Int J Clin Exp Pathol 2014;7(8):4745-4754
www.ijcep.com /ISSN:1936-2625/IJCEP0000998

Original Article

Different carcinogenic process in cholangiocarcinoma cases epidemically developing among workers of a printing company in Japan

Yasunori Sato[1], Shoji Kubo[2], Shigekazu Takemura[2], Yasuhiko Sugawara[3], Shogo Tanaka[4], Masahiro Fujikawa[5], Akira Arimoto[6], Kenichi Harada[1], Motoko Sasaki[1], Yasuni Nakanuma[1,7]

[1]Department of Human Pathology, Kanazawa University Graduate School of Medicine, Kanazawa, Japan; [2]Department of Hepato-Biliary-Pancreatic Surgery, Osaka City University Graduate School of Medicine, Osaka, Japan; [3]Artificial Organ & Transplantation Division, Department of Surgery, University of Tokyo, Tokyo, Japan; [4]Department of Surgery, Ishikiri Seiki Hospital, Osaka, Japan; [5]Department of Surgery, Nissay Hospital, Osaka, Japan; [6]Department of Hepato-Biliary-Pancreatic Surgery, Osaka Red Cross Hospital, Osaka, Japan; [7]Department of Pathology, Shizuoka Cancer Center, Shizuoka, Japan

Received June 3, 2014; Accepted July 16, 2014; Epub July 15, 2014; Published August 1, 2014

Abstract: Recently, cholangiocarcinoma has epidemically developed among young adult workers of a printing company in Japan. Exposure to organic solvents including 1,2-dichloropropane and/or dichloromethane is supposed to be associated with the carcinoma development. The metabolism of dichloromethane proceeds through a Theta-class glutathione S-transferase (GST) T1-1-catalyzed pathway, where its reactive intermediates have been implicated in genotoxicity and carcinogenicity. This study examined features of the carcinogenic process of the cholangiocarcinoma developed in the printing company. Surgically resected specimens of the cholangiocarcinoma cases were analyzed, where all cases were associated with precursor lesions such as biliary intraepithelial neoplasia (BilIN) and/or intraductal papillary neoplasm of the bile duct (IPNB). Immunohistochemical analysis confirmed constitutional expression of GST T1-1 in normal hepatobiliary tract. Immunostaining of γ-H2AX, a marker of DNA double strand break, showed that its expression was significantly increased in foci of BilIN, IPNB and invasive carcinoma as well as in non-neoplastic biliary epithelial cells of the printing company cases when compared to that of control groups. In the printing company cases, immunohistochemical expression of p53 was observed in non-neoplastic biliary epithelial cells and BilIN-1. Mutations of KRAS and GNAS were detected in foci of BilIN in one out of 3 cases of the printing company. These results revealed different carcinogenic process of the printing company cases, suggesting that the exposed organic solvents might act as a carcinogen for biliary epithelial cells by causing DNA damage, thereby contributing to the carcinoma development.

Keywords: Occupational cholangiocarcinoma, carcinogenesis, organic solvent, glutathione S-transferase, DNA damage

Introduction

Chronic biliary inflammation as occurs in primary sclerosing cholangitis and hepatolithiasis is a risk factor for the development of cholangiocarcinoma [1]. Biliary epithelial damage due to chronic inflammation can lead to the development of precursor lesions of cholangiocarcinoma such as biliary intraepithelial neoplasia (BilIN) and intraductal papillary neoplasm of the bile duct (IPNB), and cholangiocarcinoma under the condition of chronic biliary inflammation often represents a multistep carcinogenesis process [2]. The patient age over 65 years is also a risk factor of cholangiocarcinoma, and it is rarely diagnosed before 40 years of age except in patients with predisposal factors such as primary sclerosing cholangitis [1, 3].

Recently, epidemical development of cholangiocarcinoma among young adult men has been reported in Japan, in which all patients were workers of a printing company [4, 5]. At least 17 men suffered from cholangiocarcinoma arising from the large bile ducts, and their mean age was 36 years (range, 25 to 45 years) [5]. In the

