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Review

Cellular senescence in biliary pathology: Special emphasis on expression of a polycomb group protein EZH2 and a senescent marker $p16^{INK4a}$ in bile ductular tumors and lesions

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Summary. A subgroup of intrahepatic cholangiocarcinoma and combined hepatocellular- cholangiocarcinoma contain a component of cholangiolocellular carcinoma, which is composed of small bile ductular cells. Ductular reaction, a reactive lesion at the portal tract interface comprising increased bile ductules, is frequently seen in chronic advanced liver diseases. Bile duct adenoma, a benign tumor/tumorous lesion is also composed of bile ductular cells. Differential diagnosis among these bile ductular tumors/lesions is sometimes difficult. Given overexpression of a polycomb group protein EZH2 in intrahepatic cholangiocarcinoma and high expression of senescence-associated $p16^{INK4a}$ in ductular reactions, we plan to apply immunostaining for EZH2 and $p16^{INK4a}$ for differential diagnosis of these bile ductular tumors/lesions. The expression of EZH2 was seen in all cases of cholangiolocellular carcinomas, while it was not observed in bile duct adenomas or ductular reactions. In contrast, the expression of $p16^{INK4a}$ was seen in most bile duct adenomas and all ductular reactions, whereas it was barely seen in cholangiolocellular carcinomas. A borderline between cholangiolocellular carcinoma and the surrounding ductular reaction was clearly highlighted by the reverse expression pattern of EZH2 and $p16^{INK4a}$. In conclusion, immunostaining for EZH2 and $p16^{INK4a}$ may be useful for differential diagnosis for bile ductular tumors/lesions.

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Introduction

Bile ductular tumors and lesions that are composed of small bile ductular cells in the liver include cholangiolocellular carcinoma, ductular reaction, bile duct adenoma, parenchymal extinction, and so on. Differential diagnosis among these bile ductular tumors/lesions is sometimes difficult, since these tumors/ lesions show similar histological findings and cellular atypia in cholangiolocellular carcinoma is generally mild. In this review, we described an application of the immunostaining for enhancer of zeste homologue 2 (EZH2) and $p16^{INK4a}$ as a useful tool for differential diagnosis of these bile ductular tumors/lesions.

Bile ductular tumors and lesions in the liver

Cholangiolocellular carcinoma (Fig. 1A)

Intrahepatic cholangiocarcinomas arising in chronic liver diseases sometimes show histological features of cholangiolocellular carcinomas and share characteristics with cholangiocarcinoma components in combined hepatocellular and cholangiocarcinoma (Sasaki et al., 2003; Xu et al., 2011, 2012). According to the WHO classification 2010, combined hepatocellular and cholangiocarcinomas are classified into classical type

