

Original Article

# Diphenylarsinic acid exerts promotion effects on hepatobiliary carcinogenesis in a rat medium-term multiorgan carcinogenicity bioassay

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**Abstract:** We have previously demonstrated that diphenylarsinic acid (DPAA) promotes liver carcinogenesis in rats in a medium-term liver carcinogenicity bioassay. However, the effects of DPAA on other organs have not been determined. In the present study, the effects of DPAA on carcinogenesis were investigated using a rat multiorgan carcinogenicity bioassay. A total of 60 six-week-old male F344 rats were treated with the carcinogens diethylnitrosamine, N-butyl-N-(4-hydroxybutyl) nitrosamine, N-methyl-N-nitrosourea, N-bis (2-hydroxypropyl) nitrosamine, and 1,2-dimethylhydrazine dihydrochloride to initiate carcinogenesis in multiple organs. After initiation, DPAA was given at a dose of 0, 5, or 20 ppm in drinking water for 27 weeks. The incidences of moderate and severe bile duct hyperplasia were significantly increased in the 20 ppm DPAA group (29.4%, 70.6%, respectively) compared with the 0 ppm DPAA group (0%, 0%, respectively), and the incidence and multiplicity of cholangioma were significantly increased in the 20 ppm DPAA group (29.4%,  $0.4 \pm 0.8/\text{rat}$ ) compared with the 0 ppm DPAA group (0%, 0/rat). The total number and average area of glutathione S-transferase placenta form-positive foci, preneoplastic lesions in rat livers, were significantly increased in the 20 ppm DPAA group ( $10.5 \pm 2.2/\text{cm}^2$ ,  $5.3 \pm 1.7 \text{ mm}^2/\text{cm}^2$ ) compared with the 0 ppm DPAA group ( $6.2 \pm 2.9/\text{cm}^2$ ,  $2.4 \pm 1.4 \text{ mm}^2/\text{cm}^2$ ). In conclusion, our results demonstrate that DPAA promotes hepatobiliary carcinogenesis in a rat medium-term multiorgan carcinogenicity bioassay; no promotion effects were observed in other organs. (DOI: 10.1293/tox.2016-0049; J Toxicol Pathol 2017; 30: 39–45)

**Key words:** diphenylarsinic acid, liver carcinogenesis, cholangioma, bile duct hyperplasia, DMBDD

## Introduction

Diphenylarsinic acid (DPAA), an organic arsenic, is a degradation product of diphenylarsine chloride and diphenylarsine cyanide<sup>1, 2</sup>, both of which were developed as chemical warfare agents during World Wars I and II. Little attention was paid to the health effects of DPAA until neurotoxicities were reported in residents of Kamisu City, Japan, exposed chronically to DPAA through contaminated drinking well water<sup>3</sup>, possibly due to illegal dumping of chemical weapons. The health effects of chronic exposure to DPAA including the carcinogenicity of DPAA are currently of great concern.

We previously found that DPAA promoted liver carci-

nogenesis in rats via activation of aryl hydrocarbon receptor signaling and consequent induction of oxidative DNA damage<sup>4</sup>. However, no other information is available concerning cancer risk assessment of DPAA.

The purpose of the present study was to evaluate the effects of DPAA on carcinogenesis using a rat multiorgan carcinogenicity bioassay. The multiorgan carcinogenesis bioassay utilizes an initiation-promotion protocol with five types of genotoxic carcinogens (diethylnitrosamine [DEN], N-methyl-N-nitrosourea [MNU], N-butyl-N-(4-hydroxybutyl) nitrosamine [BBN], N-bis (2-hydroxypropyl) nitrosamine [DHPN], and 1,2-dimethylhydrazine dihydrochloride [DMH]) as initiators of carcinogenesis in the liver, kidneys, urinary bladder, stomach, small intestine, colon, lungs, and thyroid gland, followed by treatment with the test substance<sup>5–7</sup>. Histopathological analyses were performed to investigate development of preneoplastic lesions or cancer.

