with biliary epithelial senescence in PBC.

In conclusion, autophagy is frequently seen and correlated with cellular senescene in bile ductular cells in DRs in PBC, both in the early and advanced stage. These findings

suggest that autophagy may be involved in the pathophysiology of DRs in PBC and may precede cellular senescence of bile ductular cells in DRs in PBC. Since cellular senescence of bile ductular cells is rather frequent in the adanced stage of PBC, autophagy may precede cellular senescence of bile ductular cells in DRs in PBC.

References

1. Roskams TA, Theise ND, Balabaud C, et al. Nomenclature of the finer branches of the biliary tree: canals, ductules, and ductular reactions in human livers. *Hepatology*. 2004;39(6):1739-45.

2. Richardson MM, Jonsson JR, Powell EE, et al. Progressive fibrosis in nonalcoholic steatohepatitis: association with altered regeneration and a ductular reaction. *Gastroenterology*. 2007;133(1):80-90.

3. Clouston AD, Powell EE, Walsh MJ, Richardson MM, Demetris AJ, Jonsson JR. Fibrosis correlates with a ductular reaction in hepatitis C: roles of impaired replication, progenitor cells and steatosis. *Hepatology*. 2005;41(4):809-18.

4. Sasaki M, Ikeda H, Haga H, Manabe T, Nakanuma Y. Frequent cellular senescence in small bile ducts in primary biliary cirrhosis: a possible role in bile duct loss. *J Pathol*. 2005;205(4):451-9.

5. Sasaki M, Ikeda H, Yamaguchi J, Nakada S, Nakanuma Y. Telomere shortening in the damaged small bile ducts in primary biliary cirrhosis reflects ongoing cellular senescence. *Hepatology*. 2008;48(1):186-95.

6. Sasaki M, Ikeda H, Yamaguchi J, Miyakoshi M, Sato Y, Nakanuma Y. Bile ductular cells undergoing cellular senescence increase in chronic liver diseases along with fibrous progression. *Am J Clin Pathol*. 2010;133(2):212-23. doi:133/2/212 [pii]
10.1309/AJCPWMX47TREYWZG

7. Bartkova J, Rezaei N, Liontos M, et al. Oncogene-induced senescence is part of the tumorigenesis barrier imposed by DNA damage checkpoints. *Nature*. 2006;444(7119):633-7. doi:nature05268 [pii]
10.1038/nature05268

8. Acosta JC, O'Loghlen A, Banito A, et al. Chemokine signaling via the CXCR2 receptor reinforces senescence. *Cell*. 2008;133(6):1006-18.

9. Kuilman T, Michaloglou C, Vredeveld LC, et al. Oncogene-induced senescence relayed by an interleukin-dependent inflammatory network. *Cell*. 2008;133(6):1019-31.

10. Wajapeyee N, Serra RW, Zhu X, Mahalingam M, Green MR. Oncogenic

BRAF induces senescence and apoptosis through pathways mediated by the secreted protein IGFBP7. *Cell*. 2008;132(3):363-74.

11. Sasaki M, Miyakoshi M, Sato Y, Nakanuma Y. Modulation of the microenvironment by senescent biliary epithelial cells may be involved in the pathogenesis of primary biliary cirrhosis. *J Hepatol*. 2010;53(2):318-25. doi:S0168-8278(10)00324-7 [pii]
10.1016/j.jhep.2010.03.008

12. Mizushima N, Levine B, Cuervo AM, Klionsky DJ. Autophagy fights disease through cellular self-digestion. *Nature*. 2008;451(7182):1069-75. doi:nature06639 [pii]
10.1038/nature06639

13. Ohsumi Y. Molecular dissection of autophagy: two ubiquitin-like systems. *Nat Rev Mol Cell Biol*. 2001;2(3):211-6. doi:10.1038/35056522

14. Levine B, Kroemer G. Autophagy in the pathogenesis of disease. *Cell*. 2008;132(1):27-42. doi:S0092-8674(07)01685-6 [pii]
10.1016/j.cell.2007.12.018

15. Yin XM, Ding WX, Gao W. Autophagy in the liver. *Hepatology*. 2008;47(5):1773-85. doi:10.1002/hep.22146

16. Teckman JH, An JK, Blomenkamp K, Schmidt B, Perlmutter D. Mitochondrial autophagy and injury in the liver in alpha 1-antitrypsin deficiency. *Am J Physiol Gastrointest Liver Physiol*. 2004;286(5):G851-62. doi:10.1152/ajpgi.00175.2003
00175.2003 [pii]