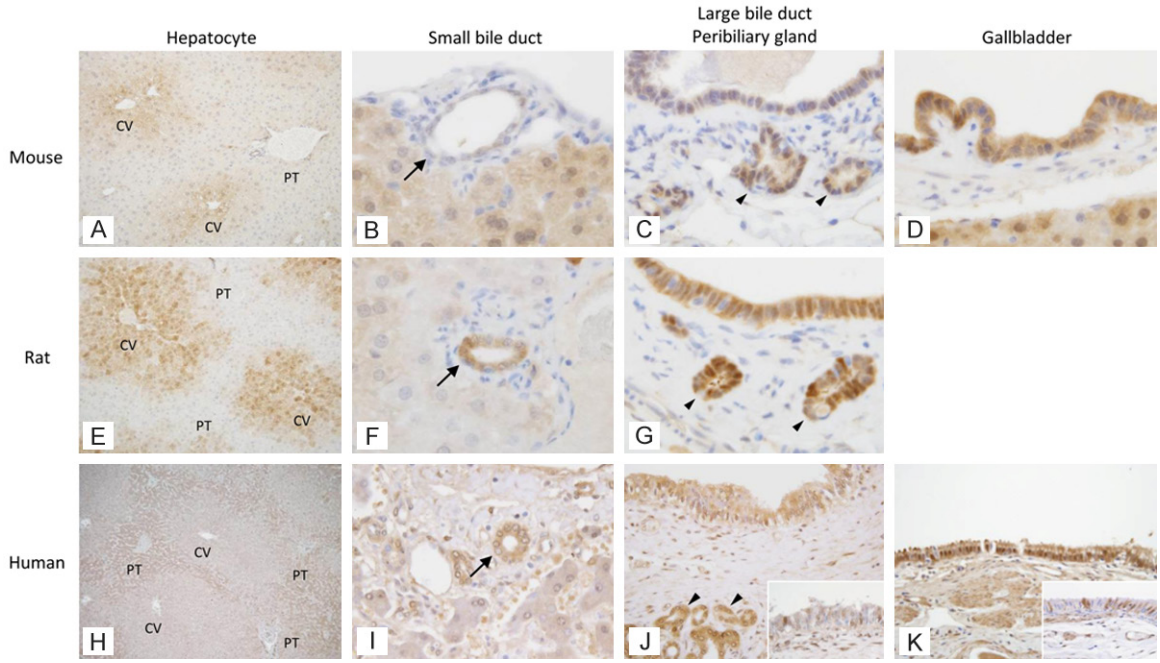

Figure 1. Distribution of GST T1-1 in normal hepatobiliary tract. Immunohistochemical expression of GST T1-1 was observed in hepatocytes and biliary epithelial cells of normal mouse, rat and human. Arrows and arrowheads indicate small bile ducts and peribiliary glands, respectively. Insets (J and K) were images taken from another part of the same case of each figure, showing heterogeneous expression of GST T1-1 in a single case. CV, central vein; PT, portal tract. Original magnifications: (A, E, H); x200: (B-D, F, G), (J) (inset), (K) (inset); x1000; (I-K); x400.

printing company, they engaged in offset color proof-printing using several organic solvents including 1,2-dichloropropane (1,2-DCP) and dichloromethane (DCM), where 1,2-DCP and DCM are classified as group 1 (carcinogenic to humans) and group 2A (probably carcinogenic to humans), respectively, according to the latest classification by the International Agency for Research on Cancer [6, 7]. In this series, DNA damage of biliary epithelial cells due to exposure to organic solvents including 1,2-DCP and/or DCM is supposed to be associated with the carcinogenic process, although the exact mechanism of the outbreak of cholangiocarcinoma remains to be determined.

In mammalian species, the metabolism of DCM proceeds through two pathways; a cytochrome P450 (CYP) 2E1 dependent oxidative pathway producing carbon monoxide, and a Theta-class glutathione S-transferase (GST) T1-1-catalyzed pathway resulting in the production of two highly reactive intermediates, formaldehyde and S-(chloromethyl) glutathione, and carbon dioxide [8]. The proportion of DCM metabolized via the GST pathway increases at higher exposures. Although CYP and GST are considered detoxification pathways for many chemicals, in the case of DCM it is the GST pathway that has been most strongly implicated in genotoxicity and carcinogenicity [9], while the involvement of the GST pathway in the metabolism of 1,2-DCP has not been fully clarified. To understand the mechanism of cholangiocarcinoma development in relation to the exposure to organic solvents, it is necessary to know the normal distribution of GST T1-1 and CYP2E1 in hepatobiliary tract. To date, however, detailed data on the distribution of the enzymes, especially GST T1-1, are lacking.

This study examined the immunohistochemical expression of GST T1-1 and CYP2E1 in normal hepatobiliary tract of mouse, rat and human. The DNA damage of biliary epithelial cells in the cholangiocarcinoma cases of the printing company was evaluated using immunohistochemistry by detecting the expression of γ-H2AX as a marker of DNA double strand break. Mutation analysis of KRAS and GNAS was also performed for the cases.

