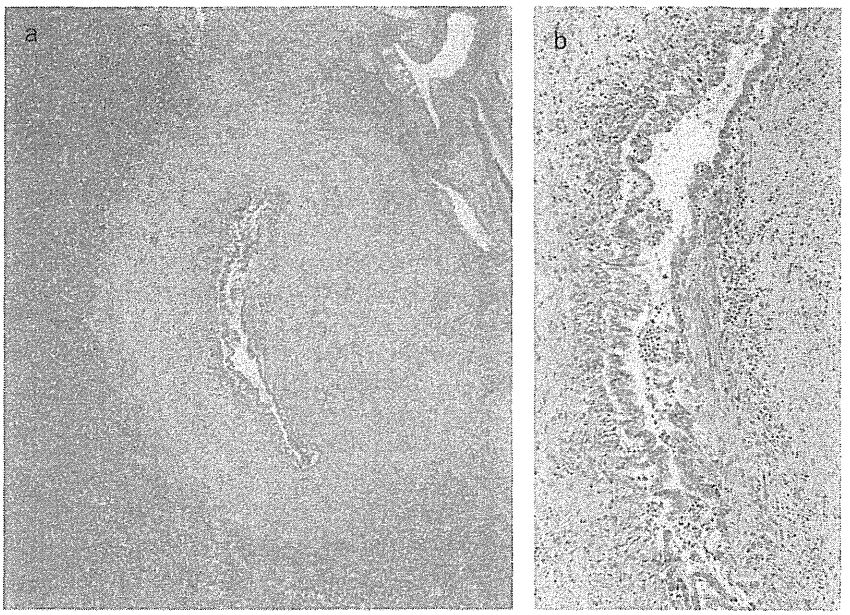

Fig. 5 Sclerotic bile duct lesion of intrahepatic bile duct with BilIN2/3 lesions
(**a**) Bile duct prominent ductal sclerosis with stenotic lumen. Patient 8, H-E staining, 150 × magnification. (**b**) Higher magnification of (**a**) Stenotic lumen covered by BilIN2/3 lesions. Beneath the epithelial lesion, mesenchymal reaction with inflammatory cells is seen. H-E staining, 150 × magnification. staining, 300 × magnification; H

administered to eight patients after surgical treatment. In two of these eight patients, radiation was administered to the bile duct stumps. In the remaining 5 of the 17 patients, chemotherapy or conservative treatment (stenting alone in one patient) was administered because of the advanced stage of the disease, as indicated by metastases to the lymph nodes around the aorta and/or intraperitoneal dissemination.

Among the 12 patients who underwent surgical treatment, intrahepatic recurrence or recurrence at the bile duct stump occurred in five patients (no. 1–3, 5, and 8), and lymph node metastasis occurred in one patient (no. 1). In one (no. 8) of the five patients, solitary intrahepatic recurrent tumor detected at 11 months after the first operation was treated with radiofrequency ablation therapy. Another four (no. 1–3, and 5) patients with recurrence died of carcinoma. Another patient (no. 11) died of hepatic failure. Furthermore, four of the five patients who underwent chemotherapy or conservative treatment died of advanced carcinoma. The median survival time from the diagnosis of cholangiocarcinoma to the death or the end of this study was 578 days.

Discussion

In this study, cholangiocarcinomas were diagnosed in relatively young workers (25–45 years old; mean, 36 years old) in the offset color proof-printing department of a printing company at an extremely high incidence (17 of 111 workers). This type of cholangiocarcinoma is newly classified as an occupational disease by the Ministry of Health, Labour and Welfare, Japan at 1 October 2013.

The peak incidence of choloangiocarcinoma occurred in patients in their sixth or seventh decade and cholangiocarcinoma is rare in relatively young patients [1–6]. Based on the data from the Osaka Cancer Registry (1975–2007), the mean age at diagnosis for intrahepatic cholangiocarcinoma in male patients ($n = 1797$) was 66.5 ± 0.21 years old and for extrahapatic cholangiocarcinoma in male patients ($n = 3638$) was 68.9 ± 0.19 years old, and the proportions of relatively young patients (25–45 years old) with cancer in intrahepatic and extrahepatic bile duct were 4.1% and 2.6%, respectively [20]. Of 228 patients who were treated for cholangiocarcinoma in the Department of Hepato-Biliary-Pancreatic Surgery in Osaka City University between December 1996 and December 2012 (cholangiocarcinoma in this study was diagnosed during this period), the mean age at diagnosis was 66.2 ± 10.3 years old and the proportion of patients whose age was less than 50 years old was 5.4% (15 patients). Of the 15 patients, five patients were the current or former workers in the printing company. Thus, the mean age of the patients in this study (36 years old) are quite younger, compared to that of typically observed patients with cholangiocarcinoma. Most workers who were exposed to high concentration of chlorinated organic solvent were relatively young, which was related to the age of the patients with cholangiocarcinoma.

Known etiologic factors for cholangiocarcinoma include hepatolithiasis, PSC, pancreaticobiliary maljunction, liver flukes, and exposure to chemical carcinogens, such as nitrosamines [1–4, 7, 8, 16–19], although the causes of most cholangiocarcinoma remain unclear. Other conditions, such as hepatitis B and C viral infections, cirrhosis, alcohol intake, and smoking, are possibly associated with cholangiocarcinoma [8, 21, 22]. Exposure to printing processes, carbon black, and some nitrogenous compounds have been

classified as group 2B (possibly carcinogenic to humans) according to reports by the International Agency for Research on Cancer (IARC) [23]. However, to date, no studies have elucidated the development of cholangiocarcinoma triggered by exposure to printing processes or associated agents. The 17 patients in this study did not exhibit known risk factors for cholangiocarcinoma, although 13 patients were smokers, three were habitual alcohol consumers, and one had a previous history of hepatitis B viral infection. Large amounts of chemicals, including organic solvents, were used in this printing department. Of the 17 patients, all were exposed to 1,2-dichloropropane, 11 were exposed to dichloromethane, and eight were exposed to 1,1,1-trichloroethane. An experiment using reproduction of the working environment by the Japanese National Institute of Occupational Safety and Health suggested that the workers in this department were exposed to high concentration of chlorinated organic solvents [24]. 1,1,1-Trichloroethane, dichloromethane, and 1,2-dichloropropane are classified as category 2 (suspected human carcinogens) according to the Globally Harmonized System of Classification and Labelling of Chemicals [25] and dichloromethane is classified as group 2B according to the IARC [23]. Conversely, other chemicals used in this department were ruled out as possible causative agents because of their lower consumption and/or shorter period of exposure [26]. In addition, other chemicals have been used in various types of industries without inducing cancer. Thus, such chlorinated organic solvents play an important role in the development of cholangiocarcinoma [26]. However, it is impossible to identify all components of the chemicals used previously in the department because the chlorinated organic solvents, such as 1,1,1-trichloroethane, dichloromethane, and 1,2-dichloropropane, have been retired until October 2006, and the organic solvents included impurities. Therefore, other unidentified chemicals might affect the development of the cholangiocarcinoma.