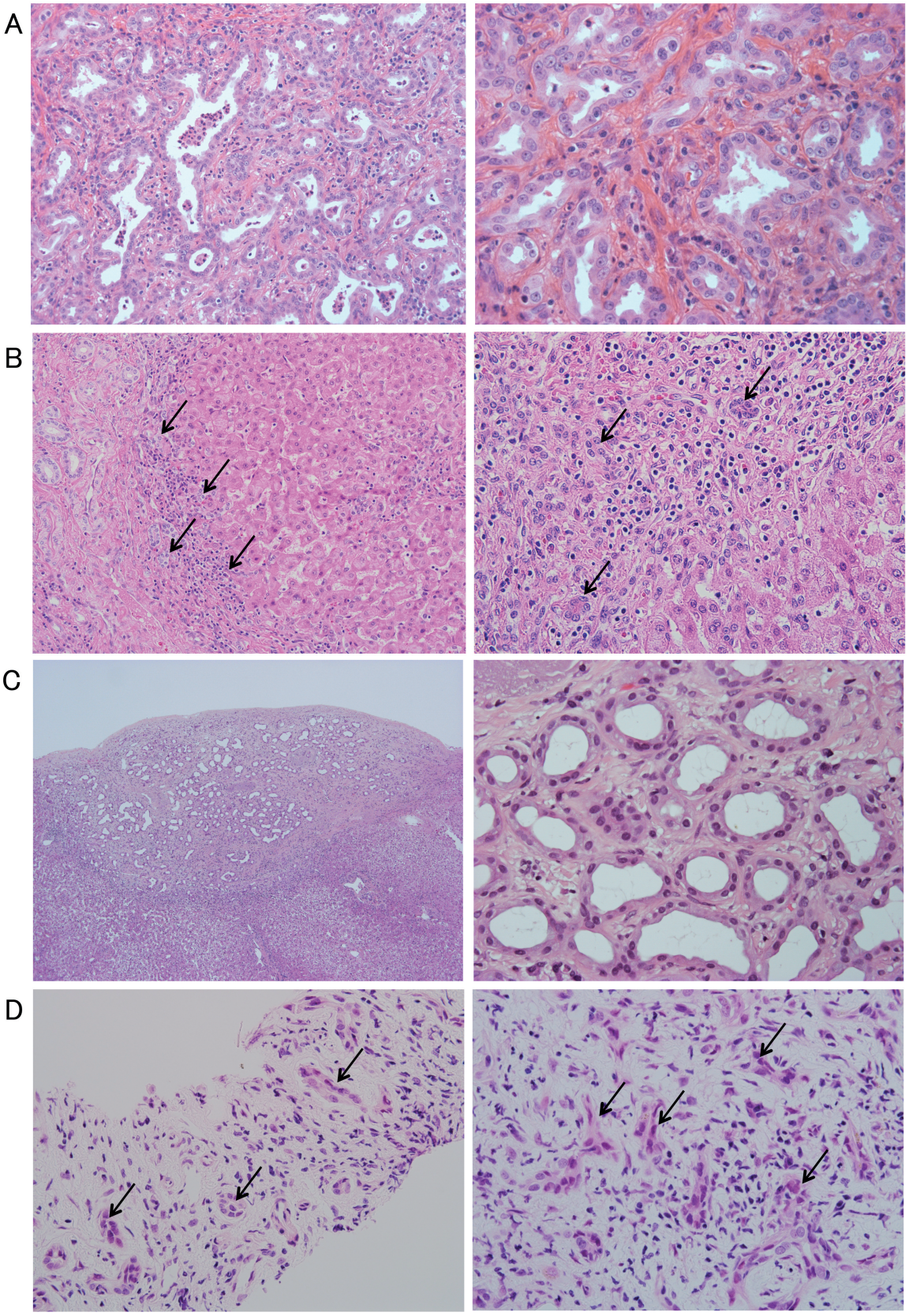

Fig. 1. Histology of bile ductular tumors and lesions in the liver. **A.** Cholangiolocellular carcinoma. Hematoxylin and eosin. x 200 (left), x 400 (right). **B.** Ductular reactions. Arrows indicate bile ductular components. Hematoxylin and eosin. x 5\10 (left), x 200 (right). **C.** Bile duct adenoma. Hematoxylin and eosin. x 50 (left), x 400 (right). **D.** Parenchymal extinction. Arrows indicate bile ductular components. Hematoxylin and eosin. x 100 (left), x 400 (right).

and subtypes with stem cell features, and the latter include the typical subtype, intermediate cell subtype and cholangiolocellular type (Theise et al., 2010). Cholangiolocellular carcinoma is composed of small bile ductular carcinoma cells resembling a ductular reaction (Roskams et al., 2004), and is also called bile ductular carcinoma (Kozaka et al., 2007). An infiltrating replacing growth pattern is a feature of cholangiolocellular carcinoma (Kozaka et al., 2007). Similar lesions include ductular reaction, parenchymal extinction and bile duct adenoma.

Ductular reaction (Fig. 1B)

Ductular reaction is a reactive lesion at the portal tract interface comprising increased bile ductules with an accompanying complex of stromal and inflammatory cells (Roskams et al., 2004). Ductular reaction is observed in the periphery of portal tracts and fibrous septa in advanced chronic liver disease (Roskams et al., 2004) and also around liver tumors such as hepatocellular carcinomas. Bile ductules are characterized by tubular or glandular structures with a poorly defined lumen, are located at the periphery of the portal tracts and are not accompanied by parallel hepatic arterial branches (Nakanuma and Sasaki, 1989; Roskams et al., 2004). Ductular cells in ductular reactions include intermediate hepatobiliary cells with heterogeneous phenotype in the diseased liver (Roskams et al., 2004). Bile ductular cells occasionally show cellular atypia due to cell injuries such as inflammation (Kozaka et al., 2007).