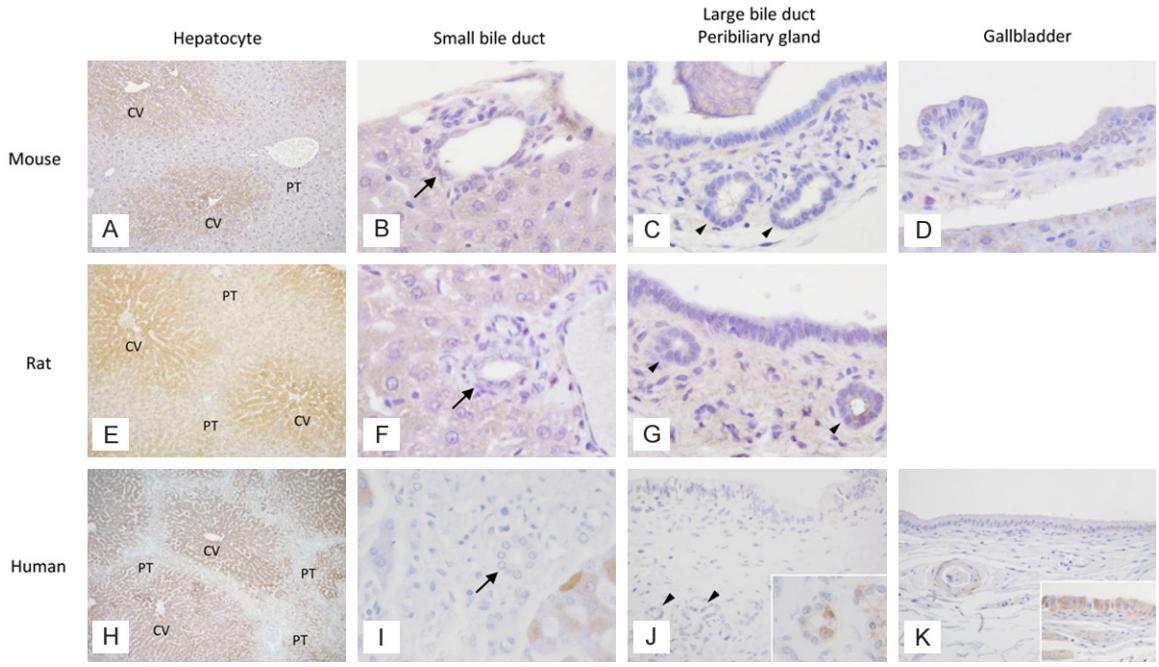

Figure 2. Distribution of CYP2E1 in normal hepatobiliary tract. Immunohistochemical expression of CYP2E1 was observed in zone 3-2 hepatocytes of normal liver of mouse, rat and human. Biliary epithelial cells typically lacked the expression of CYP2E1, but several human cases showed focal and weak immunohistochemical expression of CYP2E1 in the epithelium of peribiliary glands and gallbladder (J and K, insets). Arrows and arrowheads indicate small bile ducts and peribiliary glands, respectively. CV, central vein; PT, portal tract. Original magnifications: (A, E, H); x200: (B-D, F, G), (J) (inset), (K) (inset); x1000; (I-K); x400.

Table 1. Immunohistochemical expression of GST T1-1 and CYP2E1 in epithelial cells of normal hepatobiliary tract of mouse, rat and human

Enzyme	Species	Hepatocyte	Small bile duct	Large bile duct	Peribiliary gland	Gallbladder
GST T1-1	Mouse	+	-~+	+	+	++
	Rat	+~++	+~++	++	++	
	Human	+	+~++	+~++	+~++	+~++
CYP2E1	Mouse	+	-	-	-	-
	Rat	+	-	-	-	
	Human	+	-	-	-~+	-~+

CYP, cytochrome P450; GST, glutathione S-transferase. -, negative; +, positive (weak to moderate); ++, positive (marked).

Materials and methods

Tissue preparation

The experiments were performed in accordance with the guidelines for the care and use of laboratory animals of Kanazawa University and the World Medical Association's Declaration of Helsinki. Samples of normal liver and gallbladder were taken from 8-week-old ICR mice (n = 10), and samples of normal liver were from 8-week-old F344 rats (n = 10). Human liver samples (n = 30; mean age, 72 years) were obtained from the hilar region of the liver from autopsy files of our department. Histological examination confirmed that the human liver samples were almost normal. Human gallbladder samples (n = 15; mean age, 66 years) were obtained at the time of gastrectomy and cholecystectomy due to cholecystolithiasis, and the extent of inflammation in the gallbladder was histologically mild or minimal for all cases. The samples were formalin-fixed, and paraffin-embedded.

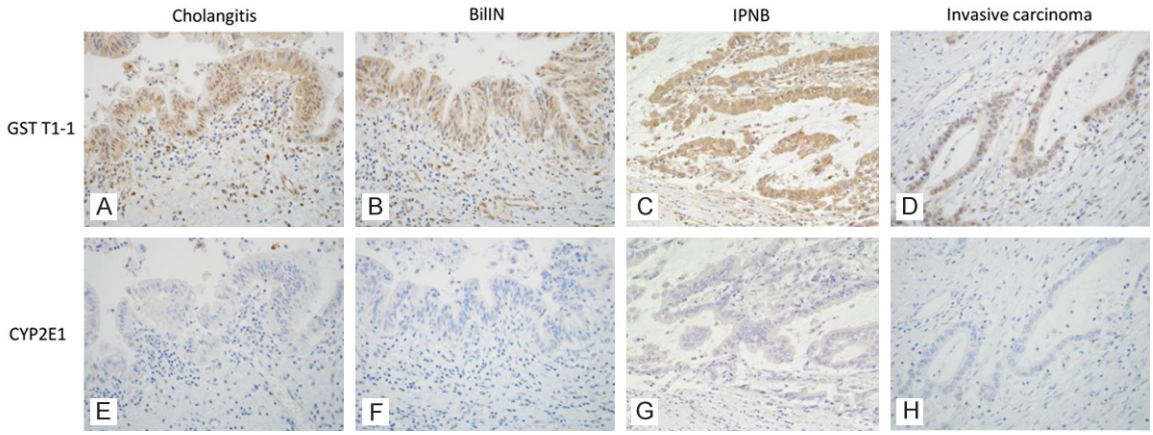

Figure 3. Expression of GST T1-1 and CYP2E1 in cholangiocarcinoma cases of the printing company. In the cases of the printing company, immunohistochemical expression of GST T1-1 was observed in biliary epithelial cells of the large bile duct with cholangitis (A), biliary intraepithelial neoplasia (BilIN) (B), intraductal papillary neoplasm of the bile duct (IPNB) (C) and cholangiocarcinoma (D). These epithelial cells lacked immunohistochemical expression of CYP2E1 (E-H). Original magnifications; (A, B, D, E, F, H); x400: (C, G); x200.

Specimens of 8 cases of cholangiocarcinoma (mean age, 36 years) that had occurred among workers of the printing company were used in this study [5]. All specimens were surgically resected, and unstained formalin-fixed, paraffin-embedded sections were provided by the associated hospitals. In all patients, cholangiocarcinoma arose from the large bile ducts, and was associated with BilIN and chronic bile duct injury. The coexistence of IPNB was recorded in 7 cases, and unstained sections including the foci of IPNB were available from 4 cases. For comparison, formalin-fixed, paraffin-embedded sections of surgically resected specimens of cholangiocarcinoma associated hepatolithiasis and BilIN (n = 16; mean age, 65 years) and conventional IPNB (n = 19; mean age 65 years) were used.