Some cholangiocarcinoma are associated with prior biliary or hepatic disease such as hepatolithiasis, PSC, and liver flukes [2–4, 7, 16–19]. In patients with hepatolithiasis, both BilIN lesions and IPNB are sometimes encountered in the large bile ducts containing stones and in the adjacent bile ducts [10]. PSC is likely an immune mediated, progressive disorder that eventually developed into cirrhosis, and some patients with PSC exhibit only BilIN lesions and not IPNB [18]. In patients with liver fluke infection, bile duct proliferation associated with periportal fibrosis is observed, resulting in cirrhosis [19]. These pathological findings in patients with hepatolithiasis, PSC, or liver flukes indicate neoplastic transformation through dysplastic changes, from bile duct hyperplasia to cholangiocarcinoma (multistep carcinogenesis) [1, 10–12, 18, 19]. In this study, chronic bile duct damage, including duct sclerosis, biliary epithelial injuries/proliferation, and focal bile duct losses, were observed in various lesions within the resected specimens in all patients for whom operative specimens were available. Furthermore, BilIN-2/3 lesions and IPNB, which are recognized as the premalignant or pre-invasive stages of cholangiocarcinoma, were also observed at various sites of the bile duct and peribiliary glands in all of the patients. The main and most invasive cholangiocarcinoma lesions were located in the large bile duct. These findings indicate that the cholangiocarcinoma detected among the printing company workers might have developed from chronic bile duct injuries into precancerous or early cancerous lesions at various sites of the bile ducts, especially large bile ducts, and eventually developed into invasive cholangiocarcinoma, similar to cholangiocarcinoma in patients with hepatolithiasis, PSC, or liver flukes. In addition, the widespread occurrence of the pathological changes without cirrhosis is an important characteristic of the patients in this study.

Surgery is the only curative treatment for cholangiocarcinoma. In this study, five patients could not be treated surgically because of advanced disease. Thus, to improve patients' long-term outcomes, regular health examinations, including measurements of serum γ-GTP activity and serum CA19-9 and CEA concentrations, are important because the early detection of cholangiocarcinoma is essential. With regard to imaging analyses, both mass lesions with or without dilatation of the peripheral bile duct as well as the dilatation and/or stenosis of the bile ducts are important findings for the detection of cholangiocarcinoma. It is also important to distinguish cholangiocarcinoma from PSC because imaging findings of cholangiography in some patients in this study were similar to those of PSC [14]. It is necessary to monitor the workers for a long time because cholanciocarcinoma was diagnosed at 9 years, 7 months after the stopping of the exposure to the chemicals including chlorinated organic solvent.

In this study, the determination of appropriate treatments was difficult because malignant and premalignant lesions were often observed at various sites in the large bile ducts. In fact, curative resection could not be performed in 5 of the 12 patients who underwent surgery. Adjuvant chemotherapy might be necessary in such patients. Liver transplantation is another treatment option for patients without lymph node involvement or distant metastases.

Acknowledgments This study was supported in part by the Health and Labour Sciences Research Grants for Research on Occupational Safety and Health "The epidemiological and cause-investigated study of cholangiocaricnoma in workers of a printing company". A part of this study was presented with the permission of the ministry at the 48th Annual Meeting of the Japanese Biliary Association (September, 2012), the 60th Annual Meeting of Japanese Society of Occupational Medicine and Traumatology (December, 2012), the 86th Annual Meeting of Japan Society for Occupational Health (May, 2013), and

for the press (March, 2013) because this event should be publicized to Japanese people to prevent subsequent occurrences. We thank Drs E. Kawamura, S. Marubashi, T. Wakasa, T. Yamamoto, S. Tanaka, and M. Kaji for their assistance with data collection.

Conflict of interest None declared.

Author contribution Study design: SK, YN, TH, and GE designed the study. Acquisition of data: SK, S. Takemura, CS, YU, AN, TN, MK, GH, HT, GT, YM, TY, HT, SN, AA, NK, MF, HF, YS, S. Tanaka, and HT. Clinical aspect of the study: SK, S. Takemura, CS, YU, AN, TN, MK, GH, NK, SU, KKS, TH, and GE analyzed data. SK, YN, NK, KKS, TH, and GE. Pathological aspect of the study: YN, YK, and MO. Data analysis: SK, S. Takemura, CS, YU, AN, TN, MK, GH, NK, SU, KKS, TH, and GE. Manuscript drafted by SK, YN, NK, KKS, TH, and GE. All authors reviewed the manuscript.