Bile duct adenoma (Fig. 1C).

Bile duct adenoma is a small, usually single, <1cm in size, and subcapsular well-circumscribed mass composed of the proliferation of small, uniform small ducts with cuboidal cells that have regular nuclei (bile ductular component) set in fibrous stroma (Hughes et al., 2010; Nakanuma et al., 2010; Goodman et al., 2012). Current evidence suggests that it is a tumor-like lesion that results from a proliferative response to a localized injury (Goodman et al., 2012). Bile duct adenoma is asymptomatic and discovered incidentally during abdominal surgery or at autopsy. The major importance of the bile duct adenoma is its possible confusion with intrahepatic cholangiocarcinoma or metastatic adenocarcinoma (Hughes et al., 2010; Nakanuma et al., 2010; Goodman et al., 2012). Some bile duct adenomas arise in chronic liver diseases such as chronic viral hepatitis and their potential as precursor lesions for cholangiocarcinoma has been implied (Hasebe et al., 1995).

Parenchymal extinction (Fig. 1D)

Parenchymal extinction is a lesion which is composed of contiguous loss of hepatocyte and formation of fibrous area (Wanless and Huang, 2012). Parenchymal extinction occurs in chronic liver disease and usually begins with small parenchymal extinction lesions (PELs). PELs aggregate into confluent regions of extinction that result in the histological pattern recognized as cirrhosis. Sometimes, a lesion called "regional parenchymal extinction" occurs in cirrhosis (Wanless and Huang, 2012). Parenchymal extinction contains preexisting portal tracts, hepatic veins and condensed parenchymal stroma with ductular reaction. Intimal fibrosis and luminal narrowing is usually seen in the hepatic vein in this type of regional parenchymal extinction (Wanless and Huang, 2012). This localized lesion sometimes resembles cholangiolocellular carcinoma, especially when it is taken by needle biopsy and differential diagnosis is needed.

Expression of EZH2 and p16^{INK4a} in hepatobiliary diseases and tumors.

Expression of a polycomb group protein (PcG) EZH2 as a marker of malignant tumor

EZH2

PcG proteins are epigenetic chromatin modifiers involved in cancer development and the roles of PcG proteins are now being evaluated in many human malignancies (Valk-Lingbeek et al., 2004). PcG proteins exist in at least two separate protein complexes: Polycomb repressive complex 2 (PRC2) and PRC1 (Lund and van Lohuizen, 2004; Valk-Lingbeek et al., 2004). PRC2 is thought to be required in the initial stage of silencing. On the other hand, PRC1 is continuously required for stable maintenance of the initiated PcG repression on specific target loci (Valk-Lingbeek et al., 2004). EZH2 is a component of PRC2, whereas Bmi1 is a representative component of PRC1 in mammals (Lund and van Lohuizen, 2004; Valk-Lingbeek et al., 2004). Several studies showed that EZH2 and Bmi1 are chromatin modifying enzymes and reportedly interact with the pathways of key elements that control cell growth and proliferation, such as *Rb* and *p16^{INK4a}* genes (Jacobs et al., 1999; Valk-Lingbeek et al., 2004), and that an abnormal expression of EZH2 and Bmi1 is involved in the processes of development and progression of malignant tumors (Varambally et al., 2002; Kleer et al., 2003; Park et al., 2004; Valk-Lingbeek et al., 2004; Tateishi et al., 2006). An aberrant expression of EZH2 is regarded as a potential marker of advanced or aggressive cancers with poor prognosis, differing from benign or indolent counterparts (Varambally et al., 2002; Kleer et al., 2003).