Immunohistochemistry

Immunostaining was performed using primary antibodies against GSTT1 (rabbit polyclonal; Proteintech Group, Inc., Chicago, IL) for mouse and rat, GSTT1 (rabbit monoclonal; Epitomics, Burlingame, CA) for human, CYP2E1 (rabbit polyclonal; Enzo Life Science, Inc., Farmingdale, NY) for mouse and rat, CYP2E1 (rabbit polyclonal; Atlas Antibodies, Stockholm, Sweden) for human, γ-H2AX (rabbit monoclonal; Novus Biologicals, Littleton, CO), and p53 (mouse monoclonal; DakoCytomation, Glostrup, Denmark). After deparaffinization, antigen retrieval was performed by microwaving the sections in Tris-ethylenediaminetetraacetic acid buffer (pH 9.0) for immunostaining of GSTT1 of mouse and rat, in 10 mmol/L citrate buffer (pH 6.0) for immunostaining of CYP2E1 of mouse and rat, γ-H2AX and p53, and in Target Retrieval Solution (DakoCytomation) for immunostaining of GSTT1 and CYP2E1 of human. The sections were then immersed in 0.3% hydrogen peroxidase in methanol for 20 minutes at room temperature to block endogenous peroxidase activity. After pretreatment with blocking serum (DakoCytomation), the sections were incubated overnight at 4°C with each primary antibody (diluted 1:100). Then, the sections were incubated with a secondary antibody conjugated to peroxidase-labeled polymer using the HISTOFINE system (Nichirei, Tokyo, Japan). Color development was performed using 3,3'-diaminobenzidine tetrahydrochloride, and the sections were lightly counterstained with hematoxylin. Negative controls were produced by substituting the primary antibody for non-immunized serum, which resulted in no signal detection.

As for the immunohistochemical analysis of the cholangiocarcinoma cases of the printing company, the expression of γ-H2AX was examined in 8 cases, whereas that of GSTT1, CYP2E1 and p53 was examined in 5, 5 and 3 cases, respectively, because of the limitation of the number of unstained sections available.

Semiquantitative analysis of the immunostained sections of γ-H2AX was performed. Foci

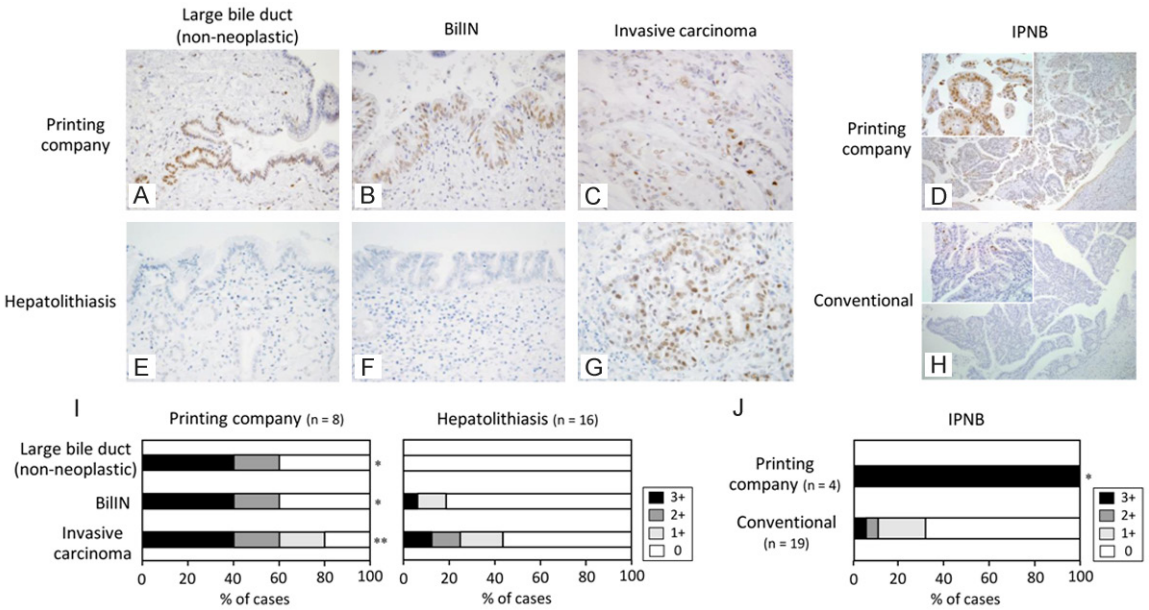

Figure 4. DNA damage in cholangiocarcinoma cases of the printing company. DNA damage was evaluated by the use of immunohistochemical staining of γ-H2AX as a marker of DNA double strand break. In cholangiocarcinoma cases of the printing company, positive expression of γ-H2AX was detected in non-neoplastic biliary epithelial cells of the large bile duct and peribiliary glands (A) as well as foci of biliary intraepithelial neoplasia (BilIN) (B) and invasive carcinoma (C). Foci of intraductal papillary neoplasm of the bile duct (IPNB) were also positive (D). In control cases of cholangiocarcinoma associated with hepatolithiasis and BilIN, several cases showed γ-H2AX expression in the invasive foci (G), while non-neoplastic biliary epithelial cells (E) and BilIN (F) were typically negative. In conventional IPNB, diffuse and intense staining was rare (H). Semiquantitative analysis of the immunostaining was performed as described in the Materials and methods. The analysis showed that the expression of γ-H2AX of each focus of the printing company cases was significantly increased when compared with that of control groups of cholangiocarcinoma associated with hepatolithiasis and BilIN (I), and conventional IPNB (J). *, $P < 0.01$; **, $P < 0.05$ vs. control groups. Original magnifications: (A), x200; (B, C, E-G), x400; (D, H); x40 (insets, x400).

of interests such as BilIN, IPNB and invasive carcinoma were observed in fields at x200 magnification, and the area of the highest labeling of γ-H2AX nuclear expression was selected for each focus in the section. The proportion of stained cells was evaluated as follows: 0, negative; 1+, 1-5% positive; 2+, 6-20% positive; and 3+, more than 20% positive.

