



Association between mitochondrial and nuclear DNA damages and cellular senescence in the patients with biliary atresia undergoing Kasai portoenterostomy and liver transplantation

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Abstract

Biliary atresia (BA) is a cholestatic disease with extrahepatic bile duct obstruction that requires early surgical intervention and occasionally liver transplantation (LT). Accumulation of toxic bile acids induces oxidative stress that results in cell damage, such as cell senescence, mitochondrial dysfunction and others. However, details of their reciprocal association and clinical significance are unexplored. Therefore, we used immuno-localization of markers for cell senescence (p16 and p21), nuclear double-strand DNA damage (γ H2AX), autophagy (p62), and mtDNA damage (mtDNA copy number) in patients with BA who underwent Kasai portoenterostomy (KP) and LT. We studied liver biopsy specimens from 54 patients with BA, 14 who underwent LT and 11 from the livers of neonates and infants obtained at autopsy. In hepatocytes, p21 expression was significantly increased in KP. In cholangiocytes, p16 expression was significantly increased in LT, and p21 expression was significantly increased in KP. p62 expression was significantly increased in the KP hepatocytes and LT cholangiocytes. Furthermore, mtDNA copy number significantly decreased in KP and LT compared with the control. Cell senescence and mitochondrial DNA damage progression were dependent on the BA clinical stages and could possibly serve as the markers of indication of LT.

Keywords Senescence · Biliary atresia · Kasai portoenterostomy · Liver · Mitochondrial damage · DNA damage

Abbreviations

| | | | |
|-------|----------------------|-------|----------------------------------|
| ALT | Alanine transaminase | FFPE | Formalin-fixed paraffin-embedded |
| BA | Biliary atresia | H&E | Hematoxylin–eosin |
| ChE | Cholinesterase | IHC | Immunohistochemistry |
| D.Bil | Direct bilirubin | KP | Kasai portoenterostomy |
| DR | Ductular reaction | LT | Liver transplantation |
| | | mtDNA | Mitochondrial DNA |

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| | |
|---------|-------------------------------|
| mtDNAcn | Mitochondrial DNA copy number |
| NL | Non-pathological liver |
| Plt | Platelet |
| ROS | Reactive oxygen species |
| T.Bil | Total bilirubin |
| 8-OHdG | 8-Hydroxydeoxyguanosine |

Introduction

Biliary atresia (BA) occurs from neonatal stage through infancy period, and is histologically characterized by cholestasis associated with sclerosing inflammation of the extrahepatic bile duct [1]. The possible etiology of biliary atresia has been proposed, including congenital bile duct dysplasia, viral infection, and immune disorders; however, none of these disorders have been established as direct causes of the disease [1, 2]. Patients with BA are usually treated with portoenterostomy, also termed Kasai portoenterostomy (KP) [3]. The clinical prognosis of patients with BA is generally evaluated in the presence or absence of jaundice disappearance and native liver survival [4]. The patient's age at surgery and jaundice clearance rate are well known to be correlated with each other [3]. The jaundice disappearance rate was reported to exceed 70% in neonatal surgery cases but reached approximately 60% in surgical cases until 80 days after birth [3]. However, this rate gradually decreased with the delay in the surgical period [3]. In addition, even though jaundice clinically disappears, the clinical condition gradually deteriorates and liver transplantation is eventually required in many patients with BA [3]. Results from the Japanese Biliary Atresia Registry revealed that 40% of patients with BA eventually required liver transplantation (LT) [3]. Therefore, liver transplantation (LT) has been performed in BA patients with persistent or recurrent jaundice following Kasai portoenterostomy (KP), or those with clinically severe BA conditions [4].

Accumulation of toxic bile acids due to extrahepatic biliary obstruction has been reported to induce oxidative stress, which leads to cellular damage or dysfunctions, such as cell senescence, mitochondrial dysfunction, and DNA damage [5, 6]. Both mitochondrial dysfunction and cellular senescence are reported to be closely correlated with each other [7, 8]. Mitochondrial dysfunction is also known to generally promote and maintain cell senescence and directly contribute to aging-related mitochondrial dysfunction (SAMD) [9–12]. Recently, mitochondrial DNA (mtDNA) damage has been studied in various diseases associated with biliary obstruction and it possibly has a role in the pathophysiology of these diseases [13]. The number of copies of mtDNA is reported to be an indicator of mtDNA quality [14–16]. Therefore, maintaining copies of mitochondrial DNA is required for maintaining mitochondrial function, and reduced mitochondrial

DNA copy numbers could reflect mitochondrial dysfunction [17, 18]. Among these factors, mitochondrial DNA copy number was reported to be reduced in hepatocytes in patients with BA and is of particular interest [19].

In the early stages of cell senescence, p53/p21 WAF1/Cip1 was reported to be activated by DNA damage as results of oxidative stress, expressional stress, and others. In addition, relatively high levels of p21 were also known to inhibit the kinase activity of cyclin D and CDK4 / 6 and to cause cell cycle arrest [20]. On the other hand, during the maintenance phase of the cell senescence, p16 was reported to be activated and inhibit the inhibition of the kinase activity of cyclin D and CDK4/6 [20]. The expression of senescence-associated cell cycle regulators including p16 INK4a and p21 WAF1/Cip1, is induced in the advanced stages of several chronic liver diseases, such as primary biliary cholangitis (PBC) and non-alcoholic steatohepatitis (NASH) [21]. p16 INK4a and p21 WAF1/Cip1 have also been reported to be induced in cholangiocytes in biliary atresia [22]. However, the association between the status of cell senescence/DNA damage and the clinical course of patients with BA remains virtually unknown. Therefore, we explored both mitochondrial and nuclear DNA damage in situ in pathological specimens of BA and their possible association with cellular senescent phenotypes, as well as their clinical significance in disease progression of BA patients who underwent KP and/or LT. BA liver samples obtained at the time of KP represented the relatively early stages and early changes in BA. In contrast, the BA liver samples obtained at the time of LT represented advanced stages of BA. In the present study, we used the livers of the same patients at KP and LT. Therefore, the changes associated with the disease course of BA in the same patients were evaluated by comparing the findings of BA liver samples taken at both KP and LT. Immuno-histochemical analysis of p16, p21, and Ki-67 was used to evaluate the status of cell senescence, and γ H2AX for double-strand breaks of nuclear DNA damage [23]. In addition, mtDNAcn in hepatocytes was studied to further explore changes in mitochondrial damage during KP and LT. In addition, p62 was also examined as an index of autophagy, which was reported to contribute to the clearance of damaged cells in the local tissue microenvironment [24, 25]. These markers were also studied in the common bile duct at KP.

Materials and methods

Patients

Fifty-four liver biopsy specimens from patients with BA who underwent Kasai portoenterostomy (KP) and extracted liver specimens from 14 patients who underwent liver

transplantation (LT) after Kasai portoenterostomy (KP) at Tohoku University Hospital from 2001 to 2017 were examined. Among the LT cases, 13 patients also underwent their first Kasai portoenterostomy surgical intervention from 2001 to 2017, during which biopsy specimens were taken and tentatively categorized as KP cases in this study. In 40 patients with BA who received KP, LT was not performed during their entire clinical course. In addition, among the cases above, 11 BA cases with residual common bile duct epithelium who underwent KP were also studied. For the control tissues of non-pathological liver (NL) in newborns and infants, 11 autopsy cases without any history of liver or metabolic diseases were retrieved from the autopsy files at Tohoku University Hospital between 2005 and 2017. The clinico-pathological characteristics of the KP, LT, and NL cases examined in this study are summarized in Supplemental Table 1. The present study protocol was approved by the Institutional Review Board of the Tohoku University School of Medicine (2019-1-996).