## Materials and Methods

### Chemicals

DPAA (purity: 99.9%) was obtained from Tri Chemical Laboratories (Yamanashi, Japan). The stability of DPAA in

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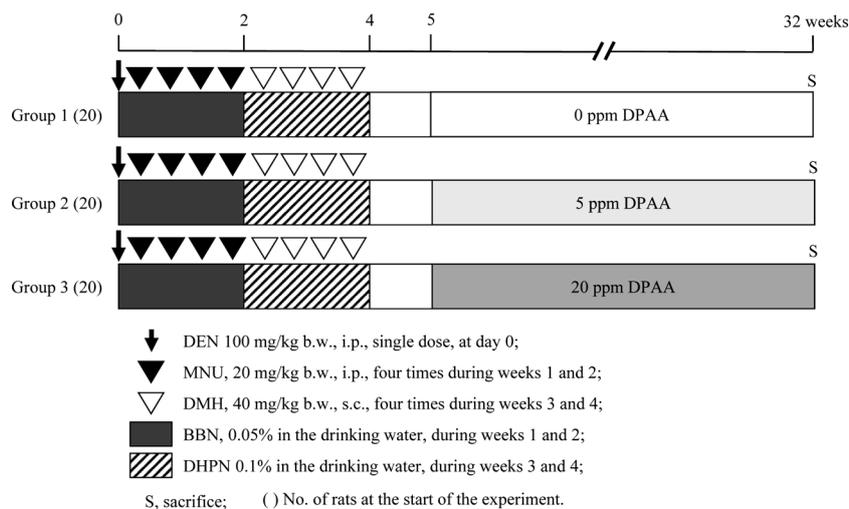
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**Fig. 1.** Experimental protocol.

tap water for 28 days at room temperature was confirmed using an ion chromatography inductively coupled plasma mass spectrometry (IC-ICP-MS) system (IC7000 ion chromatograph, Yokogawa Analytic System Inc., Tokyo, Japan; HP 4500 inductively coupled plasma mass spectrometer, Wilmington, DE, USA) at Osaka City University Graduate School of Medicine. DEN, DMH, and BBN were purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). MNU was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and DHPN was purchased from Nacal Tesque Inc. (Kyoto, Japan). Pentobarbital sodium (Somnopentyl®) was purchased from Kyoritsu Seiyaku Corporation (Tokyo, Japan).

### Animals

Five-week-old male F344 rats were purchased from Charles River Laboratories Japan, Inc. (Kanagawa, Japan). They were housed in plastic cages (three or two rats/cage) in an environmentally controlled room maintained at a temperature of  $22 \pm 2^\circ\text{C}$  and a relative humidity of  $50 \pm 10\%$ , with a 12-h light/dark cycle. Food (MF pellet diet, Oriental Yeast Co., Ltd., Tokyo, Japan) and drinking water were available ad libitum throughout the study. Experiments were initiated after a 1 week acclimation period. Body weights and food and water intake were measured weekly during the experimental period.