KRAS and GNAS mutations

Mutations of KRAS and GNAS were analyzed as previously described [10]. Briefly, foci of interests were scraped off from paraffin-embedded tissue sections. DNA was isolated using the QIAMP DNA kit (QIAGEN, Tokyo, Japan), and isolated DNA was subjected to PCR amplification of the region of the KRAS gene containing codons 12 and 13, and the GNAS gene coding codon 201. The PCR products were purified using the QIAGEN PCR purification kit (QIAGEN), and sequenced by the Big Dye cyclic sequencing kit and ABI 310 sequencer (Applied Biosystems, Forster City, CA).

Statistics

Statistical significance was determined using the Mann-Whitney *U*-test. A *P* value less than 0.05 was accepted as the level of statistical significance.

Results

Distribution of GST T1-1 and CYP2E1 in normal hepatobiliary tract

Immunohistochemical expression of GST T1-1 was observed in hepatocytes and biliary epithelial cells of normal mouse, rat and human (**Figure 1**). The positive signals of GST T1-1 were located in the cytoplasm and nuclei of the cells. In hepatocytes of mouse and rat, they were located mainly in zone 3-2 of the hepatic lobule (**Figure 1A** and **1E**). The expression of GST T1-1

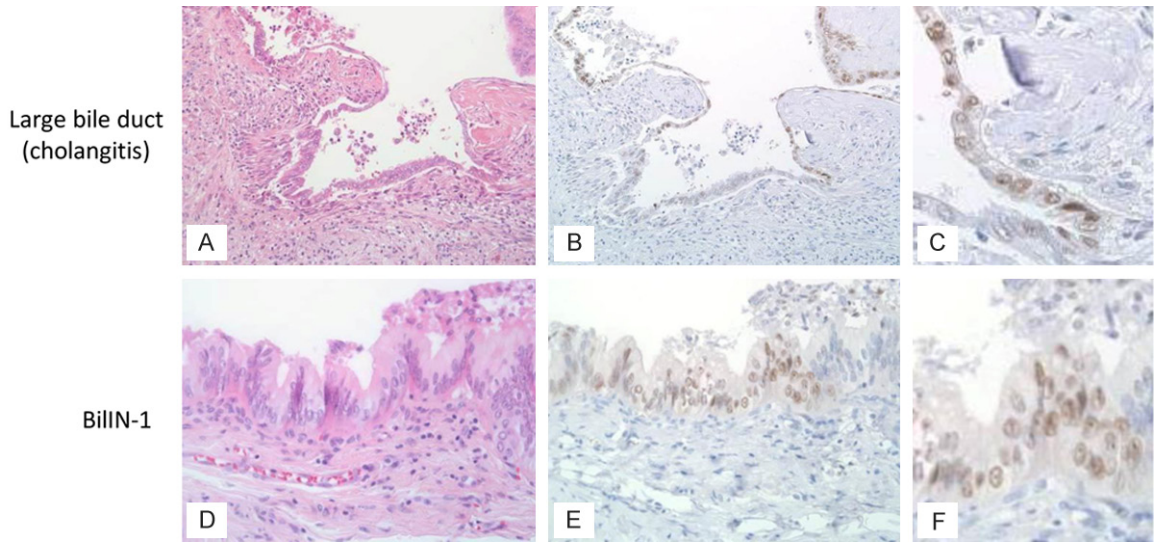

Figure 5. Expression of p53 in the printing company cases. The immunohistochemical expression of p53 was observed in non-neoplastic epithelial cells of the large bile duct associated with chronic cholangitis and bile duct damage (A-C) and the foci of biliary intraepithelial neoplasia-1 (BilIN-1) (D-F). (A, D); hematoxylin and eosin staining. Original magnifications: (A, B), x200; (C, F), x1000; (D, E), x400.

in hepatocytes of human tended to be observed diffusely in the hepatic lobule, and several cases showed the expression of GST T1-1 accentuated in zone 1 rather than zone 3-2 hepatocytes (**Figure 1H**).

The epithelium of large bile ducts and peribiliary glands of mouse, rat and human showed positive immunohistochemical signals of GST T1-1, and the gallbladder epithelium of mouse and human also expressed GST T1-1 (**Figure 1B-D**, **1F**, **1G** and **1I-K**). Although the extent of GST T1-1 expression in biliary epithelial cells was almost equal among individuals of mouse and rat, that in human biliary epithelial cells differed among individuals to some extent. In addition, there were several human cases where the expression of GST T1-1 in biliary epithelial cells was heterogeneous in a single case (**Figure 1J** and **1K**, insets).

The expression of CYP2E1 was observed in zone 3-2 hepatocytes of normal liver of mouse, rat and human, in which the positive immunohistochemical signals of CYP2E1 were located in the cytoplasm (**Figure 2A**, **2E** and **2H**).