Overexpression of EZH2 in primary liver cancer

We reported the overexpression of EZH2 in intrahepatic cholangiocarcinomas, including cholangiolocellular carcinomas and combined

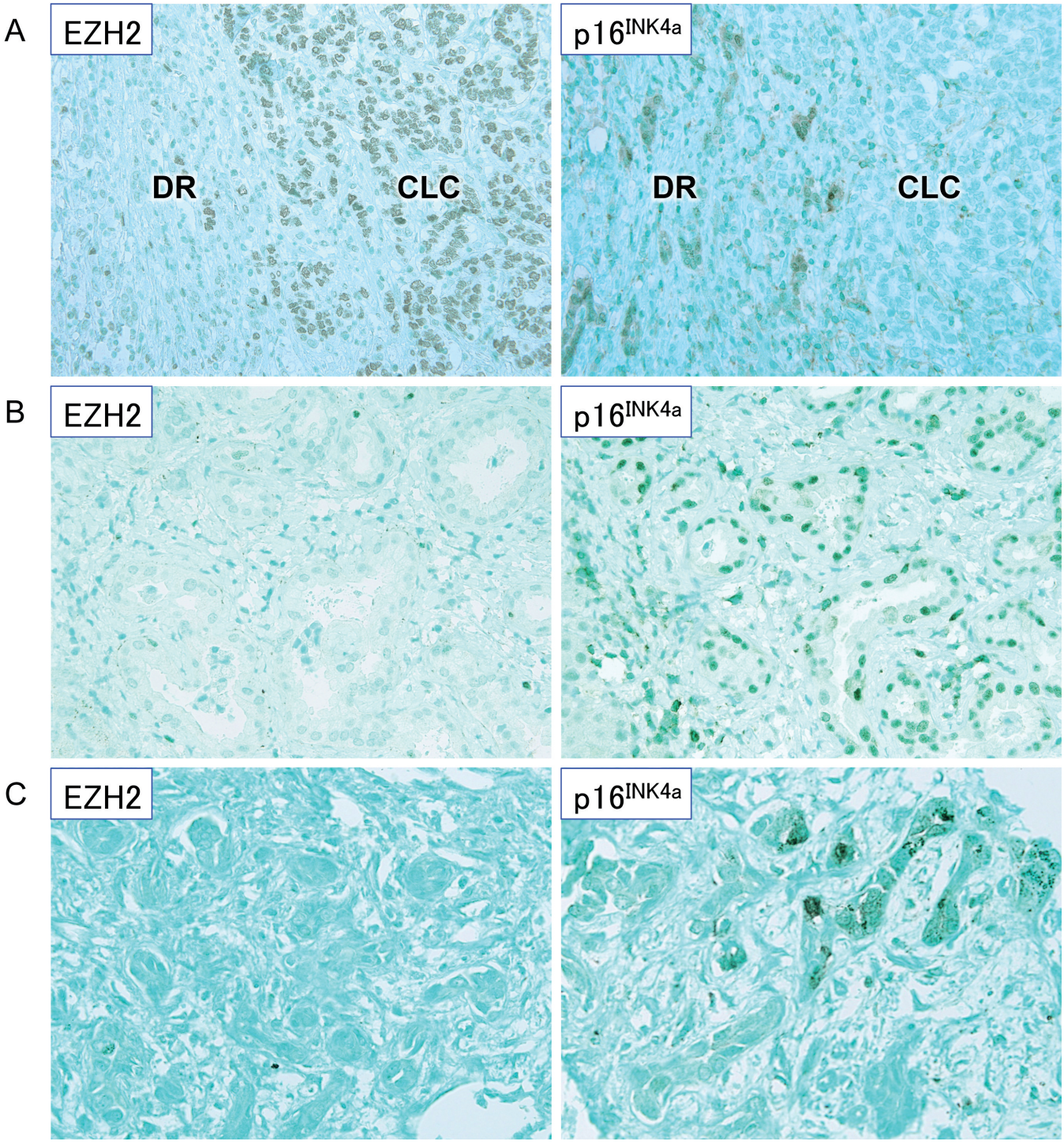

Fig. 2. Expression of p16^{INK4a} and EZH2 in bile ductular tumors and lesions in the liver. **A.** Cholangiolocellular carcinoma and ductular reactions. EZH2 is extensively expressed in the nuclei of carcinoma cells in cholangiolocellular carcinomas (CLC), whereas ductular reactions (DR) are negative for EZH2 (middle). p16^{INK4a} is extensively expressed in the nuclei and cytoplasm of cells in ductular reactions, while it is negative in carcinoma cells in cholangiolocellular carcinomas (right). The reverse expression pattern of EZH2 and p16^{INK4a} highlights the borderline of these 2 components. **B.** Bile duct adenoma. EZH2 is not expressed in bile duct adenomas (left), while p16^{INK4a} is extensively expressed (right). **C.** Parenchymal extinction. EZH2 is not expressed in bile ductular cells in parenchymal extinction (left), while p16^{INK4a} is focally expressed (right). Immunostaining for EZH2 (left) and p16^{INK4a} (right). x 200