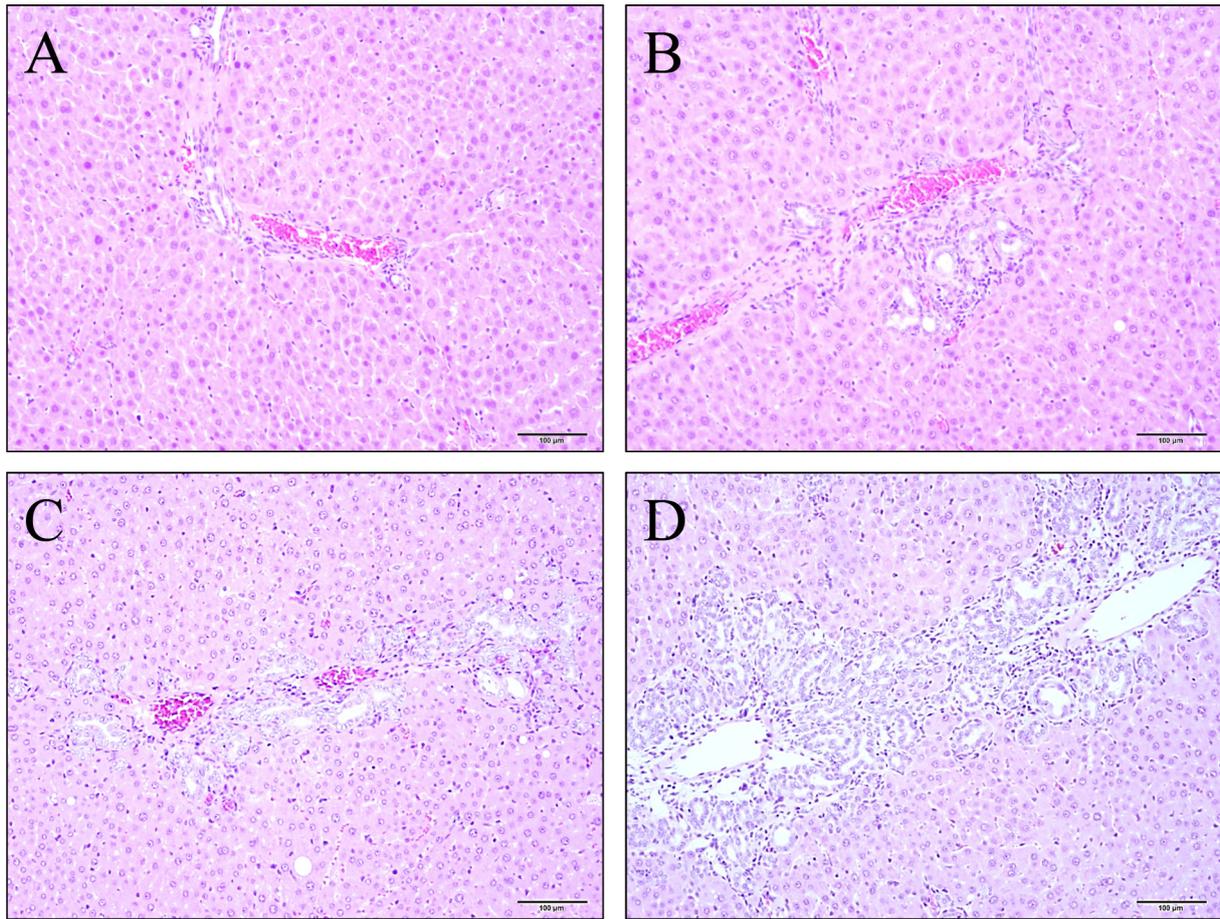
### Experimental design

The animal experimental protocols were approved by the Institutional Animal Care and Use Committee of Osaka City University Graduate School of Medicine. The experimental protocol for the rat multiorgan carcinogenicity bioassay used in this study is shown in Fig. 1. A total of 60 male F344 rats at 6 weeks of age were divided randomly into three groups. All rats were treated with the five carcinogens, DEN, MNU, BBN, DMH, and DHPN (DMBDD), as follows: A single intraperitoneal (i.p.) injection of DEN (100

mg/kg body weight [b.w.]) was given at the beginning of the experiment, followed by four i.p. injections of MNU (20 mg/kg b.w.) during weeks 1 and 2 and then four subcutaneous (s.c.) injections of DMH (40 mg/kg b.w.) during weeks 3 and 4. BBN was administered as 0.05% BBN in drinking water during weeks 1 and 2, and DHPN was administered as 0.1% DHPN in drinking water during weeks 3 and 4. One week after the initiation treatments, the animals were administered DPAA in drinking water for 27 weeks: groups 1, 2, and 3 received 0 (as the control), 5, and 20 ppm, respectively. At the end of week 32, all surviving rats were sacrificed by administration of an overdose (50 mg/kg b.w., i.p.) of pentobarbital sodium. Target organs, including the liver, colon, lung, thyroid gland, urinary bladder, and kidneys, were removed and fixed in 10% phosphate-buffered formalin and embedded in paraffin for histopathological examination. Bile duct hyperplasia was assigned a severity grade (mild, moderate, or severe). If bile duct proliferation was present in a limited area and the bile duct proliferation was slight, lesions were designated as mild hyperplasia. If the portal area was extended geographically by bile duct proliferation, the lesions were designated as severe. Intermediates between mild and severe lesions were designated as moderate bile duct hyperplasia. Fig. 2 shows representative pictures of each type of lesion. Liver tissues embedded in paraffin were processed for immunohistochemical analyses of glutathione S-transferase placenta form (GST-P)-positive foci, which are well-established preneoplastic liver lesions in rats<sup>8</sup>, and the cell proliferation marker Ki-67. Colons were excised and intraluminally injected with 0.9% saline, cut longitudinally, washed with saline, extended between two filter papers, and then fixed in 10% phosphate-buffered formalin.