Definition of native liver survival and jaundice disappearance in patients with BA

The native liver survival period was defined as the number of days from the date of the initial KP operation to that of death from liver failure or LT. The clinical definition of jaundice disappearance was defined as total serum bilirubin levels less than 2.0 mg/dl, as described previously [26].

Histological and morphological evaluation of BA liver samples

Serial tissue sections were prepared from 10% formalin-fixed paraffin-embedded (FFPE) blocks cut at 3- μ m thickness. Hematoxylin and eosin (H&E) staining, Elastica Masson (EM) stain, and CK19 immunohistochemistry (IHC) were performed for histopathological assessment of liver damage and disease progression (Supplemental Table 2).

The degree of ductular reaction (DR) was semi-quantitatively scored as 0 (negative), 1 (<30% circumference in portal tracts), 2 (\geq 30% to <60% circumference in portal tracts), or 3 (\geq 60% circumference in portal tracts), as described previously [27]. The degree of cholestasis was scored as 0 (negative), 1 (mild), 2 (moderate), or 3 (extensive), and that of giant cells was scored as 0 (negative), 1 (mild) or 2 (extensive) [22]. The degree of hepatic fibrosis was evaluated based on the criteria of the revised Inuyama classification system [28].

The status of cell senescence, DNA damage, and autophagy in BA liver samples

Serial tissue sections were analyzed using the streptavidin/biotin method. Images were captured using an Aperio

AT2 microscope (Leica Microsystems, Wetzlar, Germany). Immunoreactivity was quantitatively evaluated in hepatocytes and cholangiocytes. The expression in hepatocytes was analyzed using Halo imaging analysis software (Indica Labs, Corrales, NM, USA) [29] and that in cholangiocytes was manually evaluated by two of the authors (Y. N. and Y. Y.) who were blinded to the clinico-pathological variables of the patients.

p16 and p21 were immuno-localized to the nuclei and evaluated using a labeling index (LI) ranging from 0 to 100% [29]. Cell senescence was evaluated with p16 and p21 immunoreactivity, as well as the absence of Ki-67 positivity. p62 was immuno-localized in the cytoplasm; therefore, the results were evaluated using the H-score, which was determined by the percentage of immuno-positive cells and their relative immuno-intensity. Immuno-intensity was scored according to the following criteria: 0, negative; 1, weak; 2, moderate; and 3, marked. The H-score was then calculated by multiplying the percentage of immuno-positive cells by the immuno-intensity on a scale from 0 to 300 [30].

mtDNA copy number analysis

Subsequent sets of serial tissue sections were prepared from FFPE tissue blocks of 10- μ m thickness. Genomic DNA was extracted using the AllPrep DNA FFPE Kit (QIAGEN) following the manufacturer's protocol and as previously reported [31]. DNA extraction was performed for 24 KP cases, 12 LT cases, and 11 NL cases in which sufficient amounts of the tissues were available for evaluation. mtDNAcn was also analyzed by qRT-PCR to study the relative mtDNAcn. Primers were designed to detect ND2 in mtDNA with infrequent deletion (Fw 5'-CCATCTTTG CAGGCACACTCATC-3' and Rev 5'-ATCCACCTCAAC -TGCCTGCTATG-3') [32, 33]. The product of the RPL13A house-keeping gene was used as a nuclear-coded gene of the diploid genome (Fw 5'-CCTGGAGGAGAAGAGGAA AG-'3 and Rev 5'-TTGAGGACCTCTGTGTATTT-'3). mtDNAcn was calculated using the following formula: relative mtDNAcn/diploid genome = $2^{-\Delta\Delta Ct}$ [34].

Evaluation of immunoreactivity

Statistical analysis was performed using JMP Pro Version 15 (SAS Institute Inc., Cary, NC, USA). Multivariate analysis among individual pairs of groups was performed using the Steel–Dwass test. Paired-match analysis between the values obtained at the KP and LT periods in the same patients and paired-match analysis between cholangiocytes and common bile ducts were analyzed using the Wilcoxon signed-rank test. Correlations among clinico-pathological parameters and correlations among cholangiocytes and common bile

ducts immunoreactivity were analyzed using the Spearman's rank correlation coefficient test. Native liver survival curves were tentatively drawn by Kaplan–Meier analysis and compared between subgroups based on their median (high and low) values [35]. Differences were considered statistically significant at a p value of <0.05 .

Results

Comparison of cell senescence, DNA damage, and autophagy markers among KP, LT, and NL cases

Representative IHC images are shown in Fig. 1. In hepatocytes, p21 expression was significantly higher in KP and LT than in NL samples (KP vs. NL, $p=0.0007$; LT vs. NL, $p=0.0482$; Fig. 2b). p62 expression was also significantly higher in KP and LT than in NL samples (KP vs. NL, $p<0.0001$; LT vs. NL, $p=0.0056$) (Fig. 2d), and higher in KP than LT (KP vs. LT, $p=0.0156$; Fig. 2d).

In cholangiocytes, p16 expression was significantly higher in KP and LT than in NL (KP vs. NL, $p=0.0022$; LT vs. NL, $p<0.0001$; Fig. 2e), and higher in LT than KP (LT vs. KP, $p<0.0001$; Fig. 2e). p21 was significantly higher in KP and LT than in NL (KP vs. NL, $p<0.0001$; LT vs. NL,

$p=0.0004$; Fig. 2f); and p62 expression was significantly higher in the LT group than in the KP and NL groups (LT vs. NL, $p=0.0001$; LT vs. KP, $p<0.0001$; Fig. 2h).

We also found that p21, γ H2AX, and p62 were all significantly positively correlated between hepatocytes and cholangiocytes in the same BA patients (p21, $p<0.0001$; γ H2AX, $p=0.0293$; p62, $p=0.0036$; Fig. 2j–l). In addition, p16 tended to be positively correlated between hepatocytes and cholangiocytes in the same cases of BA, but the correlation was not statistically significant (p16; $p=0.0762$; Fig. 2i).

Association of cell senescence, DNA damage, and autophagy markers in hepatocytes and cholangiocytes with histological features of BA

With relation to intrahepatic fibrosis, p16 immunoreactivity in hepatocytes was significantly higher in group 4 than in group 2 (group 4 vs. group 2, $p=0.0241$; Fig. 3a). In cholangiocytes, p16 expression was significantly higher in group 4 than in groups 2 and 3 (group 4 vs. group 2, $p<0.0001$; group 4 vs. group 3, $p<0.0001$; Fig. 3e), while p21 expression was significantly higher in group 3 than in group 4 (group 3 vs. group 4, $p=0.0450$; Fig. 3f). We found that p62 expression in hepatocytes was significantly higher in groups 2 and 3 than in group 4 (group 2 vs. group 4, $p=0.0490$;