Biliary epithelial cells of small bile ducts, large bile ducts and peribiliary glands of mouse, rat and human, and gallbladder epithelium of mouse and human typically lacked the immunohistochemical expression of CYP2E1 (**Figure 2B-D**, **2F**, **2G**, and **2I-K**). However, there were several human cases that showed focal and weak immunohistochemical expression of CYP2E1 in the epithelium of peribiliary glands and gallbladder (**Figures 2J** and **2K**, insets). In human subjects, the expression of CYP2E1 in peribiliary glands and gallbladder was observed in 4 of 30 cases, and 5 of 15 cases, respectively. The results of immunostaining of GST T1-1 and CYP2E1 are summarized in **Table 1**.

Expression of GST T1-1 and CYP2E1 in cholangiocarcinoma and its precursor lesions

In all cases of cholangiocarcinoma of the printing company, the immunohistochemical expression of GST T1-1 was observed in foci of BilIN, IPNB and cholangiocarcinoma as well as in non-neoplastic biliary epithelial cells of the large bile duct with cholangitis and bile duct injury (**Figure 3A-D**). The expression of GST T1-1 in the background hepatobiliary tract was almost identical to that of normal human livers.

Similar to the results of normal human livers, positive immunohistochemical signals of CYP2E1 were not observed in biliary epithelial cells of the large bile duct (**Figure 3E**). The foci of BilIN, IPNB and cholangiocarcinoma also

lacked the expression of CYP2E1 in all cases of the printing company (**Figure 3F-H**).

The positive immunohistochemical expression of GST T1-1 and the lack of CYP2E1 expression in cholangiocarcinoma and its precursor lesions were not specific findings for the cases of the printing company, because similar results were observed in control groups of cholangiocarcinoma associated with hepatolithiasis and BilIN, and conventional IPNB (data not shown).

DNA damage in cholangiocarcinoma and its precursor lesions

Immunohistochemical expression of γ-H2AX, a marker of DNA double strand break, was detected in foci of invasive carcinoma in 7 of 8 cholangiocarcinoma cases of the printing company, and 6 cases further showed occasional expression of γ-H2AX in non-neoplastic biliary epithelial cells of the large bile duct and peribiliary glands as well as BilIN and IPNB (**Figure 4A-D**). The expression of γ-H2AX in small bile ducts and hepatocytes of the background liver was mostly negative.

In the control cases of cholangiocarcinoma associated with hepatolithiasis and BilIN, 7 of 16 cases showed the expression of γ-H2AX in the invasive foci (**Figure 4G**). By contrast, non-neoplastic biliary epithelial cells of the large bile duct and peribiliary glands were totally negative (**Figure 4E**). Foci of BilIN in hepatolithiasis were typically lacked γ-H2AX expression (**Figure 4F**), but 3 of 16 cases showed focal positive expression of γ-H2AX in BilIN. In cases of conventional IPNB, 6 of 19 cases showed positive immunohistochemical expression of γ-H2AX, but intense and diffuse staining was rare (**Figure 4H**).

Semiquantitative analysis showed that the expression of γ-H2AX was significantly increased in non-neoplastic biliary epithelial cells of the large bile duct, BilIN, IPNB and invasive carcinoma of the printing company cases when compared with that of control groups of cholangiocarcinoma with hepatolithiasis and BilIN, and conventional IPNB (**Figure 4I** and **4J**).

Expression of p53 in cholangiocarcinoma cases of the printing company

Immunohistochemical expression of p53 was examined in 3 cases of cholangiocarcinoma of the printing company. Invasive foci of all cases showed positive immunohistochemical expression of p53, and in one of 3 cases, positive immunohistochemical signal was observed in foci of IPNB (data not shown). It was of note that non-neoplastic epithelial cells of the large bile duct associated with chronic cholangitis and bile duct damage showed positive immunohistochemical expression of p53 (**Figure 5A-C**). In addition, immunohistochemical expression of p53 was observed in foci of BilIN-1 in one case (**Figure 5D-F**).

KRAS and GNAS mutations

KRAS and GNAS mutations were analyzed for 3 cases of cholangiocarcinoma of the printing company. From the cases, one focus of non-neoplastic biliary epithelial cells of the large bile duct, 3 foci of BilIN, 4 foci of IPNB and 3 foci of invasive carcinoma were selected and analyzed. Among them, KRAS mutation was detected in one focus of BilIN, showing mutation of GGC to GGT at codon 13. GNAS mutation was detected in one focus of BilIN, which was from the same case that had KRAS mutation but from the different focus, showing mutation of CGT to CGA at codon 201. The other foci examined here were wild type for both KRAS and GNAS.

Discussion

This study showed that GST T1-1 was constitutively expressed in normal biliary tract as well as hepatocytes, and it was also observed in cholangiocarcinoma cases of the printing company. By contrast, the immunohistochemical expression of CYP2E1 was observed in normal hepatocytes, while it was not detected in normal biliary epithelial cells as well as cholangiocarcinoma cases, with the exception of occasional expression of CYP2E1 in the non-neoplastic epithelium of peribiliary glands and gallbladder in human. In addition, DNA damage in non-neoplastic biliary epithelial cells as well as in foci of BilIN, IPNB and invasive carcinoma was found to be increased in cholangiocarcinoma cases of the printing company, which was accompanied by abnormal expression of p53 and occasional mutations of KRAS and GNAS.

There are several reports that examined the normal distribution of GST T1-1 in the liver of

mouse, rat and human [11-13]. In these studies, the expression of GST T1-1 was detected in the cytoplasm and nuclei of hepatocytes and biliary epithelial cells, although the distribution of the enzyme in relation to the different anatomical levels of the biliary tract such as small bile ducts, large bile ducts and peribiliary glands was not described in detail. Hepatocyte expression of CYP2E1 is well documented, and it is known that its expression shows individual variations in normal subjects [14].