17. Wang Y, Singh R, Xiang Y, Czaja MJ. Macroautophagy and chaperone-mediated autophagy are required for hepatocyte resistance to oxidant stress. *Hepatology*. 2010;52(1):266-77. doi:10.1002/hep.23645

18. Singh R, Kaushik S, Wang Y, et al. Autophagy regulates lipid metabolism. *Nature*. 2009;458(7242):1131-5. doi:nature07976 [pii]
10.1038/nature07976

19. Komatsu M, Kurokawa H, Waguri S, et al. The selective autophagy substrate p62 activates the stress responsive transcription factor Nrf2 through inactivation of Keap1. *Nat Cell Biol*. 2010;12(3):213-23. doi:ncb2021 [pii]
10.1038/ncb2021

20. Sasaki M, Miyakoshi M, Sato Y, Nakanuma Y. Autophagy mediates the process of cellular senescence characterizing bile duct damages in primary biliary cirrhosis. *Lab Invest*. 2010;90(6):835-43. doi:labinvest201056 [pii]
10.1038/labinvest.2010.56

21. Mathew R, Karp CM, Beaudoin B, et al. Autophagy suppresses tumorigenesis through elimination of p62. *Cell*. 2009;137(6):1062-75. doi:S0092-8674(09)00391-2 [pii]
10.1016/j.cell.2009.03.048

22. Young AR, Narita M, Ferreira M, et al. Autophagy mediates the mitotic senescence transition. *Genes Dev*. 2009;23(7):798-803. doi:gad.519709 [pii]
10.1101/gad.519709

23. White E, Lowe SW. Eating to exit: autophagy-enabled senescence revealed. *Genes Dev*. 2009;23(7):784-7. doi:23/7/784 [pii]
10.1101/gad.1795309

24. Nakanuma Y, Sasaki M. Expression of blood-group-related antigens in the intrahepatic biliary tree and hepatocytes in normal livers and various hepatobiliary diseases. *Hepatology*. 1989;10:174-8.

25. Portmann B, Nakanuma Y. Diseases of the bile ducts. In: MacSween R, Burt A, BC P, Ishak K, Scheuer P, Anthony P, eds. *Pathology of the Liver*. 4th Eds ed. London: Churchill Livingstone; 2001. p. 435-506.

26. Ludwig J. Small-duct primary sclerosing cholangitis. *Semin Liver Dis*. 1991;11(1):11-7.

27. Desmet V, Gerber M, Hoofnagle J, Manns M, Scheuer P. Classification of chronic hepatitis: diagnosis, grading and staging. *Hepatology*. 1994;19:1513-20.

28. Brunt EM, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. *Am J Gastroenterol*. 1999;94(9):2467-74.

29. Bjorkoy G, Lamark T, Brech A, et al. p62/SQSTM1 forms protein aggregates degraded by autophagy and has a protective effect on huntingtin-induced cell death. *J Cell Biol*. 2005;171(4):603-14. doi:jcb.200507002 [pii]
10.1083/jcb.200507002

30. Komatsu M, Waguri S, Koike M, et al. Homeostatic levels of p62 control

cytoplasmic inclusion body formation in autophagy-deficient mice. *Cell*. 2007;131(6):1149-63. doi:S0092-8674(07)01354-2 [pii]
10.1016/j.cell.2007.10.035

31. Sasaki M, Ikeda H, Sato Y, Nakanuma Y. Decreased expression of Bmi1 is closely associated with cellular senescence in small bile ducts in primary biliary cirrhosis. *Am J Pathol*. 2006;169(3):831-45.

32. Lamark T, Kirkin V, Dikic I, Johansen T. NBR1 and p62 as cargo receptors for selective autophagy of ubiquitinated targets. *Cell Cycle*. 2009;8(13):1986-90. doi:8892 [pii]

33. Ichimura Y, Kumanomidou T, Sou YS, et al. Structural basis for sorting mechanism of p62 in selective autophagy. *J Biol Chem*. 2008;283(33):22847-57. doi:M802182200 [pii]
10.1074/jbc.M802182200

34. Pankiv S, Clausen TH, Lamark T, et al. p62/SQSTM1 binds directly to Atg8/LC3 to facilitate degradation of ubiquitinated protein aggregates by autophagy. *J Biol Chem*. 2007;282(33):24131-45. doi:M702824200 [pii]
10.1074/jbc.M702824200