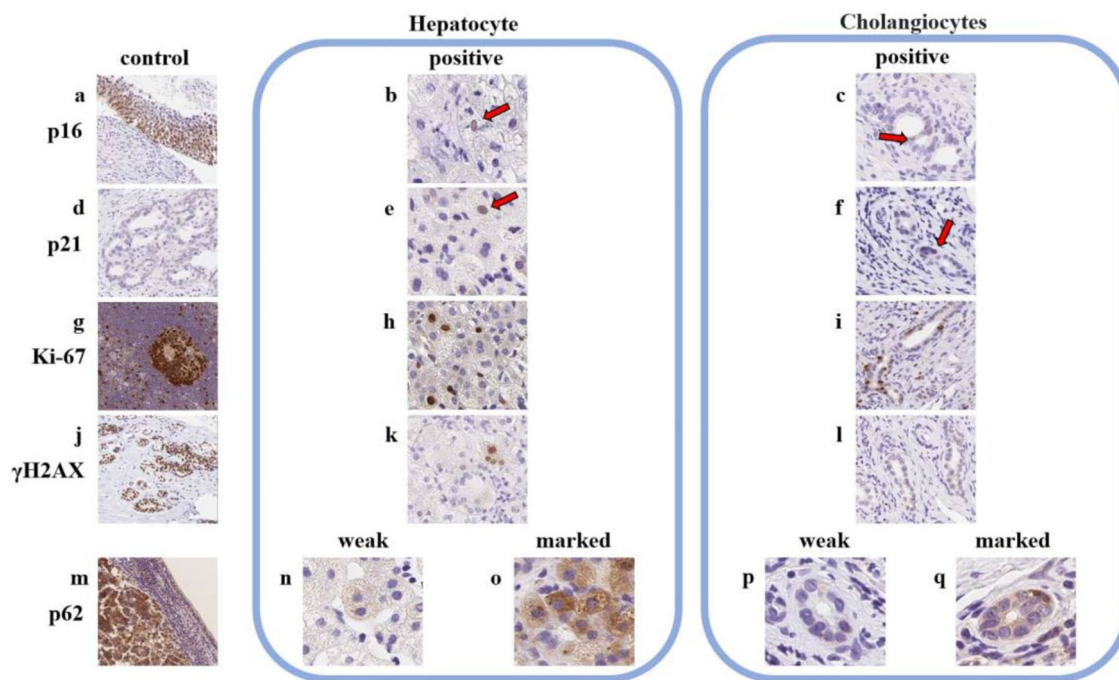


Fig. 1 Representative IHC images of the liver tissues from patients with BA. The positive control tissue for p16 IHC was that of cervical cancer (a), p16-positive hepatocytes (b), and cholangiocytes (c). The positive control tissue for p21 IHC was breast cancer (d), p21-positive hepatocytes (e), and hepatocytes (f). The positive control of Ki-67 was lymph node tissue (g). Ki67-positive hepatocytes (h) and

cholangiocytes (i). The positive control tissue for γ H2AX was breast cancer (j). γ H2AX-positive hepatocytes (k) and cholangiocytes (l). The positive control tissue for p62 IHC was lung cancer (m). Hepatocytes with weak (n) and marked (o) p62 immunoreactivity. Cholangiocytes with weak (p) and marked (q) p62 immunoreactivity

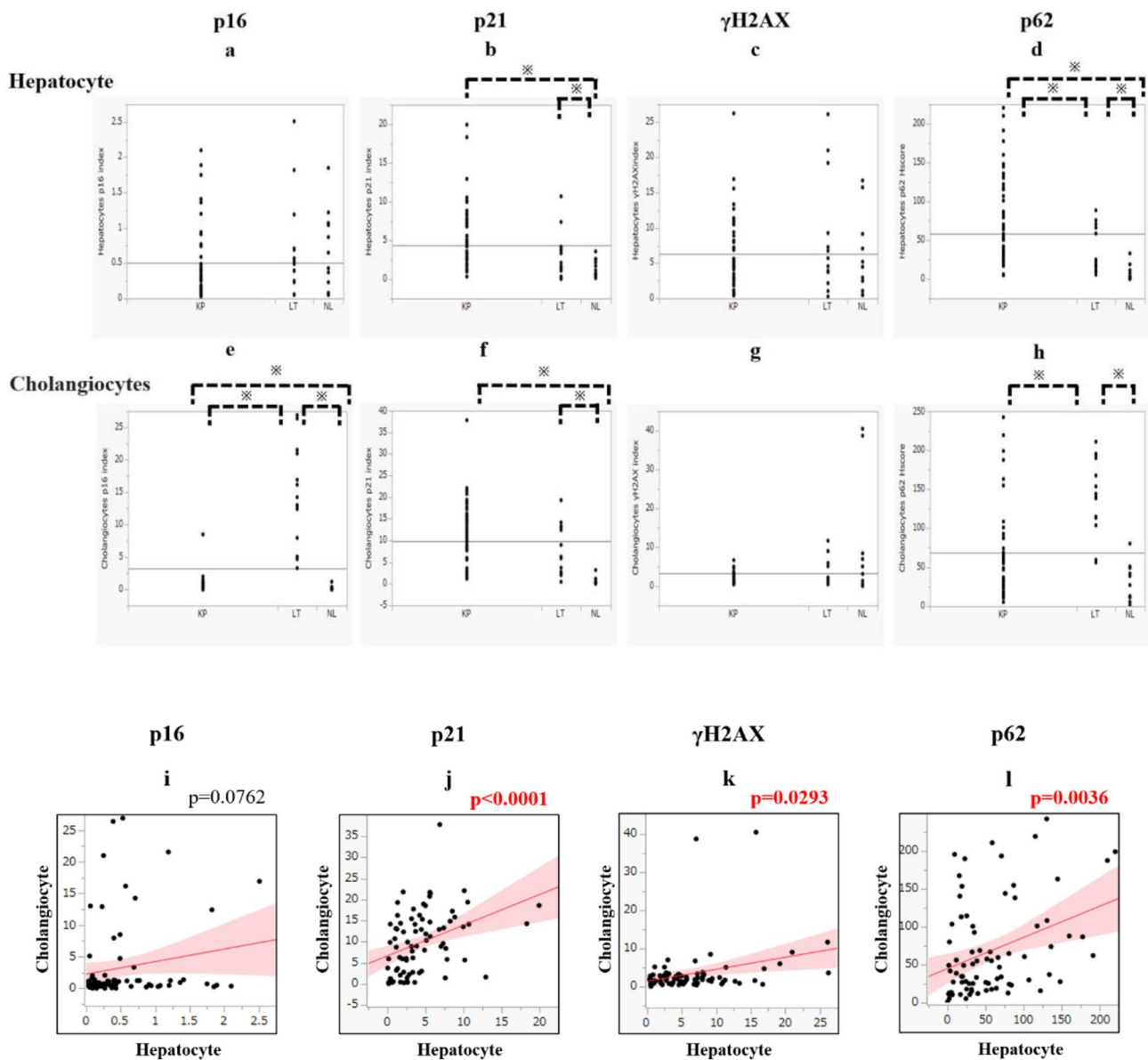


Fig. 2 Comparison of the markers examined in hepatocytes and cholangiocytes among KP, LT, and NL samples. * Indicates $p < 0.05$. Comparison of p16 (a) and p21 (b) in hepatocytes. KP and LT had significantly higher immunoreactivity than NL. Comparison of γ H2AX (c) and p62 (d) in hepatocytes. KP and LT had significantly higher immunoreactivity than NL, while KP had significantly higher immunoreactivity than LT. Comparison of p16 in cholangiocytes (e). KP and LT had significantly higher expression than NL, while LT had significantly higher immunoreactivity than KP. Comparison of p21 in cholangiocytes (f). KP and LT had significantly higher immunoreactivity than NL. Comparison of γ H2AX in cholangiocytes (g). LT

had significantly higher immunoreactivity than KP and NL(h). Comparison of p16 examined in hepatocytes and cholangiocytes (i). Comparison of p21 examined in hepatocytes and cholangiocytes showed that p21 was significantly positively correlated between hepatocytes and cholangiocytes in the same patient cases (j). Comparison of γ H2AX examined in hepatocytes and cholangiocytes, showing γ H2AX was significantly positively correlated between hepatocytes and cholangiocytes in the same patient cases (k). Comparison of p62 examined in hepatocytes and cholangiocytes showing p62 was significantly positively correlated between hepatocytes and cholangiocytes in the same cases (l).

group 3 vs. group 4, $p = 0.0185$; Fig. 3b); while in cholangiocytes, p62 expression was significantly higher in group 4 than in groups 2 and 3 (group 2 vs. group 4, $p < 0.0001$; group 3 vs. group 4, $p = 0.0040$; Fig. 3h).