hepatocellular and cholangiocarcinomas (Sasaki et al., 2008a,b). The expression of EZH2 in non-neoplastic components such as bile ducts and hepatocytes was low in previous studies (Sasaki et al., 2008; Sasaki et al., 2008b,c). Several other studies also demonstrated overexpression of EZH2 in primary liver carcinoma (Au et al., 2012; Hajosi-Kalcakosz et al., 2012). Overexpressed EZH2 is thought to repress the expression of senescence-associated p16^{INK4a} in the process of multi-step cholangio-carcinogenesis through intraepithelial neoplasm in large bile ducts, for example, in cholangiocarcinoma associated with hepatolithiasis and cholangiocarcinoma associated with pancreatico-biliary malformation (Sasaki et al., 2008b,c; Yamaguchi et al., 2009).

Expression of p16^{INK4a} as a marker of cellular senescence

Cellular senescence

Cellular senescence is defined as a condition in which a cell no longer has the ability to proliferate (Collado et al., 2007). Senescent cells are irreversibly arrested at the G1 phase of the cell cycle and do not respond to various external stimuli, but remain metabolically active (Collado et al., 2007). Cellular senescence is implicated in several pathological responses in the adult, with important repercussions in tumor suppression, wound healing, and aging (Collado et al., 2007). There are two types of cellular senescence, replicative senescence and stress-induced, or premature senescence. Normal cells can only divide a finite number of times before they reach a state of replicative cellular senescence. Cellular senescence can be triggered by multiple mechanisms, including telomere shortening, epigenetic derepression of the INK4a/ARF locus, and DNA damage (Collado et al., 2007). Cellular senescence imposes a potent barrier to tumor genesis and contributes to the cytotoxicity of certain anti-cancer agents (Braig et al., 2005; Collado et al., 2007). Senescent cells display several characteristics, including histological changes in vitro and in vivo (Brodsky and Uryvaeva, 1977; Sigal et al., 1999), shortened telomeres, increased activity of senescence-associated β-galactosidase (SA-β-gal) and increased expression of p16^{INK4} and p21$^{WAF1/CIP1}$ (Dimri et al., 1995). Autophagy is induced during the process of senescence and facilitates cellular senescence (Young et al., 2009; Sasaki et al., 2010b).

Recent studies suggest that senescent cells play an important role in modulating the microenvironment by secreting biological active molecules, called senescence-associated secretory phenotypes (SASPs). SASPs include cytokines (IL-6, IL-1 and so on), chemokines (CXCL8/IL-8, CCL2/monocyte chemotactic protein-1 (MCP)-1) and so on), growth factors and profibrogenic factors (Shelton et al., 1999; Acosta et al., 2008; Coppe et al., 2008; Kuilman et al., 2008; Wajapeyee et al., 2008).

Biliary epithelial senescence.

Recent progress in the field of hepatology has disclosed that cellular senescence is involved in the pathophysiology of various chronic liver diseases (Lunz et al., 2001; Paradis et al., 2001; Wiemann et al., 2002; Sasaki et al., 2005, 2006, 2008; Krizhanovsky et al., 2008) and hepatocarcinogenesis (Plentz et al., 2005, 2007). Accumulating evidence suggests that cellular senescence is also involved in bile duct lesions in PBC and ductular reaction in various chronic advanced liver diseases (Sasaki et al., 2005, 2008a, 2010a,b; Chiba et al., 2011).

Damaged small bile duct in PBC: We have reported the cellular senescence of BECs with shortened telomeres, the expression of SA-β-gal, and the augmented expression of p16^{INK4a} and p21$^{WAF1/Cip1}$ in damaged small bile ducts in PBC (Sasaki et al., 2005, 2008a). This suggests that cellular senescence may be involved in the pathogenesis of progressive bile duct loss in PBC. Oxidative stress due to inflammation may play a role for the induction of cellular senescence (Sasaki et al., 2005, 2006, 2008c,d). Furthermore, senescent BECs may be involved in modulation of the inflammatory microenvironment around affected small bile ducts by recruiting monocytes and possibly other types of inflammatory cells via SASP, such as CCL2 and CX3CL1 in PBC (Sasaki et al., 2010b, 2014a).