### Immunohistochemical analysis

Liver tissues embedded in paraffin were examined for GST-P-positive foci and Ki-67 by immunohistochemical staining using the avidin-biotin-peroxidase complex (ABC)



**Fig. 2.** Representative appearance of the normal portal area (A) and mild (B), moderate (C), and severe (D) bile duct hyperplasia. A and B are livers of rats from the 0 ppm DPAA group, and C and D are livers of rats from the 20 ppm DPAA group. Each picture is shown at the same magnification.

method. Ki-67 immunostaining was performed in the livers of 10 rats randomly chosen from each group, including livers with cholangiomas. Briefly, paraffin sections were deparaffinized in xylene and then hydrated in graded concentrations of ethanol for 5 min each. Endogenous peroxidase activity was blocked with 3% hydrogen peroxidase in distilled water for 5 min. After blocking nonspecific binding with serum for 15 min, sections were incubated overnight at 4°C with rabbit anti-rat GST-P polyclonal antibody (Medical & Biological Laboratories Co., Ltd., Aichi, Japan) diluted 1:1,000 or rabbit anti-Ki-67 polyclonal antibody (Epitomics, Inc., Burlingame, CA, USA) diluted 1:500. Immunoreactivity was detected using a VECSTAIN Elite ABC Kit (Rabbit IgG; Vector Laboratories, Inc., Burlingame, CA, USA).

#### *Quantitative analysis of GST-P-positive foci in the liver*

Numbers and areas of GST-P-positive foci larger than 0.2 mm in diameter and the total areas of liver sections examined were measured blindly using a color image processor (IPAP-WIN, Sumika Technoservice Corporation, Hyogo, Japan), and the number and area of GST-P-positive foci

**Table 1.** Final Body Weights and Organ Weights

	Group 1	Group 2	Group 3
DMBDD treatment	+	+	+
DPAA (ppm)	0	5	20
Initial no. of rats	20	20	20
Final no. of rats	16	17	16
Survival rate (%)	80	85	80
Final body weight (g)	333.6 ± 29.1	337.5 ± 19.6	317.2 ± 19.5
Absolute (g)			
Liver	7.3 ± 1.0	7.5 ± 0.6	8.8 ± 1.0*
Kidneys	2.5 ± 0.9	2.4 ± 0.4	3.1 ± 1.1
Relative (g/100 g b.w.)			
Liver	2.2 ± 0.2	2.2 ± 0.1	2.8 ± 0.3*
Kidneys	0.8 ± 0.3	0.7 ± 0.1	1.0 ± 0.4*

\*  $p < 0.05$ , vs. 0 ppm DPAA group (Group 1).

per square centimeter of liver tissue were calculated.