References

1. World Health Organization Classifications of Tumours : International Agency for Research on Cancer (IARC). 4th edn. WHO classification of tumours of the digestive system. Bosman FT, Carneiro F, Hruban RH, Theise ND, editors. Lyon: World Health Organization Classifications of Tumours : International Agency for Research on Cancer (IARC); 2010. p. 195–278.
2. Parkin DM, Ohshima H, Srivatanakul P, Vatanasapt V. Cholangiocarcinoma: epidemiology, mechanisms of carcinogenesis and prevention. Cancer Epidemiol Biomarkers Prevention. 1993;2:537–44.
3. Shaib Y, El-Serag HB. The epidemiology of cholangiocarcinoma. Semin Liver Dis. 2004;24:115–25.
4. Khan SA, Thomas HC, Davidson BR, Taylor-Robinson AD. Cholangiocarcinoma. Lancet. 2005;366:1303–14.
5. Ikai I, Arii S, Okazaki M, Okita K, Omata M, Kojiro M, et al. Report of the 17th mationwaide follow-up survey of primary liver cancer in Japan. Hepatol Res. 2007;27:676–91.
6. Miyagawa S, Ishihara S, Horiguchi S, Takada T, Miyazaki M, Nagakawa T. Biliary tract cancer treatment: 5,584 results from the biliary tract cancer statistics registry from 1998 to 2004 in Japan. J Hepatobiliary Pancreat Surg. 2009;16:1–7.
7. Khan SA, Toledano MB, Taylor-Robinson SD. Epidemiology, risk factors, and pathogenesis of cholangiocarcinoma. HPB (Oxford). 2008;10:77–82.
8. Shaib YH, El-Serag HB, Davila JA, Morgan R, McGlynn KA. Risk factors of intrahepatic cholangiocarcinoma in the United States: a case-control study. Gastroenterology. 2005;128:620–6.
9. Kumagai S, Kurumatani N, Arimoto A, Ichihara G. Cholagiocarcinoma among offset colour proof-printing workers exposed to 1,2-dichloropropane and/or dichloromethane. Occup Environ Med. 2013;70:508–10.
10. Zen Y, Sasaki M, Fujii T, et al. Different expression patterns of mucin core proteins and cytokeratins during intrahepatic cholangiocarcinogenesis from biliary intraepithelial neoplasia and intraductal papillary neoplasm of the bile duct—an immunohistochemical study of 110 cases of hepatolithiasis. J Hepatol. 2006;44:350–8.
11. Zen Y, Adsay NV, Bardadin K, et al. Biliary intraepithelial neoplasia: an international interobserver agreement study and proposal for diagnostic criteria. Mod Pathol. 2007;20:701–9.
12. Itatsu K, Zen Y, Ohira S, et al. Immunohistochemical analysis of the progression of flat and papillary preneoplastic lesions in intrahepatic cholangiocarcinogenesis in hepatolithiasis. Liver Int. 2007;27:1174–84.
13. Lelbach WK. Cirrhosis in the alcoholic and its relation to the volume of alcohol abuse. Ann N Y Acad Sci. 1975;252:85–105.
14. Vitellas KM, Keogan MT, Freed KS, et al. Radiologic manifestations of sclerosing cholangitis with emphasis on MR cholangiopancreatography. RadioGraphics. 2000;20:959–75.
15. 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.
16. Kubo S, Kinoshita H, Hirohashi K, Hamba H. Hepatolithiasis associated with cholangiocarcinoma. World J Surg. 1995;19:637–41.
17. Burak K, Angulo P, Pasha TM, Egan K, Petz J, Lindor KD. Incidence and risk factors for cholangiocarcinoma in primary sclerosing cholangitis. Am J Gastroentrol. 2004;99:523–6.
18. Chen TC, Nakanuma Y, Zen Y, et al. Intraductal papillary neoplasia of the liver associated with hepatolithiasis. Hepatology. 2001;34:651–8.
19. Watanapa P, Watanapa WB. Liver fluke-associated cholangiocarcinoma. Br J Surg. 2002;89:962–70.
20. Ikeda A, Miyashiro I, Nakayama T, Ioka A, Tabuchi T, Ito Y, et al. Descriptive epidemiology of bile duct carcinoma in Osaka. Jpn J Clin Oncol. 2013;43:1150–5.
21. Yamamoto S, Kubo S, Hai S, et al. Hepatitis C virus infection as a likely etiology of intrahepatic cholangiocarcinoma. Cancer Sci. 2004;95:592–5.
22. Tyson GL, El-Serag HB. Risk factors for cholangiocarcinoma. Hepatology. 2011;54:173–84.
23. 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
24. National Institute of Occupational Safety and Health, Japan. Industrial accident in a printing company in Osaka [in Japanese]. Report A-2012-02, 2012. http://www.mhlw.go.jp/stf/houdou/2r9852000002ioeh-att/2r9852000002ioik.pdf
25. United Nations Economic Commission for Europe. Chapter 3.6 : carcinogenicity, part 3 : health hazards. Globally harmonized system of classification and labeling of chemicals (GHS). Third revised edition. 2009. http://www.unece.org/trans/danger/publi/ghs/ghs_rev03/03files_e.html
26. Ministry of Health, Labour and Welfare: occupational biliary tract cancer cases in Japan. 2013. http://www.mhlw.go.jp/english/policy/employ-labour/labour-standards/Occupational.html

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1 Time course of the patients showing the duration of employment, the time of diagnosis of cholangiocarcinoma, and prognosisSolid line, the duration of employment with exposure to chlorinated organic solvent; dotted line, the duration of employment without exposure to chlorinated organic solvent; open arrow, diagnosis of cholangiocarcinoma; closed arrow, death.

Table S1 Chemicals used in the offset proof-printing department.

Figure S1 Time course of the patients showing the duration of employment, the time of diagnosis of cholangiocarcinoma, and prognosis