We next asked which of these markers correlated with DR, and found that p16 immunoreactivity in cholangiocytes was significantly higher in group 3 than in group 2 (group 3 vs. group 2, $p = 0.0020$; Fig. 3e), and p62 expression in cholangiocytes was significantly higher in group 3 than in

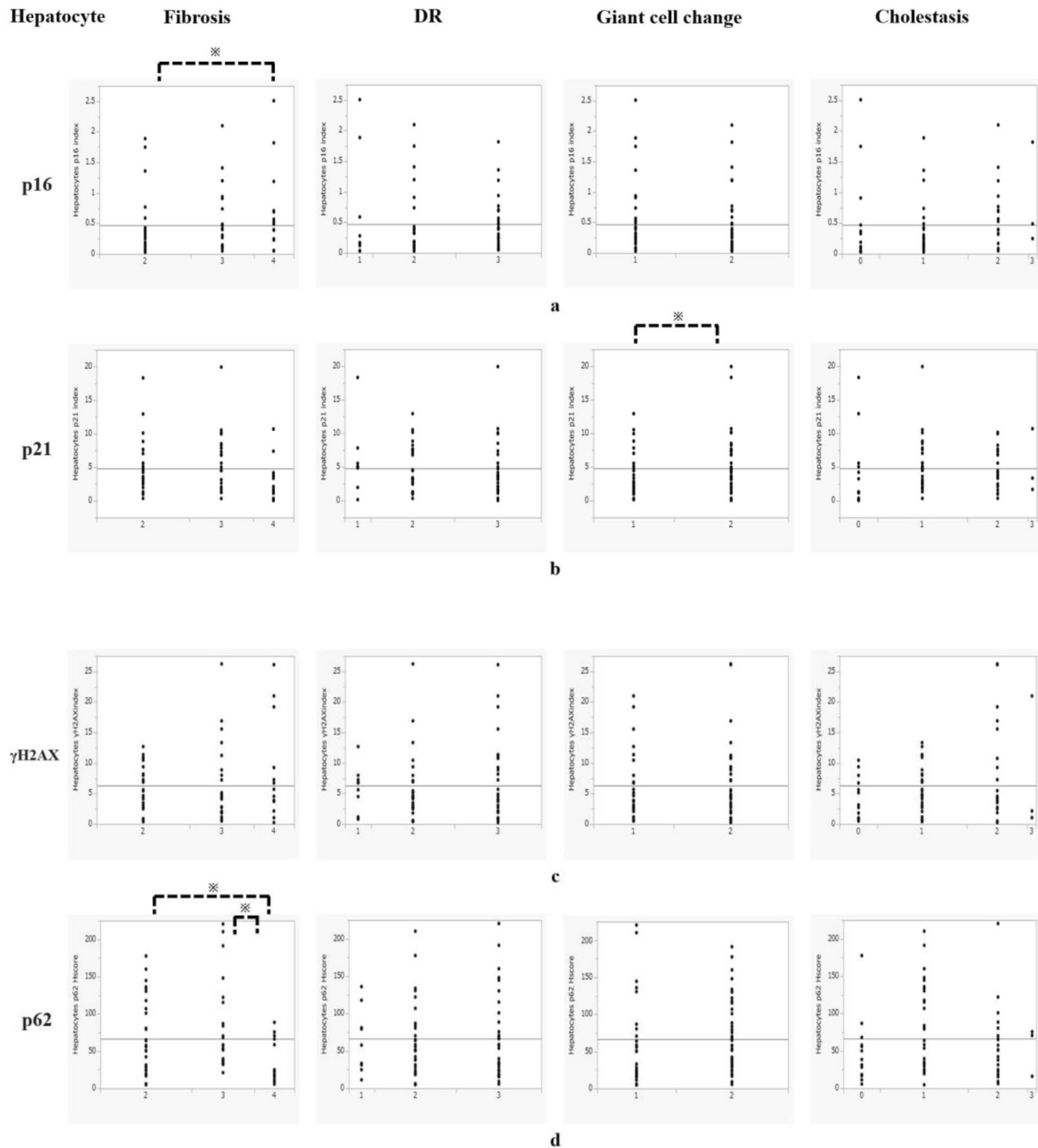


Fig. 3 Comparison of immunoreactivity of each marker examined in hepatocytes and cholangiocytes among the pathological factors examined in BA liver samples. ※ Indicates $p < 0.05$. Comparison of p16 in hepatocytes and fibrosis, DR, giant cell change, and cholestasis, indicating group 4 had significantly higher immunoreactivity than group 2 for fibrosis (a). Comparison of p21 in hepatocytes and fibrosis, DR, giant cell change and cholestasis, indicating group 2 had significantly higher immunoreactivity than group 1 for giant cell change (b). Comparison of γ H2AX in hepatocytes and fibrosis, DR, giant cell change and cholestasis (c). Comparison of p62 in hepatocytes and fibrosis, DR, giant cell change and cholestasis, showing that group 2 and group 3 had significantly higher immunoreactivity than group 4 for fibrosis (d). Comparison of p16 in cholangiocytes and fibrosis, DR,

giant cell change, and cholestasis indicating group 4 had significantly higher immunoreactivity for fibrosis than groups 2 and 3, while in the DR group, group 3 showed significantly higher immunoreactivity than group 2; and in cholangiocytes, group 3 showed significantly higher expression than group 1 (e). Comparison of p21 in cholangiocytes and fibrosis, DR, giant cell change, and cholestasis showing group 4 had significantly higher immunoreactivity for fibrosis than group 3 (f). Comparison of γ H2AX in cholangiocytes and fibrosis, DR, giant cell change, and cholestasis (g). Comparison of p62 in cholangiocytes and fibrosis, DR, giant cell change, and cholestasis, which shows that group 4 had significantly higher immunoreactivity for fibrosis than groups 2 and 3; while in the DR, group 3 had significantly higher immunoreactivity than groups 1 and 2 (h).