#### *Statistical analysis*

All mean values are reported as mean ± SD values. Statistical analyses were performed using the StatLight program (Yukms Co., Ltd., Tokyo, Japan) and IBM SPSS Sta-

**Table 2.** Histopathological Findings

Site and type of lesion DPAA (ppm)		Incidence (%)			Multiplicity (No./rat)			
		0	5	20	0	5	20	
Effective no. of rats <sup>a</sup>		19	20	17	19	20	17	
Liver	Hepatocellular adenoma	5 (26.3)	3 (15.0)	2 (11.8)	0.3 ± 0.5	0.2 ± 0.4	0.1 ± 0.3	
	Hepatocellular carcinoma	1 (5.3)	0 (0)	2 (11.8)	0.1 ± 0.2	0	0.1 ± 0.3	
	Total hepatocellular tumors	5 (26.3)	3 (15.0)	4 (23.5)	0.3 ± 0.6	0.2 ± 0.4	0.2 ± 0.4	
	Bile duct hyperplasia	mild	19 (100)	19 (95.0)	0 (0)*			
		moderate	0 (0)	1 (5.0)	5 (29.4)*			
		severe	0 (0)	0 (0)	12 (70.6)*			
	Cholangioma	0 (0)	0 (0)	5 (29.4)*	0	0	0.4 ± 0.8*	
Hemangiosarcoma	0 (0)	0 (0)	1 (5.9)	0	0	0.1 ± 0.2		
Colon	Adenoma	7 (36.8)	11 (55.0)	8 (47.1)	0.7 ± 1.1	0.9 ± 1.1	1.0 ± 1.4	
	Adenocarcinoma	5 (26.3)	7 (35.0)	1 (5.9)	0.4 ± 0.7	0.6 ± 0.9	0.1 ± 0.5	
	Total tumors	10 (52.6)	13 (65.0)	8 (47.1)	1.1 ± 1.4	1.5 ± 1.4	1.1 ± 1.6	
Lung	Adenoma	19 (100.0)	19 (95.0)	17 (100)	3.7 ± 2.7	4.5 ± 2.3	3.8 ± 2.4	
	Adenocarcinoma	8 (42.1)	4 (20.0)	9 (52.9)	0.5 ± 0.6	0.3 ± 0.7	1.0 ± 1.1	
	Total tumors	19 (100.0)	19 (95.0)	17 (100)	4.2 ± 2.8	4.5 ± 2.8	4.8 ± 2.9	
Thyroid gland <sup>b</sup>	Follicular cell hyperplasia	15 (78.9)	12 (63.2)	8 (47.1)	1.6 ± 1.5	1.4 ± 1.3	0.5 ± 0.6*	
	Follicular cell adenoma	5 (26.3)	5 (26.3)	2 (11.8)	0.3 ± 0.6	0.3 ± 0.6	0.2 ± 0.5	
	Follicular cell carcinoma	6 (31.6)	5 (26.3)	6 (35.3)	0.4 ± 0.6	0.4 ± 0.7	0.4 ± 0.5	
	Total tumors <sup>c</sup>	10 (52.6)	10 (52.6)	8 (47.1)	0.7 ± 0.7	0.7 ± 0.7	0.5 ± 0.6	
Urinary bladder	PN hyperplasia	5 (26.3)	9 (45.0)	5 (29.4)	0.4 ± 0.7	0.7 ± 0.9	0.4 ± 0.6	
	Papilloma	5 (26.3)	5 (25.0)	3 (17.6)	0.4 ± 0.7	0.4 ± 0.8	0.2 ± 0.4	
	Urothelial carcinoma	2 (10.5)	1 (5.0)	2 (11.8)	0.1 ± 0.3	0.1 ± 0.2	0.2 ± 0.5	
	Total tumors <sup>d</sup>	6 (31.6)	6 (30.0)	5 (29.4)	0.5 ± 0.8	0.5 ± 0.8	0.4 ± 0.6	
Kidney	Renal cell carcinoma	4 (21.1)	4 (20.0)	1 (5.9)	0.2 ± 0.4	0.3 ± 0.6	0.1 ± 0.2	
	Nephroblastoma	14 (73.7)	17 (85.0)	17 (100)*	1.7 ± 1.4	1.5 ± 0.9	2.3 ± 1.5	
	Renal pelvic carcinoma (urothelial carcinoma)	1 (5.3)	3 (15.0)	1 (5.9)	0.1 ± 0.2	0.2 ± 0.4	0.1 ± 0.2	
	Hemangiosarcoma	0 (0)	1 (5.0)	0 (0)	0	0.1 ± 0.2	0	