Ductular reaction: Senescence-associated p16^{INK4a} and p21$^{WAF1/Cip1}$ are frequently expressed in ductular cells in ductular reaction in the advanced stage of various chronic liver diseases, including PBC and nonalcoholic steatohepatitis (NASH) (Sasaki et al., 2005, 2008a, 2010a). A proportion of ductular cells expressing p16^{INK4a} and p21$^{WAF1/Cip1}$ are particularly frequent in PBC at the advanced stages (Sasaki et al., 2010a). Ductular cells expressing p16^{INK4a} and p21$^{WAF1/Cip1}$ are always positive for cyclin D, but negative for cyclin A, and show no Ki-67 labeling, suggesting that some ductular cells in ductular reactions in chronic liver diseases are at G1-arrest and undergoing cellular senescence (Sasaki et al., 2005, 2008a, 2010a).

Senescent bile ductular cells in ductular reactions may be involved in the progression of fibrosis in these diseases (Sasaki et al., 2008a, 2010a). For example, bile ductular cells in ductular reactions express CCL2 as SASP, which may be responsible for chemoattraction of activated hepatic stellate cells (HSCs) and inflammatory cells and subsequent fibrosis in PBC and NASH (Chiba et al., 2011; Sasaki et al., 2012). In fact, the migration of cultured mouse HSCs was significantly facilitated in the presence of cultured senescent mouse BECs, and this migration was mediated by CCL2 secreted from senescent BECs (Chiba et al., 2011).

Application of immunostaining for EZH2 and p^{16INK4a} for differential diagnosis of bile ductular tumors/lesions

Given the high expression characteristically seen in cholangiolocellular carcinomas and ductular reaction, respectively, we examined whether immunostaining for EZH2 and p16^{INK4a} would be useful for the differential diagnosis between cholangiolocellular carcinomas and ductular reactions (Sasaki et al., 2014b). Furthermore, we examined the expressions of EZH2 and p16^{INK4a} in bile duct adenoma to clarify the nature of this lesion. In addition, we examined the expression of EZH2 and p16^{INK4a} in cholangiolocellular carcinoma.

Cholangiolocellular carcinoma

The expression of EZH2 was seen in the nuclei of carcinoma cells in all cases (Fig. 2). About three quarter cases of cholangiolocellular carcinomas showed extensive expression (score 2) (Fig. 3). The expression of EZH2 was significantly higher in cholangiolocellular carcinomas than ductular reactions and bile duct adenomas ($p<0.05$) (Fig. 3). The expression of p16^{INK4a} was seen in the nuclei and cytoplasm of cells, when present (Fig. 2). Cell cycle inhibitor p16^{INK4a} is expressed both in the nucleus and cytoplasm, and nuclear immunoreactivity implied the active function of p16^{INK4a} in association with senescence (Witkiewicz et al., 2011). The expression of p16^{INK4a} was seen in about one third of cases, but extensive expression of p16^{INK4a} was seen only in limited cases in cholangiolocellular carcinomas. The expression of p16^{INK4a} was significantly lower in cholangiolocellular carcinomas than ductular reactions and bile duct adenomas ($p<0.05$) (Fig. 3).

Ductular reactions

Ductular reactions were seen around carcinoma in most cases with cholangiolocellular carcinomas, in parallel to the degree of fibrosis. The expression of EZH2 was not observed in bile ductular cells in ductular reactions (Figs. 2, 3). In contrast, the expression of p16^{INK4a} was seen in all cases of ductular reactions, and more than half of ductular reactions showed extensive expression of p16^{INK4a} (Figs. 2, 3). The borderline between cholangiolocellular carcinomas and surrounding ductular reactions is clearly highlighted by the reverse expression pattern of EZH2 and p16 INK4a (Fig. 2) in about two-thirds of cholangiolocellular carcinomas with surrounding ductular reactions.