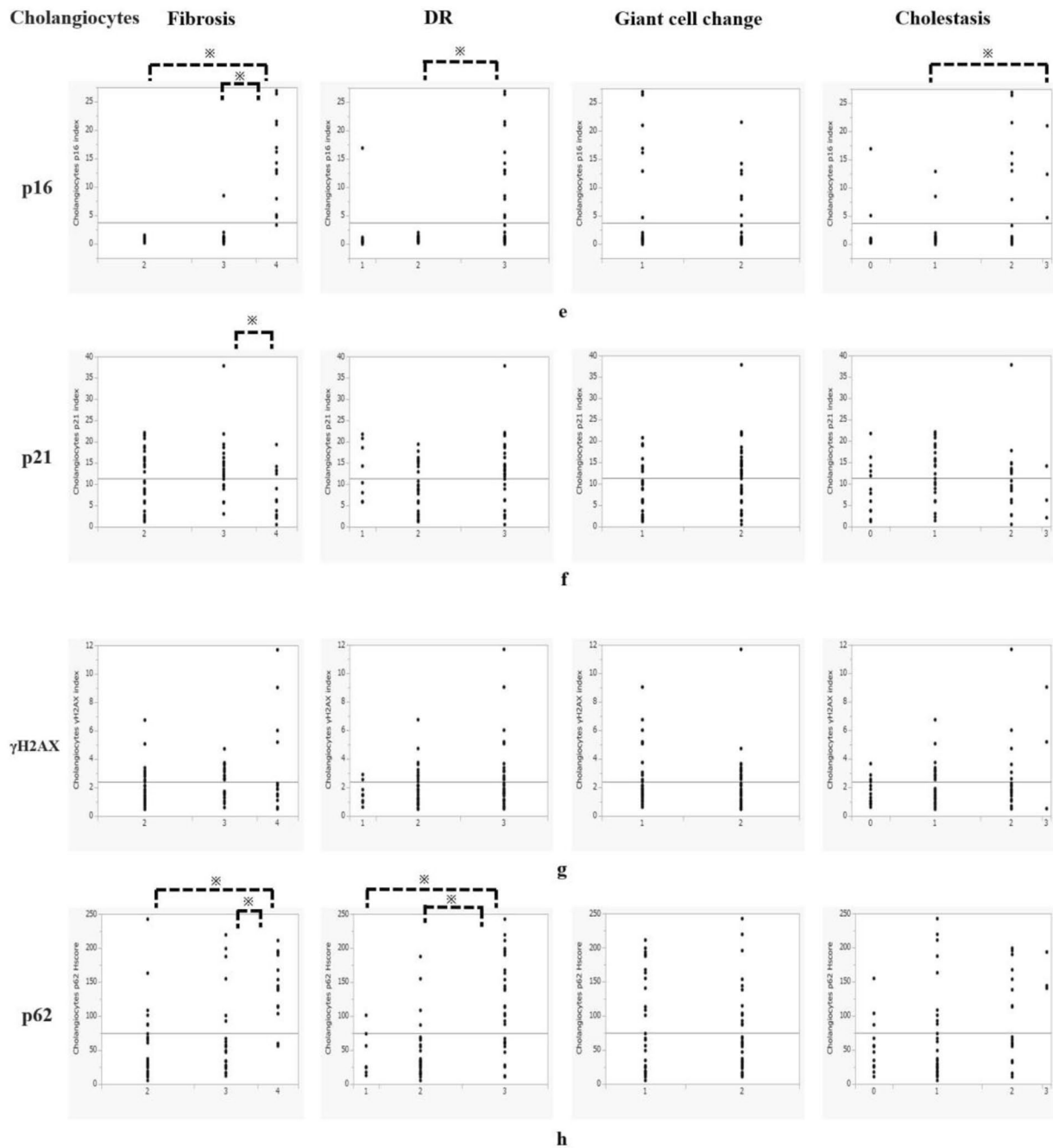


Fig. 3 (continued)

groups 1 and 2 (group 3 vs. group 1, $p=0.0135$; group 3 vs. group 2, $p=0.0006$; Fig. 3h).

Regarding the correlation of each markers with giant cell changes in the liver, p21 immunoreactivity in hepatocytes was significantly higher in group 2 than in group 1 (group 2 vs. group 1, $p=0.0326$; Fig. 3b). With respect to cholestasis, p16 expression in cholangiocytes was significantly higher in group 3 than in group 1 (group 3 vs. group 1, $p=0.0447$; Fig. 3e).

Comparison of cell senescence, DNA damage, and autophagy markers among cholangiocytes and common bile ducts

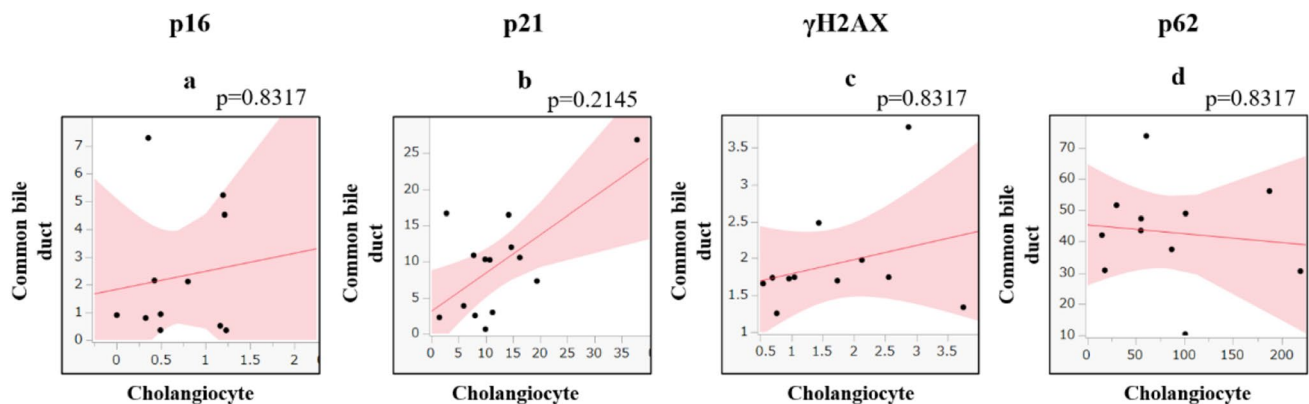
Markers of cell senescence, DNA damage, and autophagy markers between cholangiocytes and common bile ducts in the same patients were also examined. p16 immunoreactivity in common bile ducts was significantly higher than cholangiocytes ($p=0.0166$; Table 1). However,

Table 1 Comparison of cell senescence, DNA damage, and autophagy markers among cholangiocytes and common bile ducts

| | Cholangiocytes | Common bile duct | <i>p</i> value |
|-------|----------------|------------------|--|
| p16 | 0–2.01 | 0.32–7.29 | <i>p</i> = 0.0166 <i>S</i> = 37.500 |
| p21 | 1.45–37.83 | 0.64–26.83 | <i>p</i> = 0.1762 <i>S</i> = – 22.500 |
| γH2AX | 0.55–3.75 | 1.25–3.79 | <i>p</i> = 0.2783 <i>S</i> = 13.000 |
| p62 | 15.09–219.43 | 10.44–73.68 | <i>p</i> = 0.2061 <i>S</i> = – 15.000 |

Bold indicates *p* < 0.05

no significant correlation was detected between cholangiocytes and common bile ducts in the same cases (Fig. 4a–d).

**Fig. 4** Comparison of the markers examined in cholangiocytes and common bile ducts. * Indicates *p* < 0.05. Comparison of p16 examined in cholangiocytes and common bile ducts (a). Comparison of p21 examined in cholangiocytes and common bile ducts (b). Compar-

Matched analysis of individual markers in the same BA patients with BA between their first KP and subsequent LT

Cell senescence, DNA damage, and autophagy markers were compared between the time of the first surgical intervention with KP and that of the subsequent LT. We found that p16 immunoreactivity in both hepatocytes and cholangiocytes was significantly higher at the time of LT than KP (LT vs. KP, hepatocytes: *p* = 0.0164, cholangiocytes: *p* = 0.0001; Table 2), while p62 expression in cholangiocytes was significantly higher in LT than in KP (LT vs. KP, *p* = 0.0001; Table 2).