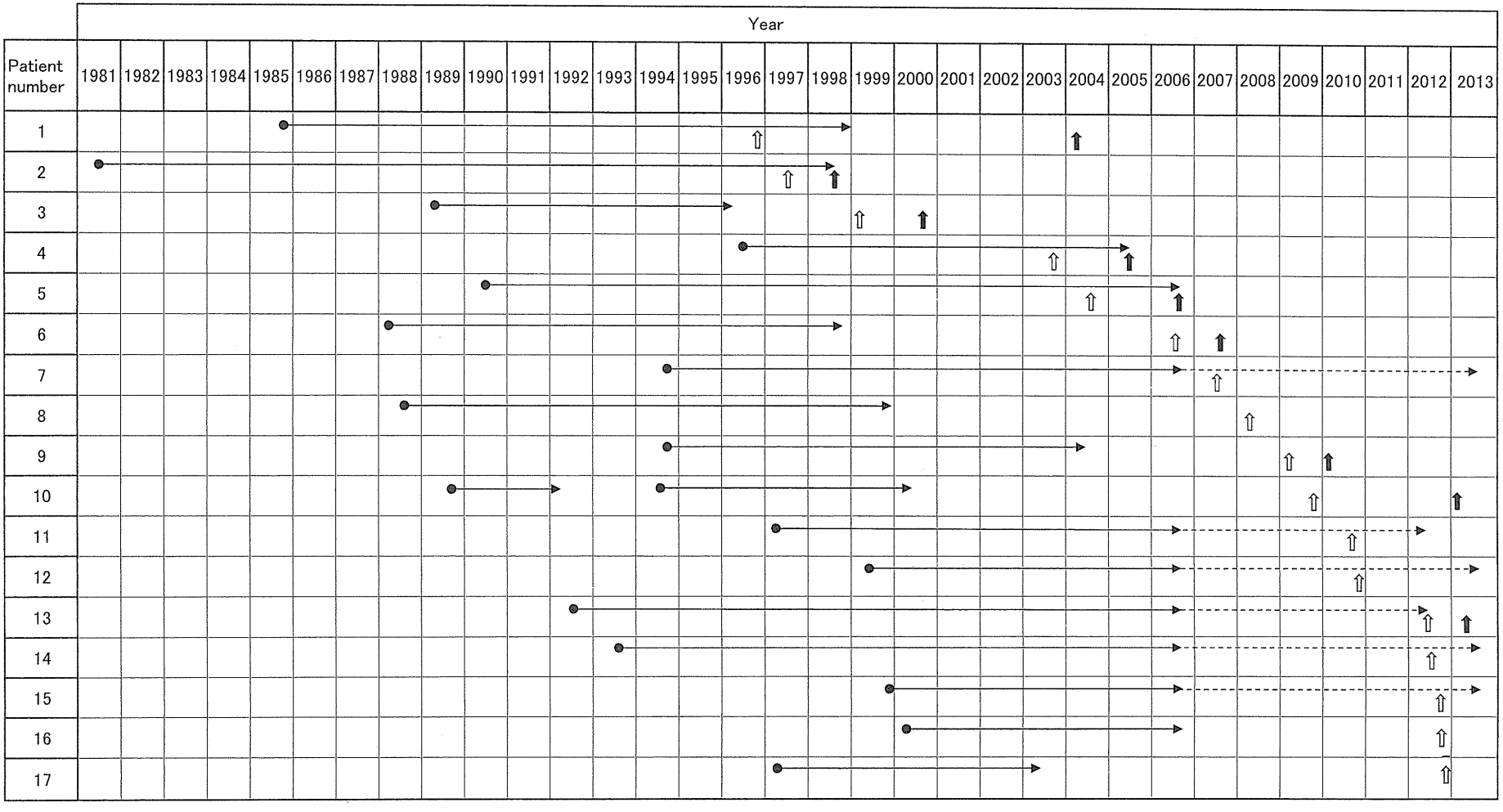

Table S1 Chemicals used in the offset proof-printing department

1,1,1-Trichloroethane	Diethylene glycol monobutyl ether
1,2-Dichloropropane	Propyleneglycol monomethyl ether
Dichloromethane	2-Methyl-2.4pentadiol
Diclorofluoroethane	3-Methyl-3-methoxybutanol
2- Buthanol	Solvent naphtha (coal)
2-Methylpentane	Xylene
3-Methylpentane	Kerosene
N-Hexane	Mineral oil
Cyclohexan	Hydrocarbons
Isopropyl alcohol	Aromatic hydrocarbons
Ethanol	Inks

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

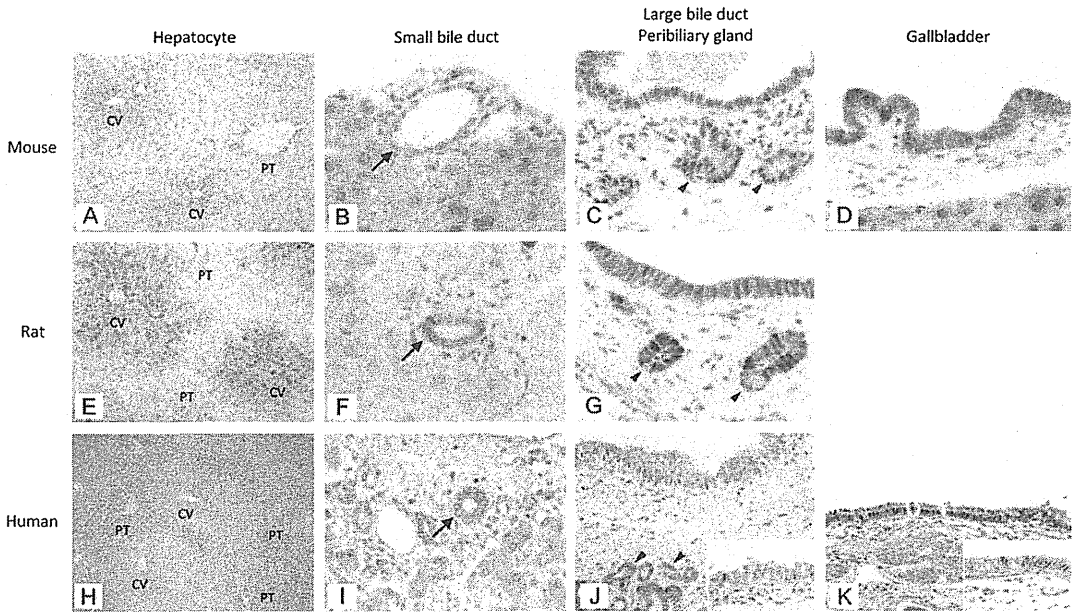

Figure 1. Distribution of GST T1-1 in normal hepatobiliary tract. Immunohistochemical expression of GST T1-1 was observed in hepatocytes and biliary epithelial cells of normal mouse, rat and human. Arrows and arrowheads indicate small bile ducts and peribiliary glands, respectively. Insets (J and K) 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.

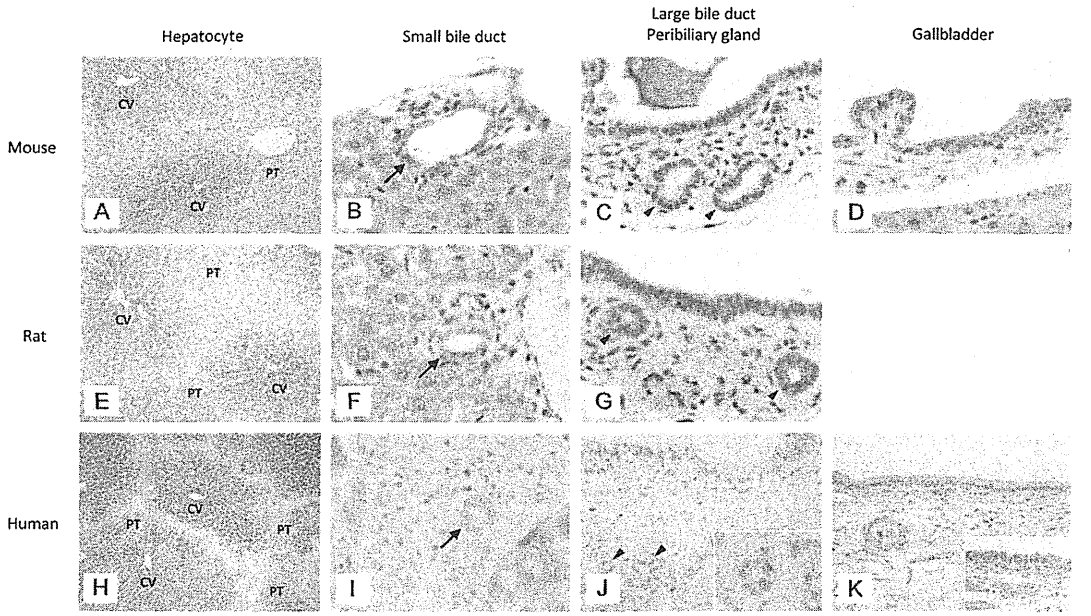

Figure 2. Distribution of CYP2E1 in normal hepatobiliary tract. Immunohistochemical expression of CYP2E1 was observed in zone 3-2 hepatocytes of normal liver of mouse, rat and human. Biliary epithelial cells typically lacked the expression of CYP2E1, but several human cases showed focal and weak immunohistochemical 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.