Bile duct adenoma. The expression of EZH2 was not observed in bile ductular cells in bile duct adenomas (Figs. 2, 3). The expression of p16^{INK4a} was seen in most cases and extensive expression was observed in about one third of cases (Figs. 2, 3). Taken together, bile duct adenoma showed a similar expression pattern of EZH2 and p16^{INK4a} to ductular reactions, which is different from cholangiolocellular carcinomas.

Parenchymal extinction. Marked ductular reactions were occasionally observed in the area of collapse or extinction associated with cirrhosis. In addition, a florid ductular reaction was observed in the setting of hepatic

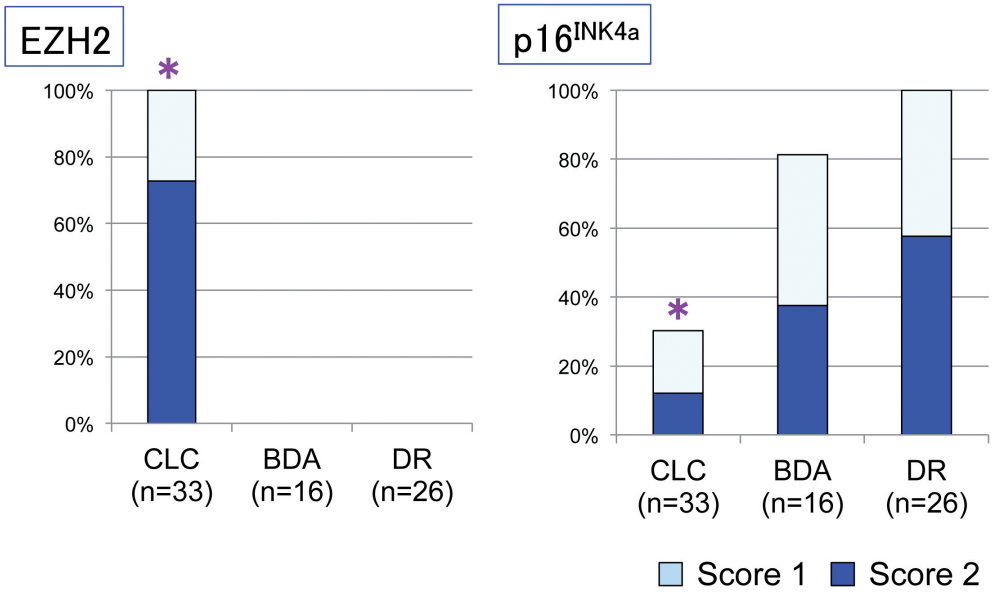

Fig. 3. Semi-quantitative analysis of EZH2 and p16^{INK4a} expression in cholangiolocellular carcinoma (CLC), ductular reactions (DR) and bile duct adenomas (BDA). Half-tone, score 1 (focal expression); black, score 2 (extensive expression); *$p<0.05$ vs. ductular reactions and bile duct adenomas (Sasaki et al., 2014).

regeneration following fulminant hepatitis. In our study, ductular cells in parenchymal extinction associated with cirrhosis and in fulminant hepatitis showed expression of p16^{INK4a}, whereas there was no expression of EZH2 (Fig. 2), similarly to the ductular reactions.

Discussion

Our studies demonstrate that the immunostaining for EZH2 and p16^{INK4a} highlighted the difference between cholangiolocellular carcinoma and ductular reactions (Sasaki et al., 2008c, 2010a; Sasaki et al., 2014b). Furthermore, the expression pattern of these markers is different in cholangiolocellular carcinomas from bile duct adenomas and parenchymal extinction (Sasaki et al., 2014b). Taken together, immunostaining for EZH2 and p16^{INK4a} may be useful to make a differential diagnosis between cholangiolocellular carcinomas, ductular reactions, bile duct adenomas and parenchymal extinction in cirrhosis. As expected, a borderline between cholangiolocellular carcinomas and ductular reactions was clearly highlighted by the reverse expression pattern of EZH2 and p16^{INK4a} in two thirds of cases with cholangiolocellular carcinomas and ductular reactions (Sasaki et al., 2014b).