Comparison of mtDNAcn among KP, LP and NL cases

mtDNAcn was significantly lower in KP and LT than in NL (KP vs. NL, *p* = 0.0244; LT vs. NL, *p* = 0.0141; Fig. 5); however, mtDNAcn in the LT group tended to be lower than that in the KP group, although this difference was not statistically significant.

ison of γH2AX examined in cholangiocytes and common bile ducts (c). Comparison of p62 examined in cholangiocytes and common bile ducts (d)

Table 2 Matched analysis of individual markers in the same BA patients with BA between their first KP and subsequent LT

| | Hepatocytes | | | Cholangiocytes | | |
|-------|-------------|------------|--|----------------|--------------|--|
| | KP | LT | <i>p</i> value | KP | LT | <i>p</i> value |
| p16 | 0.03–1.20 | 0.06–2.51 | <i>p</i> = 0.0164 <i>S</i> = 30.500 | 0–8.48 | 3.32–26.91 | <i>p</i> = 0.0001 <i>S</i> = 45.500 |
| p21 | 0.36–18.24 | 0.03–10.71 | <i>p</i> = 0.0955 <i>S</i> = – 19.500 | 1.44–18.58 | 0.51–19.33 | <i>p</i> = 0.1082 <i>S</i> = – 18.500 |
| γH2AX | 0.51–13.35 | 0.28–26.10 | <i>p</i> = 0.0955 <i>S</i> = 19.500 | 0.89–3.11 | 0.50–11.69 | <i>p</i> = 0.3777 <i>S</i> = 5.500 |
| p62 | 6.24–117.90 | 9.19–88.65 | <i>p</i> = 0.1698 <i>S</i> = – 14.500 | 10.65–101.26 | 56.32–211.01 | <i>p</i> = 0.0001 <i>S</i> = 45.500 |

Bold indicates *p* < 0.05

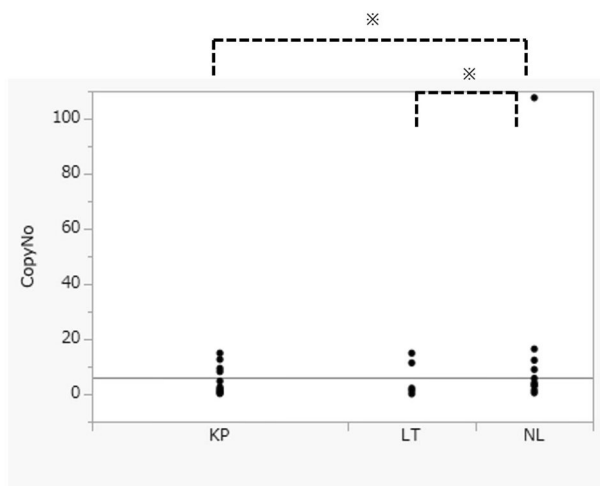


Fig. 5 Comparison of mtDNAcn among KP, LT, and NL. * Indicates $p < 0.05$. KP and LT were significantly lower than NL

Correlation between clinical characteristics and cell senescence, DNA damage, and autophagy marker expression in patients with BA

The expression of markers for cell senescence, DNA damage, and autophagy and mtDNAcn was compared using clinical data from patients with BA to evaluate their potential relationships (Table 3). Total bilirubin (T.Bil) levels were significantly and positively correlated with mtDNAcn ($p = 0.0005$), while direct bilirubin (D.Bil) levels were significantly positively correlated with p21 ($p = 0.0142$) in hepatocytes. In addition, p21 expression in cholangiocytes ($p = 0.0394$), and mtDNAcn ($p = 0.0083$) was also significantly correlated to D.Bil. Alanine transaminase (ALT) levels were also significantly positively correlated with p21 ($p = 0.0020$) and p62 ($p = 0.0211$) expression in hepatocytes, as well as with p21 expression in cholangiocytes ($p = 0.0033$). Cholinesterase (ChE) was

significantly inversely correlated with the expression of p16 ($p = 0.0248$) and γ H2AX ($p = 0.0073$) in hepatocytes, and p16 ($p = 0.0001$) and p62 ($p = 0.0033$) expression in cholangiocytes. Platelet (Plt) was also significantly inversely correlated with p16 expression in hepatocytes ($p = 0.0406$) and p16 ($p = 0.0028$) and p62 ($p = 0.0200$) expression in cholangiocytes; as well as with mtDNAcn ($p = 0.0049$).

Association between post-operative prognosis and native liver survival and cell senescence, DNA damage, and autophagy marker expression in patients with BA

Figure 6 shows the Kaplan–Meier curves of the individual factors examined in this study. No significant correlation was detected among the individual markers in terms of cell senescence, DNA damage, and autophagy in either hepatocytes or cholangiocytes and the date of native liver survival (Fig. 6a–h). However, native liver survival was significantly prolonged in BA cases with lower mtDNAcn (Fig. 6i), although no significant differences were detected when examining only those associated with jaundice disappearance after KP (Fig. 6j).

Discussion

This is one of the first studies to simultaneously evaluate markers of cell senescence, DNA damage, and autophagy in both hepatocytes and cholangiocytes, as well as mtDNA copy number in patients with BA who underwent KP and/or LT during their clinical course. The results indicated a clinical significance of the relationship between the expression of these markers and mtDNA damage with BA disease progression. In addition, although few studies have examined liver tissues at KP and LT in the same patients, this is

Table 3 Correlation between clinical characteristics and cell senescence, DNA damage, and autophagy marker expression in patients with BA

| | Hepatocytes | | | | Cholangiocytes | | | | mtDNAcn (N=47) |
|-------|--|---|--|---|--|---|----------------------------------|--|---|
| | p16 Index | p21 Index | γ H2AX index | p62 Index | p16 Index | p21 Index | γ H2AX index | p62 score | |
| T.Bil | $\rho = 0.0361$ $p = 0.7703$ | $\rho = 0.1802$ $p = 0.1413$ | $\rho = 0.0762$ $p = 0.5367$ | $\rho = 0.2097$ $p = 0.0861$ | $\rho = -0.0340$ $p = 0.7831$ | $\rho = 0.2374$ $p = 0.0513$ | $\rho = 0.0085$ $p = 0.9452$ | $\rho = -0.0557$ $p = 0.6519$ | $\rho = 0.5476$ $p = 0.0005$ |
| DBil | $\rho = 0.0587$ $p = 0.6346$ | $\rho = 0.2961$ $p = 0.0142$ | $\rho = 0.1189$ $p = 0.3343$ | $\rho = 0.2344$ $p = 0.0544$ | $\rho = -0.0517$ $p = 0.6757$ | $\rho = 0.2505$ $p = 0.0394$ | $\rho = 0.1525$ $p = 0.2145$ | $\rho = -0.0499$ $p = 0.6862$ | $\rho = 0.4332$ $p = 0.0083$ |
| ALT | $\rho = 0.0174$ $p = 0.8880$ | $\rho = 0.3690$ $p = 0.0020$ | $\rho = -0.0129$ $p = 0.9169$ | $\rho = 0.2792$ $p = 0.0211$ | $\rho = -0.0119$ $p = 0.9232$ | $\rho = 0.3514$ $p = 0.0033$ | $\rho = -0.0001$ $p = 0.9991$ | $\rho = 0.0803$ $p = 0.5151$ | $\rho = 0.2810$ $p = 0.0968$ |
| ChE | $\rho = -0.2721$ $p = 0.0248$ | $\rho = -0.0206$ $p = 0.8675$ | $\rho = -0.3228$ $p = 0.0073$ | $\rho = 0.0934$ $p = 0.4488$ | $\rho = -0.4524$ $p = 0.0001$ | $\rho = 0.0783$ $p = 0.5259$ | $\rho = -0.1566$ $p = 0.2023$ | $\rho = 0.3513$ $p = 0.0033$ | $\rho = 0.1534$ $p = 0.3717$ |
| Plt | $\rho = -0.2490$ $p = 0.0406$ | $\rho = -0.1591$ $p = 0.1949$ | $\rho = -0.1753$ $p = 0.1526$ | $\rho = 0.0586$ $p = 0.6353$ | $\rho = -0.3565$ $p = 0.0028$ | $\rho = -0.1105$ $p = 0.3697$ | $\rho = -0.0631$ $p = 0.6092$ | $\rho = -0.2816$ $p = 0.0200$ | $\rho = 0.4582$ $p = 0.0049$ |