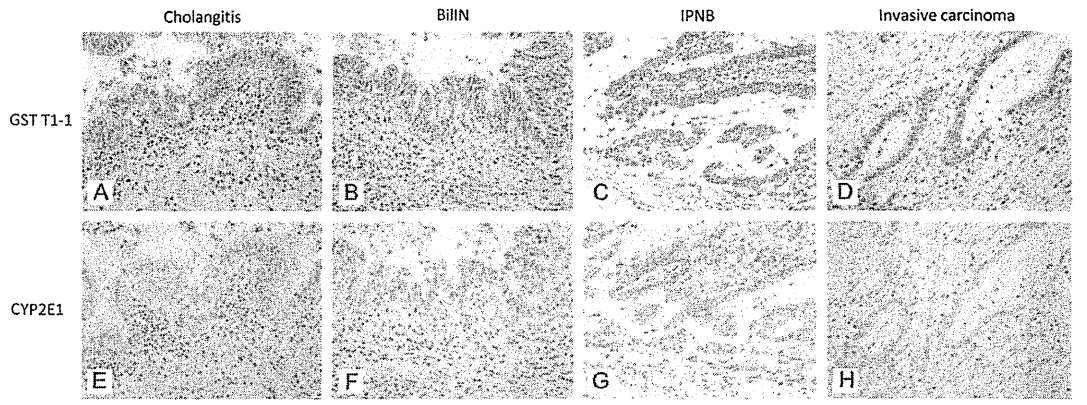

Figure 3. Expression of GST T1-1 and CYP2E1 in cholangiocarcinoma cases of the printing company. In the cases of the printing company, immunohistochemical expression of GST T1-1 was observed in biliary epithelial cells of the large bile duct with cholangitis (A), biliary intraepithelial neoplasia (BilIN) (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

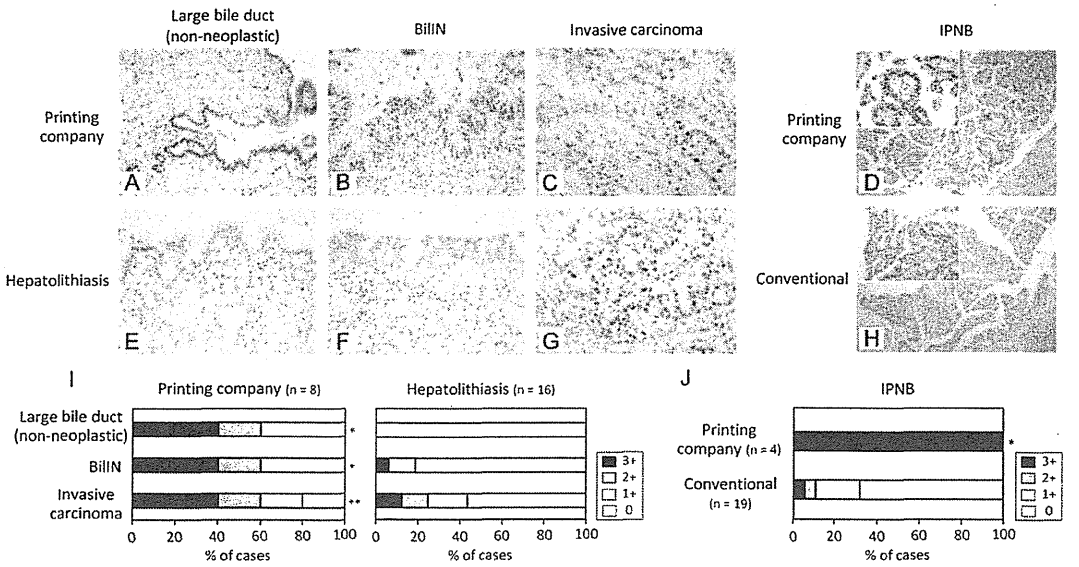

Figure 4. DNA damage in cholangiocarcinoma cases of the printing company. DNA damage was evaluated by the use of immunohistochemical staining of γ-H2AX as a marker of DNA double strand break. In cholangiocarcinoma cases of the printing company, positive expression of γ-H2AX was detected in non-neoplastic 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

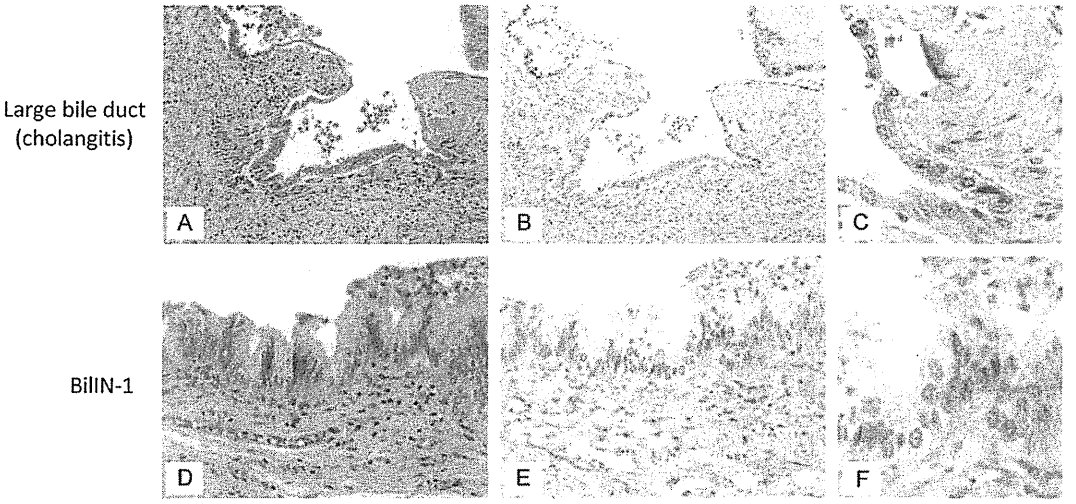

Figure 5. Expression of p53 in the printing company cases. The immunohistochemical expression of p53 was observed in non-neoplastic epithelial cells of the large bile duct associated with chronic cholangitis and bile duct damage (A-C) and the foci of biliary intraepithelial neoplasia-1 (BilIN-1) (D-F). (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.