There are 2 possibilities to explain the significance of this reverse expression pattern EZH2 and p16^{INK4a} in cholangiolocellular carcinomas and ductular reactions (Fig. 4). One possibility is that the microenvironment around tumor nodules, such as ischemia and/or starvation due to altered blood supplies, may further accelerate cellular senescence in ductular reactions around tumor nodules (Fig. 4). Alternatively, an extensive expression of senescence-associated p16^{INK4a} in ductular reactions and bile duct adenomas may suggest that these lesions are premalignant lesions harboring genetic alterations such as K-ras mutation (oncogene-induced senescence) (Braig et al., 2005) (Fig. 4). We have previously reported that cellular senescence may be involved in the development of cholangiocarcinoma arising in the large bile duct via biliary intraepithelial neoplasia (Sasaki et al., 2008b; Yamaguchi et al., 2009); that is, the expression of senescence-associated p16^{INK4a} occurs in the early stage, then overexpression of EZH2 plays a role in the bypass/escape from senescence followed by the development of overt carcinoma (Sasaki et al., 2008; Yamaguchi et al., 2009). The carcinogenesis pathways and precursor lesions regarding the peripheral type of intrahepatic cholangiocarcinomas and cholangiolocellular carcinomas remain unclear, so far. The senescent cells in ductular reactions and bile duct adenomas may reflect premalignant conditions harboring genetic alterations, and EZH2 may be involved in the bypass/escape of cellular senescence in carcinogenesis of cholangiolocellular carcinoma, similarly to that in the large bile ducts (Sasaki et al., 2008b; Yamaguchi et al., 2009). This hypothesis seems to be attractive, although further studies are needed to confirm this point.

Concluding remarks

In conclusion, immunostaining for EZH2 and p16 INK4a may be a useful tool for the differential diagnosis of bile ductular tumors and lesions, including cholangiolocellular carcinomas, bile duct adenomas, ductular reactions and parenchymal extinctions. The expression pattern of EZH2 and p16 INK4a may represent an involvement of cellular senescence in the carcinogenesis of cholangiolocellular carcinoma, similarly to cholangiocarcinoma arising in large bile ducts via premalignant lesion, biliary intraepithelial

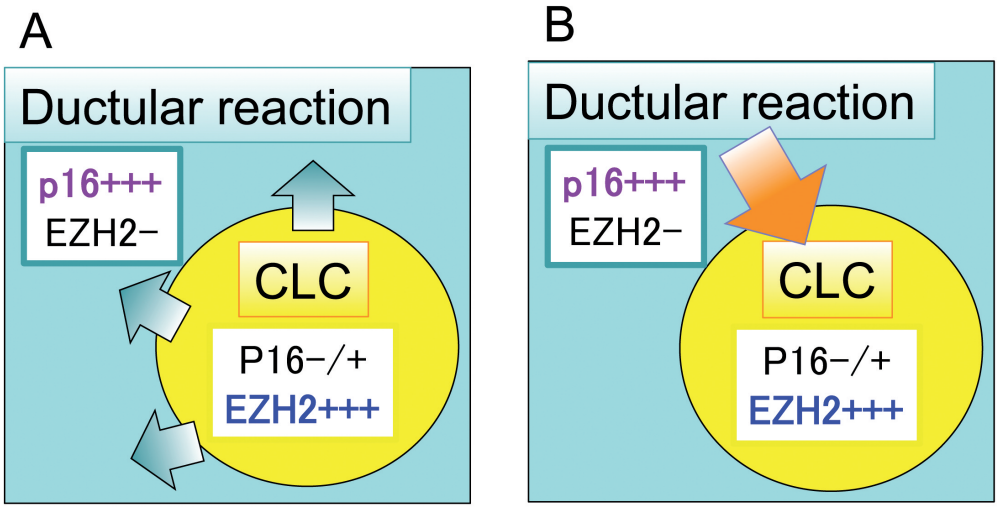

Fig. 4. Two possibilities to explain the significance of expression pattern of EZH2 and p16^{INK4a} in cholangiolocellular carcinoma (CLC) and ductular reaction (DR). **A.** Microenvironment changes around tumor nodules, such as ischemia and/or starvation due to altered blood supplies, may further accelerate cellular senescence in ductular reactions around tumor nodules. **B.** Extensive expression of senescence-associated p16^{INK4a} in ductular reactions suggests that ductular reactions around a cholangiolocellular carcinoma may be premalignant lesions harboring genetic alterations such as K-ras mutation (oncogene-induced senescence), in which the cholangiolocellular carcinoma arises.

neoplasia.

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