DCM is a potent hepatic and pulmonary carcinogen in mouse [15]. By contrast, DCM exposure showed no increase in the incidence of hepatic or pulmonary tumors in rat, which might be partially due to the lower catalytic activity of rat GST T1-1 toward DCM than that of mouse [16]. The risk posed to human health by DCM is uncertain because no long-term adverse effects have been seen following occupational exposure. A number of cohort studies have not provided any epidemiological evidence to link DCM exposure with a higher incidence of human cancer [17-19].

It has been suggested that, compared to the catalytic activity of GST T1-1 toward DCM in the mouse, humans do not have a sufficiently high capacity to activate DCM for this compound to be considered to represent a carcinogenic risk [11]. However, the outbreak of cholangiocarcinoma among workers of the printing company suggested the causal relation between the development of cholangiocarcinoma and exposure to organic solvents of 1,2-DCP and/or DCM [4, 5].

As shown in this study, biliary epithelial cells of the normal human biliary tract expressed GST T1-1 and they lacked the expression of CYP2E1. The biliary epithelium of the hepatobiliary tract is supplied by arterial vessels originating from hepatic arterial branches [20]. In these circumstances, 1,2-DCP and/or DCM inhaled in the lung may directly affect the biliary epithelial cells via arterial blood, where GST T1-1-catalyzed pathway may result in the production of highly reactive intermediates related to the carcinogenesis in the absence of CYP2E1, thereby causing BilIN, IPNB and cholangiocarcinoma. In fact, DNA damage was significantly increased in non-neoplastic biliary epithelial cells of the large bile duct and peribiliary glands of the printing company cases.

Although small bile ducts expressed GST T1-1 in this study, cholangiocarcinoma developing in the peripheral portion of the liver has not been recorded in the series of the printing company cases [4, 5]. Because cholangiocytes in the large and small intrahepatic bile ducts have different functions and responses to injuries [21], the heterogeneity of cholangiocytes in the liver may explain for the reason, but the exact mechanism remains unclear.

It seems to be interesting that gallbladder cancer never occurred in the workers of the printing company who developed cholangiocarcinoma [4, 5]. In this study, the expression of CYP2E1 was observed in human gallbladder epithelial cells in several cases. A previous study also demonstrated the expression of CYP2E1 in the gallbladder epithelial cells of human [22]. These observations suggest that CYP2E1-dependent oxidative pathway may have a function of detoxification of 1,2-DCP and/or DCM in the gallbladder in some patients. In addition, lung cancer has not occurred in the cases of epidemic cholangiocarcinoma patients, although DCM is pulmonary carcinogen in mouse [15]. Since GST T1-1 was found in only low levels in human lung, it is suggested that in man the lung has little capacity to activate DCM in human [23].

According to our previous study, immunohistochemical expression of p53 was observed in neither non-neoplastic biliary epithelial cells of the large bile duct nor BilIN-1 in hepatolithiatic livers [24, 25]. In addition, GNAS mutation was not observed in any grade of BilIN as well as intrahepatic cholangiocarcinoma [25]. Although the number of the cases examined in this study was small, the results showed that the expression of p53 in non-neoplastic biliary epithelial cells and BilIN-1, and the occurrence of GNAS mutation in BilIN in the cases of the printing company. These results indicate the different and characteristic carcinogenic process of the printing company cases.

In summary, this study suggests that the exposed organic solvents reaching to the biliary epithelial cells via arterial blood may act as a carcinogen for the cells through the GST T1-1-catalyzed pathway. The resultant DNA damage may lead to the development of cholangiocarcinoma. Although detailed mechanism of the carcinogenesis requires to be further addressed,

this study provides evidence that may support the causal relation between organic solvent exposure and cholangiocarcinoma development in the patients.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Yasuni Nakanuma, Department of Human Pathology, Kanazawa University Graduate School of Medicine, 13-1 Takaramachi, Kanazawa 920-8640, Japan. Tel: +81-76-265-2195; Fax: +81-76-234-4229; E-mail: nakanuma@staff.kanazawa-u.ac.jp

References

[1] Lazaridis KN, Gores GJ. Cholangiocarcinoma. Gastroenterology 2005; 128: 1655-67.

[2] Nakanuma Y, Sasaki M, Sato Y, Ren X, Ikeda H, Harada K. Multistep carcinogenesis of perihilar cholangiocarcinoma arising in the intrahepatic large bile ducts. World J Hepatol 2009; 1: 35-42.

[3] Charbel H, Al-Kawas FH. Cholangiocarcinoma: epidemiology, risk factors, pathogenesis, and diagnosis. Curr Gastroenterol Rep 2011; 13: 182-7.

[4] Kumagai S, Kurumatani N, Arimoto A, Ichihara G. Cholangiocarcinoma among offset colour proof-printing workers exposed to 1,2-dichloropropane and/or dichloromethane. Occup Environ Med 2013; 70: 508-10.

[5] Kubo S, Nakanuma Y, Takemura S, Sakata C, Urata Y, Nozawa A, Nishioka T, Kinoshita M, Hamano G, Terajima H, Tachiyama G, Matsumura Y, Yamada T, Tanaka H, Nakamori S, Arimoto A, Kawada N, Fujikawa M, Fujishima H, Sugawara Y, Tanaka S, Toyokawa H, Kuwae Y, Ohsawa M, Uehara S, Sato KK, Hayashi T, Endo G. Case series of 17 patients with cholangiocarcinoma among young adult workers of a printing company in Japan. J Hepatobiliary Pancreat Sci 2014; 21: 479-88.