J Hepatobiliary Pancreat Sci (2014) 21:809–817
DOI: 10.1002/jhbp.137

ORIGINAL ARTICLE

Characteristics of printing company workers newly diagnosed with occupational cholangiocarcinoma

Shoji Kubo · Masahiko Kinoshita · Shigekazu Takemura · Shogo Tanaka · Hiroji Shinkawa · Takayoshi Nishioka · Genya Hamano · Tokuji Ito · Makoto Abue · Masaru Aoki · Kei Nakagawa · Michiaki Unno · Susumu Hijioka · Toshihisa Fujiyoshi · Yasuhiro Shimizu · Toru Mizuguchi · Ken Shirabe · Akihiro Nishie · Yoshinao Oda · Kenji Takenaka · Tomonari Kobarai · Terumasa Hisano · Akio Saiura · Hiroshi Numao · Mayura Toda · Yuko Kuwae · Yasuni Nakanuma · Ginji Endo

Published online: 3 August 2014

S. Kubo (✉) · M. Kinoshita · S. Takemura · S. Tanaka · H. Shinkawa · T. Nishioka · G. Hamano · T. Ito
Department of Hepato-Biliary-Pancreatic Surgery, Osaka City University Graduate School of Medicine, 1-4-3 Asahimachi, Abeno-ku, Osaka 545-8585, Japan
e-mail: m7696493@msic.med.osaka-cu.ac.jp

M. Abue
Department of Gastroenterology, Miyagi Cancer Center, Natori, Japan

M. Aoki
Department of Digestive Surgery, Nippon University School of Medicine, Tokyo, Japan

K. Nakagawa · M. Unno
Division of Hepato-Biliary-Pancreatic Surgery, Department of Surgery, Tohoku University Graduate School of Medicine, Sendai, Japan

S. Hijioka · T. Fujiyoshi
Department of Gastroenterology, Aichi Cancer Center Hospital, Nagoya, Japan

Y. Shimizu
Department of Surgery, Aichi Cancer Center Hospital, Nagoya, Japan

T. Mizuguchi
Department of Surgery 1, Sapporo Medical University School of Medicine, Sapporo, Japan

K. Shirabe
Department of Surgery and Sciences, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

A. Nishie
Department of Clinical Radiology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

Y. Oda
Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

K. Takenaka
Department of Surgery, Fukuoka City Hospital, Fukuoka, Japan

T. Kobarai
Department of Surgery, HaraSanshin Hospital, Fukuoka, Japan

T. Hisano
Department of Hepato-Biliary-Pancreatology, National Hospital Organization Kyushu Cancer Center, Fukuoka, Japan

A. Saiura
Department of Surgery, Cancer Institute Hospital of Japanese Foundation for Cancer Research, Tokyo, Japan

H. Numao
Department of Gastroenterology, Aomori Prefectural Central Hospital, Aomori, Japan

M. Toda
Department of Gastroenterology and Hepatology, Kansai Rosai Hospital, Amagasaki, Japan

Y. Kuwae
Department of Diagnostic Pathology, Osaka City University Graduate School of Medicine, Osaka, Japan

Y. Nakanuma
Department of Human Pathology, Kanazawa University Graduate School of Medicine, Kanazawa, Japan

G. Endo
Department of Preventive Medicine and Environmental Health, Osaka City University Graduate School of Medicine, Osaka, Japan

Abstract
Background Cholangiocarcinoma has been reported in workers exposed to chlorinated organic solvents and has consequently been classified as an occupational disease (occupational cholangiocarcinoma) by the Japanese Ministry of Health, Labour and Welfare. This study aimed to identify the characteristics of nine workers newly diagnosed with occupational cholangiocarcinoma.
Methods This study was a retrospective study conducted in 13 hospitals and three universities. Clinicopathological findings of nine occupational cholangiocarcinoma patients from seven printing companies in Japan were investigated and compared with 17 cholangiocarcinoma patients clustered in a single printing company in Osaka.
Results Patient age at diagnosis was 31–57 years. Patients were exposed to 1,2-dichloropropane and/or dichloromethane. Serum γ-glutamyl transpeptidase activity was elevated in all patients. Regional dilatation of the intrahepatic bile ducts without tumor-induced obstruction was observed in two patients. Four patients developed intrahepatic cholangiocarcinoma and five developed hilar cholangiocarcinoma. Biliary intraepithelial neoplasia and/or intraductal papillary neoplasm of the bile duct was observed in four patients with available operative or autopsy specimens.
Conclusions Most of these patients with occupational cholangiocarcinoma exhibited typical findings, including high serum γ-glutamyl transpeptidase activity, regional dilatation of the bile ducts, and precancerous lesions, similar to findings previously reported in 17 occupational cholangiocarcinoma patients in Osaka.

Keywords γ-glutamyl transpeptidase · Biliary intraepithelial neoplasia · Intraductal papillary neoplasm of the bile duct · Occupational cholangiocarcinoma · Organic solvent

Introduction

Recently, an outbreak of cholangiocarcinoma was reported among workers in an offset color proof-printing department at a printing company in Osaka, Japan [1, 2]. These 17 patients with cholangiocarcinoma had been exposed to chemicals, including 1,2-dichloropropane (DCP) and/or dichloromethane (DCM), at the company. This type of cholangiocarcinoma was recently recognized as an occupational disease by the Japanese Ministry of Health, Labour and Welfare, on 1 October 2013 [3]. Until the end of 2013, the Ministry confirmed "occupational cholangiocarcinoma" in the 17 former or current workers of the company [2], along with nine workers of seven other printing companies. We have previously reported the clinicopathological findings of the first 17 workers [2]. The aim of this study was to investigate the clinical and pathological findings of the nine patients employed at the other printing companies who were also diagnosed with occupational cholangiocarcinoma.

Subjects and methods

The subjects of this study were nine patients with occupational cholangiocarcinoma, which was newly recognized as an occupational disease by the Japanese Ministry of Health, Labour and Welfare until the end of 2013. Patients with cholangiocarcinoma diagnosed between March 1988 and November 2011 were enrolled in this study. The nine patients were former or current workers at seven printing companies in Hokkaido, Aomori, Miyagi, Saitama, Aichi, Osaka, and Fukuoka. All nine had been exposed to chemicals, including a high concentration of chlorinated organic solvents, such as 1,1,1-trichloroethane (TCE), DCM, and/or DCP, over a long period of time. These organic solvents are used to remove ink residues. We investigated the clinical findings, laboratory test results, diagnostic imaging results, pathologic findings, treatments, and prognoses of the nine patients. The clinicopathological findings of nine occupational cholangiocarcinoma patients from seven printing companies were compared with those in our previous study, which examined 17 cholangiocarcinoma patients clustered in a single printing company in Osaka.

Patient data, including history of alcohol intake and smoking, and clinical findings were obtained from the medical records from each hospital and/or interviews with the patients or their family members. The results of laboratory tests and diagnostic imaging were obtained from the medical records and/or films from each hospital. For all nine patients, the diagnosis of cholangiocarcinoma was confirmed at the individual hospitals by pathologists who analyzed surgically resected specimens in four patients and biopsy specimens in five patients.