Bold indicates $p < 0.05$

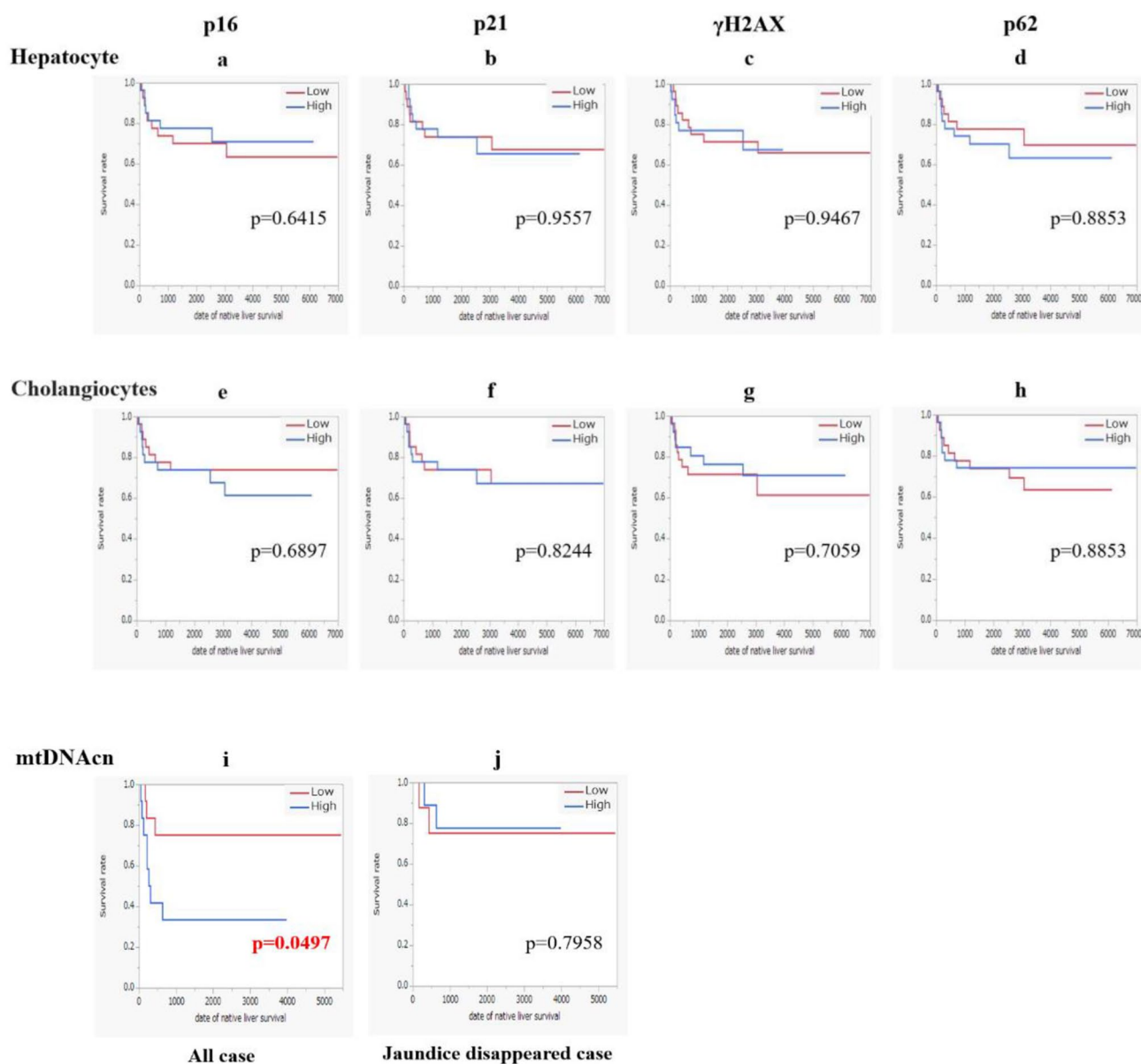


Fig. 6 Native liver survival date analysis using Kaplan–Meier curve in patients with BA. * Indicates $p < 0.05$. p16 (a), p21 (b), γ H2AX (c), and p62 (d) status in hepatocytes. p16 (e), p21 (f), γ H2AX (g), and p62 (h) status in cholangiocytes. mtDNAcn status in all patient

cases indicated that native liver survival date was significantly prolonged when mtDNAcn was low (i). mtDNAcn status in cases where jaundice disappeared (j)

the first report to show that mitochondrial DNA damage and change occur during the clinical course of patients with BA.

The majority of senescence-inducing stressors have been reported to activate either the p16Ink4a and/or p53/p21 intracellular signaling, which have emerged as biomarkers of cellular senescence [36–38]. Bile salt was taken up by transporter Ntcp and accumulated in hepatocytes. Elevated levels of bile salt in hepatocytes caused endoplasmic reticulum stress and mitochondrial damage. This resulted in the release of inflammatory chemokines through Tlr9-dependent signaling pathways. Then reactive oxygen species (ROS)

were released from the induced inflammatory cells, resulting in cytotoxicity [39]. During cell senescence, p21 can contribute to the early stages of the process that are associated with cell stress, making it a marker for the initiation of cell senescence; while p16 plays an important role in maintaining cell senescence [20, 40–43]. Therefore, increased p16 expression at the time of LT in the same BA case could reflect the progression of cell senescence during the clinical development of BA. With respect to hepatocytes, p21 expression increased in livers that received KP, while in cholangiocytes, p16 expression was high in LT and p21 was

high in KP, which was consistent with previously reported findings [22]. The progression of cellular senescence in BA hepatocytes and cholangiocytes was similar following this period in the clinical course of BA; however, the profiles of the phenotypes were partially different. These results will need further investigation to understand how these cells senesce during BA progression.

The correlation of these findings with clinical data from patients with BA also revealed that p16 expression in both hepatocytes and cholangiocytes was significantly inversely correlated with that of ChE and Plt, which could represent viability or biological synthetic activities of hepatocytes. In addition, p16 expression in hepatocytes was significantly positively correlated with the stages of fibrosis, while that in cholangiocytes correlated with the stages of fibrosis and the degree of DR and cholestasis. We found that p21 expression was also significantly positively correlated with giant cell changes in hepatocytes, while decreased expression in cholangiocytes correlated with the progression of fibrosis. These results indicated the synchronization of the progressive status between the disease period and cellular senescence in both hepatocytes and cholangiocytes. Giant cell changes in the liver were reported to be closely associated with cellular senescence [44], which is consistent with the results of this study.