[6] IARC (International Agency for Research on Cancer). List of classification by cancer sites with sufficient or limited evidence in humans, Volume 1 to 105. 2012 (http://monographs.iarc.fr/ENG/Classification/index.php).

[7] International Agency for Research on Cancer. Volume 110: Perfluoro-octanoic acid, tetrafluoroethylene, dichloromethane, 1,2-dichloropropane, 1,3-propane sultone. IARC Working Group; Lyon, June 3-10, 2014. IARC Monogr Eval Carcinog Risk Chem Hum (in press).

[8] Gargas ML, Clewell HJ 3rd, Andersen ME. Metabolism of inhaled dihalomethanes in vivo: differentiation of kinetic constants for two independent pathways. Toxicol Appl Pharmacol 1986; 82: 211-23.

[9] Cooper GS, Scott CS, Bale AS. Insights from epidemiology into dichloromethane and cancer risk. Int J Environ Res Public Health 2011; 8: 3380-98.

[10] Sasaki M, Matsubara T, Nitta T, Sato Y, Nakanuma Y. GNAS and KRAS mutations are common in intraductalpapillary neoplasms of the bile duct. PLoS One 2013; 8: e81706.

[11] Sherratt PJ, Williams S, Foster J, Kernohan N, Green T, Hayes JD. Direct comparison of the nature of mouse and human GST T1-1 and the implications on dichloromethane carcinogenicity. Toxicol Appl Pharmacol 2002; 179: 89-97.

[12] Mainwaring GW, Williams SM, Foster JR, Tugwood J, Green T. The distribution of theta-class glutathione S-transferases in the liver and lung of mouse, rat and human. Biochem J 1996; 318: 297-303.

[13] Quondamatteo F, Schulz TG, Bunzel N, Hallier E, Herken R. Immunohistochemical localization of glutathione S-transferase-T1 in murine kidney, liver, and lung. Histochem Cell Biol 1998; 110: 417-23.

[14] Hata S, Miki Y, Fujishima F, Sato R, Okaue A, Abe K, Ishida K, Akahira J, Unno M, Sasano H. Cytochrome 3A and 2E1 in human liver tissue: Individual variations among normal Japanese subjects. Life Sci 2010; 86: 393-401.

[15] Graves RJ, Coutts C, Eyton-Jones H, Green T. Relationship between hepatic DNA damage and methylene chloride-induced hepatocarcinogenicity in B6C3F1 mice. Carcinogenesis 1994; 15: 991-6.

[16] Burek JD, Nitschke KD, Bell TJ, Wackerle DL, Childs RC, Beyer JE, Dittenber DA, Rampy LW, McKenna MJ. Methylene chloride: a two-year inhalation toxicity and oncogenicity study in rats and hamsters. Fundam Appl Toxicol 1984; 4: 30-47.

[17] Hearne FT, Pifer JW, Grose F. Absence of adverse mortality effects in workers exposed to methylene chloride: an update. J Occup Med 1990; 32: 234-40.

[18] Heineman EF, Cocco P, Gómez MR, Dosemeci M, Stewart PA, Hayes RB, Zahm SH, Thomas TL, Blair A. Occupational exposure to chlorinated aliphatic hydrocarbons and risk of astrocytic brain cancer. Am J Ind Med 1994; 26: 155-69.

[19] Tomenson JA, Bonner SM, Heijne CG, Farrar DG, Cummings TF. Mortality of workers exposed to methylene chloride employed at a plant producing cellulose triacetate film base. Occup Environ Med 1997; 54: 470-6.

[20] Nakanuma Y, Hoso M, Sanzen T, Sasaki M. Microstructure and development of the normal and pathologic biliary tract in humans, including blood supply. Microsc Res Tech 1997; 38: 552-70.
[21] Ueno Y, Alpini G, Yahagi K, Kanno N, Moritoki Y, Fukushima K, Glaser S, LeSage G, Shimosegawa T. Evaluation of differential gene expression by microarray analysis in small and large cholangiocytes isolated from normal mice. Liver Int 2003; 23: 449-59.
[22] Lakehal F, Wendum D, Barbu V, Becquemont L, Poupon R, Balladur P, Hannoun L, Ballet F, Beaune PH, Housset C. Phase I and phase II drug-metabolizing enzymes are expressed and heterogeneously distributed in the biliary epithelium. Hepatology 1999; 30: 1498-506.
[23] Sherratt PJ, Pulford DJ, Harrison DJ, Green T, Hayes JD. Evidence that human class Theta glutathione S-transferase T1-1 can catalyse the activation of dichloromethane, a liver and lung carcinogen in the mouse. Comparison of the tissue distribution of GST T1-1 with that of classes Alpha, Mu and Pi GST in human. Biochem J 1997; 326: 837-46.
[24] Nakanishi Y, Zen Y, Kondo S, Itoh T, Itatsu K, Nakanuma Y. Expression of cell cycle-related molecules in biliary premalignant lesions: biliary intraepithelial neoplasia and biliary intraductal papillary neoplasm. Hum Pathol 2008; 39: 1153-61.
[25] Hsu M, Sasaki M, Igarashi S, Sato Y, Nakanuma Y. KRAS and GNAS mutations and p53 overexpression in biliary intraepithelial neoplasia and intrahepatic cholangiocarcinomas. Cancer 2013; 119: 1669-74.