The pathological findings were recorded and described according to the World Health Organization classification of intrahepatic and extrahepatic cholangiocarcinoma [4]. Intrahepatic cholangiocarcinoma was grossly classified as a mass-forming, periductal infiltrating, or intraductal growth. Extrahepatic cholangiocarcinoma was grossly classified as a papillary, nodular, scirrhous constricting, or diffusely infiltrating tumor. Preneoplastic or early preinvasive neoplastic lesions of the biliary tree were classified as flat dysplastic epithelial tumors (biliary intraepithelial neoplasia [BilIN]) or grossly visible papillary tumors (intraductal papillary neoplasm of the bile duct [IPNB]) [4–7]. BilIN lesions were histologically classified according to their cellular and structural features as BilIN-1 (mild atypia), BilIN-2 (moderate atypia), or BilIN-3 (severe atypia corresponding to *in situ*

Table 1 Clinical findings in patients with cholangiocarcinoma

Patient no.	Clinical findings		Exposure to chlorinated organic solvents
	Age	Symptom(s) or health examination	Organic solvents and periods of exposure[a]
1	37	Liver dysfunction	DCP (16 years), TCE, DCM[b]
2	42	Jaundice	DCP (16 years), TCE, DCM[b]
3	49	Liver dysfunction	DCM (12 years), TCE
4	57	Jaundice	DCP (11 years), DCM (11 years)
5	31	Abdominal pain	DCP (3 years 10 months), DCM (3 years 10 months)
6	47	Abdominal pain, back pain	DCP (12 years), DCM (12 years)
7	47	Nausea	DCP (16 years), DCM (6 years)
8	46	Epigastralgia, nausea	DCP (13 years), DCM (19 years)
9	41	Jaundice, abdominal pain	DCM (11 years), TCE

DCM dichloromethane, *DCP* 1.2-dichloropropane, *TCE* 1,1,1-trichloroethane

[a] The period of exposure to TCE was not evaluated because TCE is not considered to be a main causative agent

[b] The period of exposure to DCM was not evaluated because the amount of DCM used was small

carcinoma). BilIN-1 lesions presented with mild atypical cellular and nuclear features, such as nuclear membrane irregularities or nuclear enlargements with only minimal disturbances to cellular polarity. BilIN-2 lesions had evident aberrant cellular and nuclear features not sufficient to suggest overt carcinoma; these lesions also had focal disturbances in cellular polarity. BilIN-3 lesions presented with diffuse disturbances in cellular polarity with or without distinct atypical cellular and nuclear features that corresponded to carcinoma *in situ*. In this study, primarily BilIN-2 and BilIN-3 lesions were examined because it is controversial whether BilIN-1 lesions contain any reactive hyperplastic changes. Other pathological terms used in this study were characterized or defined as follows [2]. "Chronic bile duct injury" was used as a collective term to describe duct injuries such as epithelial damage, fibrosis of the duct wall and periductal tissue, and chronic inflammatory cell infiltration, in various combinations. "Proliferative changes of bile ducts" was used to describe bile ducts with non-neoplastic biliary epithelial proliferation. "Bile duct sclerosis" was used to describe fibrous thickening of the duct wall with or without additional periductal fibrosis.

This study was approved by the ethics committee of Osaka City University, and all subjects or their legally authorized representatives (for deceased patients) provided written informed consent. This multicenter occupational cholangiocarcinoma study group consisted of investigators at 13 hospitals (including five university hospitals) and three universities.

Statistical analysis

Student's *t*-test or the Mann–Whitney *U*-test were used to determine significant difference in age and results of laboratory tests. The χ^2 test or Fisher's exact tests was used to evaluate significant differences in the categorical data between groups. Differences with $P < 0.05$ were considered to be statistically significant. Statistical analysis was performed with JMP 9.0 (SAS Institute, Cary, NC, USA).

Results

Clinical findings

The age of the patients at diagnosis was between 31 and 57 years (mean, 44 years), and all patients were men (Table 1). Of the nine patients, five (patients 4–8) had been exposed to DCP and DCM; two (patients 1, 2) had been exposed to DCP, DCM, and TCE; and two patients (patients 3, 9) had been exposed to DCM and TCE. The period of exposure to chlorinated organic solvents ranged from 3 years and 10 months to 19 years. No patient was a habitual alcohol user (≥80 g of ethanol consumed daily), and six patients (patients 1, 5–9) were smokers. Seven patients experienced abdominal pain, back pain, nausea, and/or jaundice. Abnormal liver function was noted in two patients (patients 1, 3) during regular health examinations.

Laboratory test results

At the time of cholangiocarcinoma diagnosis, the serum concentrations of total bilirubin were elevated in six patients (Table 2). Serum aspartate aminotransferase (AST) activity was elevated in seven patients, and alanine aminotransferase (ALT) activity was elevated in eight patients. Serum γ-glutamyl transpeptidase (γ-GTP) activity was elevated in all nine patients. In two (patients 1, 3) of the three patients for whom laboratory test results from regular health

Table 2 Results of laboratory test and diagnostic imaging

Patient no.	T-Bil (mg/dL)	Laboratory tests at diagnosis					Diagnostic imaging			
		AST (IU/L)	ALT (IU/L)	γ-GTP (U/L)	CEA (ng/mg)	CA19-9 (U/ml)	Space-occupying lesion or mass lesion	Bile duct stenosis or obstruction	Dilated bile ducts due to tumor-induced obstruction	Dilated bile ducts without tumor-induced obstruction
1	1.0	114 (h)	212 (h)	526 (h)	2.5	2418 (h)	Yes	Yes	Yes	Yes
2	10.0 (h)	188 (h)	321 (h)	1404 (h)	3.1	23253 (h)	Yes	Yes	Yes	No
3	1.2	84 (h)	137 (h)	2034 (h)	0.7	565.3 (h)	Yes	Yes	Yes	No
4	22.8 (h)	34	27	105 (h)	1.4	1283 (h)	Yes	Yes	Yes	No
5	5.2 (h)	96 (h)	145 (h)	270 (h)	49.2 (h)	2392 (h)	Yes	No	Yes	Yes
6	0.6	26	36 (h)	346 (h)	11.2 (h)	3.3	Yes	Yes	No	No
7	1.8 (h)	167 (h)	391 (h)	1109 (h)	1.5	16	No	Yes	Yes	No
8	1.8 (h)	261 (h)	301 (h)	1120 (h)	1.4	8.6	Yes	Yes	Yes	No
9	9.2 (h)	55 (h)	133 (h)	437 (h)	1.6	144.6 (h)	Yes	Yes	Yes	No

γ-GTP γ-glutamyl transpeptidase, *ALT* alanine aminotrasferase, *AST* aspartate aminotrasferase, *CA19-9* carbohydrate antigen 19-9, *CEA* carcinoembryonic antigen, *h* higher than the reference range, *T-Bil* total bilirubin