35. Monick MM, Powers LS, Walters K, et al. Identification of an autophagy defect in smokers' alveolar macrophages. *J Immunol*. 2010;185(9):5425-35. doi:jimmunol.1001603 [pii]
10.4049/jimmunol.1001603

Figure legends

1. Expression of LC3 and p62 in ductular reaction (DR) in PBC and control liver.

A) Coarse vesicular expression of LC3 in DRs in PBC. Coarse vesicular expression of LC3 is seen in the cytoplasm of ductular cells in PBC (left, arrows), whereas no expression of LC3 is found in ductular cells in CVH, C (right, arrows). Immunostaining for LC3. Original magnification, x400. B) Coarse vesicular expression of p62 is seen in the cytoplasm of ductular cells in PBC (left, arrows), whereas no or faint expression of p62 is found in ductular cells in CVH, C (right, arrows). Immunostaining for p62. Original magnification, x400.

2. Increased expression of LC3 and p62 in ductular reaction (DR) in PBC.

A) The frequency and extent of LC3 expression. White column, focal expression (1+), gray columns, extensive expression (2+). a, $p<0.01$ vs other groups, b, $p<0.05$ vs normal liver (NL). B) The frequency and extent of p62 expression. White column, focal expression (1+), gray columns, extensive expression (2+). a, $p<0.01$ vs normal liver (NL) and nonalcoholic steatohepatitis (NASH), stage (st)1 and 2, b, $p<0.05$ vs chronic viral hepatitis (CVH), F1 and F2 and F3 and F4. C) Correlation between expression of LC3 and p62 in ductular reaction (DR) in primary biliary cirrhosis (PBC) and control diseases. In PBC, both molecules are expressed frequently and extensively, while expression of these two molecules are infrequent or focal in control livers (chronic viral hepatitis (CVH) and extrahepatic biliary obstruction (EBO)). There is a statistical correlation in the

distribution of these two molecules ($p<0.01$, rs=0.558).

3. Correlation between autophagy markers and senescent markers.

A) Correlation between expression of LC3 and p16 in ductular reaction (DR) in primary biliary cirrhosis (PBC) and control diseases. In PBC, both molecules are expressed frequently and extensively, while expression of these two molecules are infrequent or focal in control livers (chronic viral hepatitis (CVH) and extrahepatic biliary obstruction (EBO)). There is a statistical correlation in the distribution of these two molecules ($p<0.01$, rs=0.574).

B) Correlation between expression of LC3 and p21 in ductular reaction (DR) in primary biliary cirrhosis (PBC) and control diseases. In PBC, both molecules are expressed frequently and extensively, while expression of these two molecules are infrequent or focal in control livers (CVH and EBO). There is a statistical correlation in the distribution of these two molecules ($p<0.01$, rs=0.462).

C) Correlation between expression of p62 and p16 in ductular reaction (DR) in primary biliary cirrhosis (PBC) and control diseases. In PBC, both molecules are expressed frequently and extensively, while expression of these two molecules are infrequent or focal in control livers (CVH and EBO). There is a statistical correlation in the distribution of these two molecules ($p<0.01$, rs=0.335).

D) Correlation between expression of p62 and p21 in ductular reaction (DR) in primary

biliary cirrhosis (PBC) and control diseases. In PBC, both molecules are expressed frequently and extensively, while expression of these two molecules are infrequent or focal in control livers (CVH and EBO). There is a statistical correlation in the distribution of these two molecules ($p<0.05$, $rs=0.285$).

Table 1. Primary antibodies used in this study.

Primary antibody	***Type (clone)***	***Pre-treatment***	***dilution***	***Source***
LC3	Goat poly	MW-CB (95℃, 20min)	1:50	Santa-Cruz, Santa-Cruz, CA
p62	Rabbit poly	eARI –BA (121℃, 5min)	1:1000	MBL, Nagoya, Japan
$p16^{INK4a}$	Mouse mono (JC8)	eARI –BA (121℃, 5min)	1:100	Neomarkers, Freemont, CA
$p21^{WAF1/Cip1}$	Mouse mono (70)	eARI –BA (121℃, 5min)	1:100	BD Transduction, San Jose, CA

p62, p62/sequestosome-1; LC3, microtubule-associated proteins-light chain 3β; LAMP-1, lysosome-associated membrane protein-1; MW, microwave treatment; CB, 0.05M citric buffer (pH 6); eARI, electronic antigen retrieval instrument (pascal, Dako); BA, 0.05M boric acid buffer (pH 8).