We also used γ H2AX as a marker of DNA double-strand breaks caused by genotoxic substances, such as UV and radiation [45], to further explore the association between the status of DNA damage and senescence status of BA liver samples. DNA damage and altered replication are integral components of the aging phenotype [38, 46]. Our results demonstrated that the number of γ H2AX-positive cells was very small at every stage of the disease, suggesting that double-stranded DNA damage did not frequently occur in BA liver cells, despite abundant noxious stimuli. This is possibly because of more stable DNA in BA cells in contrast to those of various diseases that present with genomic instability, such as neoplastic diseases. 8-hydroxydeoxyguanosine (8-OHdG), a marker of DNA damage due to oxidative stress [47], was reported to be present in hepatocytes of BA liver [48]. 8-OHdG was reported to be produced by reactive oxygen species and induce point mutations during DNA replication [47, 49], whereas γ H2AX was a DNA double-strand breaks marker generally reported as the most deleteriously cytotoxic event caused by irradiation and other noxious stimuli. [45]. Therefore, biliary obstruction could promote ROS oxidative stress in BA liver, subsequently inflicting genotoxicity on the hepatocytes as previously reported but could not reach to the double-stranded DNA cleavage. However, further investigation is required to test this interesting hypothesis.

Autophagy represents genetically regulated mechanisms that involve intracellular turnover of cellular proteins and damaged organelles. p62 is an adaptor protein involved in

the delivery of ubiquitin-bound cargo to the auto-phagosome and helps to regulate the formation of protein aggregates [25, 50–52]. Accumulation of p62 was reported to reflect dysfunctional autophagy [53–55]. In this study, BA livers that received KP had significantly higher levels of p62 expression in hepatocytes, while those that received LT showed a significant increase in cholangiocytes. In addition, hepatocyte p62 expression demonstrated a significant inverse correlation with fibrosis progression. Therefore, in hepatocytes, induction of autophagy occurs in the early stages of BA in the liver, while the relative dysfunction of autophagy could result in cytosolic p62 accumulation. In advanced stages of BA, liver damage is considered irreversible, possibly beyond the autophagy-regulating clearance. In contrast, the induction of autophagy in cholangiocytes was considered to be maintained even at the stage of LT.

Mitochondrial dysfunction due to mtDNA damage has been reported in various disorders, including neurodegenerative diseases, myopathy, and diabetes [56–59]. Mitochondrial dysfunction usually results in cellular dysfunction and senescence [9–11]. Of particular interest, mtDNA was found to be more susceptible to stress-induced damage, including oxidative DNA damage, than nuclear DNA because of its proximity to the potential intracellular sites of oxidative phosphorylation and its intrinsic lack of histones [60, 61]. The TCA cycle is highly active in hepatocytes and mitochondrial function is closely related to hepatocyte function [62, 63]. Therefore, mtDNA damage due to cholestasis may represent a very early stage of hepatocyte dysfunction and cell senescence. In this study, a significant decrease in mtDNAcn was detected in BA livers that had received KP and LT compared to normal livers. Although no significant difference was detected, mtDNAcn tended to decrease in BA livers that had received LT compared to KP livers. These results indicate that mtDNA degradation could occur during cell aging and stages of BA progression in the liver. In addition, when we assessed the correlation of the results with native liver survival in patients with BA, the liver survival rate tended to be higher in those that had a lower mtDNAcn. However, no significant correlation was detected between the mtDNAcn and liver survival time when examined in samples from patients whose jaundice disappeared after KP. Therefore, the effects of other potential factors, such as patient age at time of surgery, could also contribute to the clinical progression of BA in the liver.

BA is a disease caused by obstruction of the extrahepatic bile duct due to sclerosing inflammation of unknown cause [1]. In this study, there were no significant correlation between cholangiocytes and common bile ducts in the same patients in cell senescence, DNA damages, and autophagy markers. Those findings indicated that the degree of sclerosing inflammation of the extrahepatic bile duct, which could represent the major pathophysiology of BA, was not necessarily

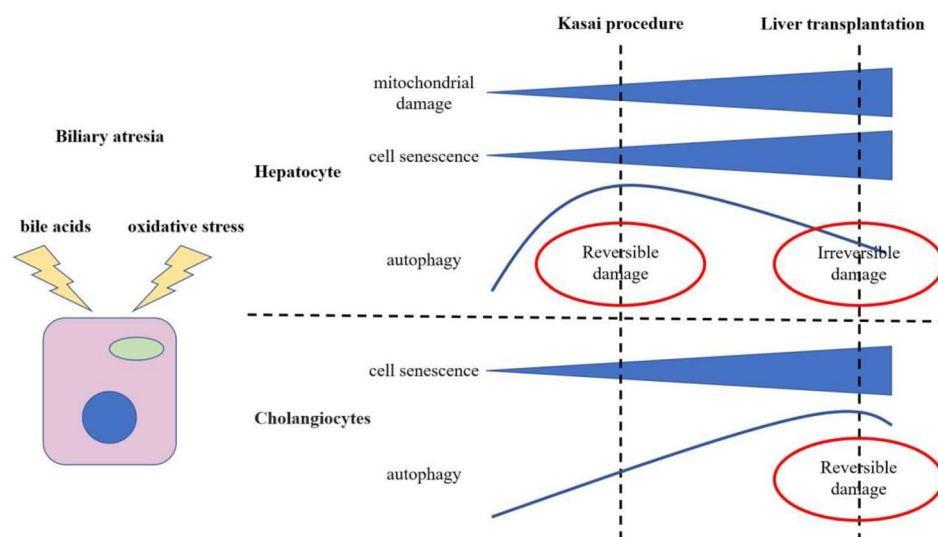


Fig. 7 Predicted cellular injury or damage processes in the liver during biliary atresia (BA). Bile acid accumulation and oxidative stress occur in hepatocytes and cholangiocytes due to cholestasis. In hepatocytes, cellular senescence and mitochondrial damage occur as liver damage progresses. At early stages of BA in livers that undergo KP, the cell clearance mechanisms can be initiated; however, when the liver damage is advanced such that LT was required, the clear-

ance mechanism is decreased. Liver damage can be reversed at early stages of BA in livers that receive KP, but is considered irreversible at advanced stages of BA in livers that require LT. In cholangiocytes, cell senescence progresses similarly to hepatocytes, but the clearance mechanism can be maintained at advanced clinical stages of BA in livers that receive LT

correlated with that of liver injury. Only p16 immunoreactivity was higher in the common bile duct ($p=0.0166$; Table 1). These results therefore suggested that cell senescence in the common bile duct could be more advanced compared to the intrahepatic bile duct and reached the maintenance phase [20, 40–43]. However, this hypothesis needs further investigation.

Figure 7 presents a hypothetical scheme that depicts the association between the senescence, DNA damage, and autophagy markers we tested and the progression of the BA disease period based on the findings of our present study. As demonstrated in Fig. 7, cell senescence might be induced in both hepatocytes and cholangiocytes at relatively early stages of the disease and senescence phenotypes become pronounced as the disease progresses into the advanced stages of BA in the liver. In addition, mtDNA degradation can become more pronounced with disease progression, but can also be detected at earlier stages of the disease, which possibly reflects damage inflicted on hepatocytes. Moreover, cytosolic p62 accumulation becomes pronounced as the BA progresses; however, we detected a discrepancy between hepatocytes and cholangiocytes in the BA liver samples. This finding could reflect a potential discrepancy in the phase of autophagy impairment between hepatocytes and cholangiocytes in the BA liver, but further investigation is required for clarification of this interpretation.

The age matching in this study was not obtained between the control group and BA cases, especially in the LT cases. In addition, BA disease specificity was not

evaluated in the present study; however, our results suggest that cell senescence, mitochondrial DNA damage, and autophagy dysfunction are all associated with the progression of liver cellular damage in patients with BA.

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Declarations

Conflicts of interest The authors have no conflicts of interest.

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