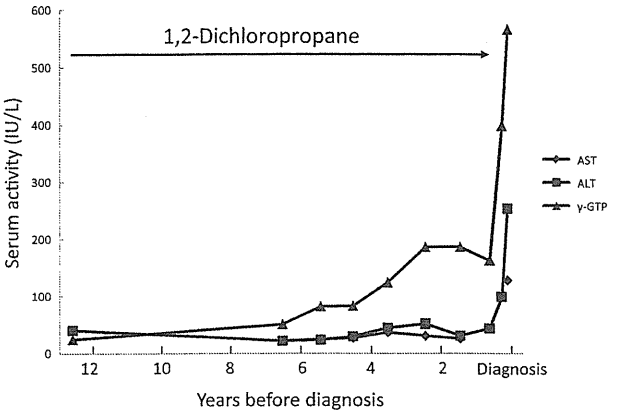

Fig. 1 Changes in laboratory test results before cholangiocarcinoma diagnosis (patient no. 1)

examinations for several years prior to the diagnosis of cholangiocarcinoma were available, the serum γ-GTP activity was observed to have increased gradually (Fig. 1). The serum concentration of carcinoembryonic antigen was elevated in two patients, and the serum concentration of carbohydrate antigen 19-9 (CA19-9) was elevated in six patients. The serum concentration of Dupan-2 was elevated in three of four patients examined, and the serum concentration of Span-1 was elevated in all three patients examined. All nine patients were negative for serum hepatitis B surface antigen and hepatitis C virus antibodies.

Diagnostic imaging results

Space-occupying lesions were observed in eight patients on computed tomography, magnetic resonance imaging, and/or ultrasonography (Table 2 and Fig. 2a). Stenosis or obstruction of the bile duct was observed in eight patients (Fig. 2b) on magnetic resonance cholangiopancreatography and/or direct cholangiography. Dilatation of peripheral bile ducts due to tumor-induced bile duct obstruction was observed in eight patients on ultrasonography, computed tomography, and/or magnetic resonance imaging (Fig. 2b). Regional dilatation of intrahepatic bile ducts without tumor-induced obstruction, a characteristic finding in the previous study [2], was observed in two patients (patients 1, 5) through magnetic resonance cholangiopancreatography (Fig. 2c).

Diagnosis and pathological findings of cholangiocarcinoma

Considering the radiological and pathological findings, four patients (patients 1, 4–6) were diagnosed with intrahepatic cholangiocarcinoma, and the remaining five patients (patients 2, 3, 7–9) were diagnosed with extrahepatic

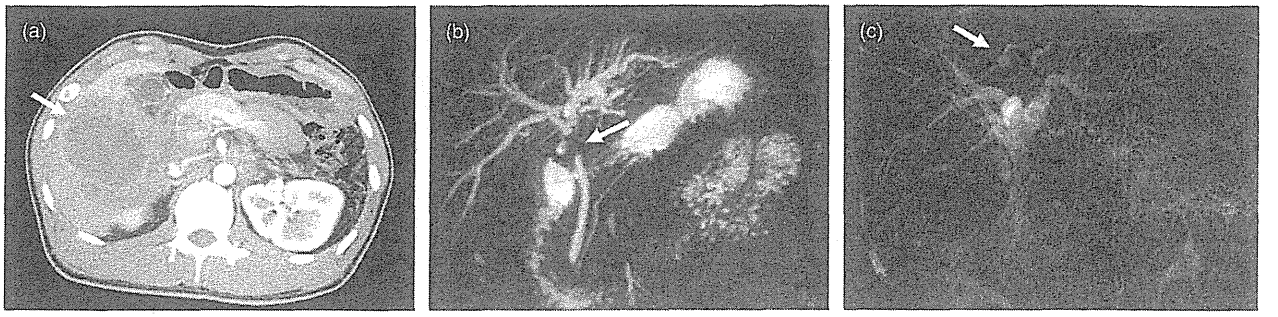

Fig. 2 Diagnostic imaging results of patients with cholangiocarcinoma. (**a**) Intrahepatic cholangiocarcinoma of the mass-forming type (arrow) in patient 5. (**b**) Extrahepatic (hilar) cholangiocarcinoma in patient 7; stenosis of the bile ducts in the hepatic hilum is observed (arrow). (**c**) Dilated intrahepatic bile ducts without tumor-induced obstruction (arrow) in patient 5

Table 3 Pathological findings of cholangiocarcinoma

Patient no.	Location	Type	Pathological examination		Stage by TNM classification
			Specimens	Histology of tumor and BilIN or IPNB in non-cancerous tissue	
1	Intrahepatic	Mass-forming	Biopsy during ERCP; autopsy	Adenocarcinoma with papillary component; adenocarcinoma, BilIN and IPNB	IVB
2	Extrahepatic (hilar)	Scirrhous constricting	Surgically resected	Tubular adenocarcinoma and BilIN	IIIB
3	Extrahepatic (hilar)	Unknown	Biopsy during ERCP	Papillary adenocarcinoma with compact pattern	I
4	Intrahepatic	Mass-forming	Surgically resected	Well to moderately differentiated adenocarcinoma and BilIN	IVB
5	Intrahepatic	Mass-forming	Needle liver biopsy	Adenocarcinoma	IVA
6	Intrahepatic	Mass-forming	Surgically resected (2 nodules)	Well to moderately differentiated adenocarcinoma with tubular or papillary component, poorly differentiated adenocarcinoma and BilIN	IVA
7	Extrahepatic (hilar)	Unknown	Biopsy during ERCP	Well differentiated adenocarcinoma with papillary component	II
8	Extrahepatic (hilar)	Diffusely infiltrating	Surgically resected	Moderately differentiated tubular adenocarcinoma	IIIA
9	Extrahepatic (hilar)	Nodular	Biopsy during ERCP	Adenocarcinoma	II

BilIN biliary intraepithelial neoplasia, *ERCP* endoscopic retrograde cholangiopancreatograpy, *IPNB* intraductal papillary neoplasm of the bile duct

cholangiocarcinoma (hilar cholangiocarcinoma, Table 3). The four cases of intrahepatic cholangiocarcinoma were classified as the mass-forming type. Comprehensive imaging studies and gross analyses demonstrated that the primary and most invasive cholangiocarcinoma lesions were located in the common hepatic duct, the left or right hepatic duct, or the first to third branches of the intrahepatic bile duct (also known as the large bile duct) [8]. Lymph node metastsis in three patients (patients 1, 5, 6) was observed on diagnostic imaging scans at the time of cholangiocarcinoma diagnosis.

In four (patients 1, 3, 7, 9) of the nine patients, adenocarcinoma was identified in biopsy specimens obtained during endoscopic retrograde cholangiopancreatography. Three (patients 1, 3, 7) of these specimens exhibited papillary proliferation (Fig. 3a). In one patient (patient 5),