\*  $p < 0.05$ , vs. the 0 ppm DPAA group (Group 1), PN, papillary or nodular; <sup>a</sup> number of rats surviving to week 9 and used for tissue analysis, other than the thyroid gland; <sup>b</sup> effective number of rats for thyroid gland analysis are 19, 19 and 17 in the 0, 5 and 20 ppm DPAA groups, respectively (see text for explanation); <sup>c</sup> follicular cell adenoma + follicular cell carcinoma; <sup>d</sup> papilloma + urothelial carcinoma.

tistics (IBM Japan Co., Ltd., Tokyo, Japan). Homogeneity of variance analysis was performed by the F-test or Bartlett test. Differences in mean values among the groups were evaluated by the Dunnett test when variances were homogeneous and the Steel test when variances were heterogeneous. Differences between the 0 ppm DPAA group (mild bile duct hyperplasia) and 20 ppm DPAA group (moderate hyperplasia, severe hyperplasia, and cholangioma) in the mean values of the Ki-67 index were evaluated by Student's t-test when variances were homogeneous and the Welch's t-test when variances were heterogeneous. Incidence was assessed by Fisher's exact probability test.  $P$  values less than 0.05 were considered significant.

## Results

### General observations

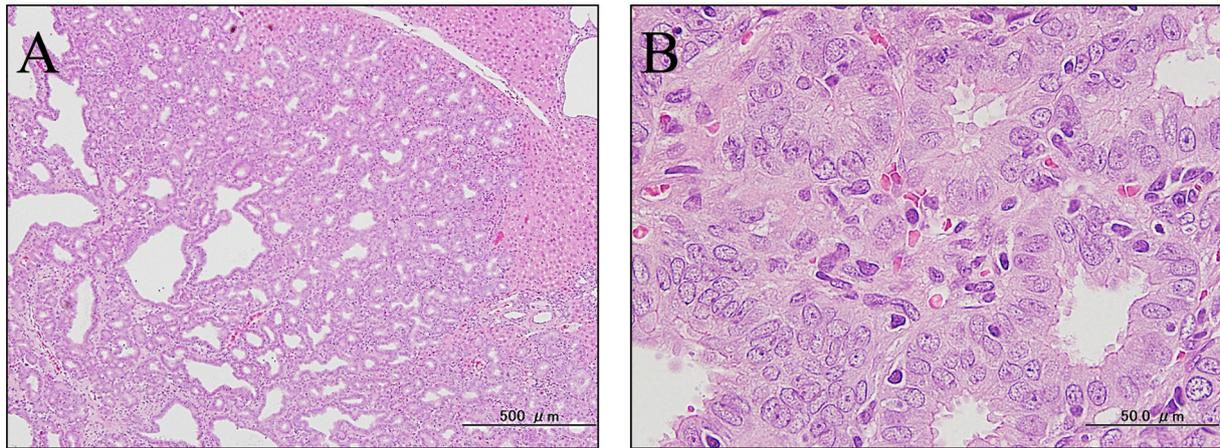
The number of rats surviving to the end of the study, final body weights, and absolute and relative liver and kidney weights are summarized in Table 1. Four animals died during weeks 7 and 8, possibly due to DMBDD treatment-related reasons, and were therefore not included in the analyses. Therefore, the numbers of rats surviving to week 9 (i.e., the effective number of rats) were 19, 20, and 17 in

the 0, 5, and 20 ppm DPAA groups, respectively. However, the effective numbers of rats for examination of the thyroid gland were 19, 19, and 17 in the 0, 5, and 20 ppm DPAA groups, respectively, due to sampling error. The numbers of rats that survived until scheduled sacrifice were 16, 17, and 16 in the 0, 5, and 20 ppm DPAA groups, respectively, and there were no significant differences in survival rates among the groups. Final body weights did not significantly differ among the groups. Absolute and relative liver weights and relative kidney weights of the 20 ppm DPAA group were significantly higher than those of the 0 ppm DPAA group. These factors may be associated with the severity of bile duct hyperplasia and the high incidence of cholangioma and nephroblastoma, as described below.

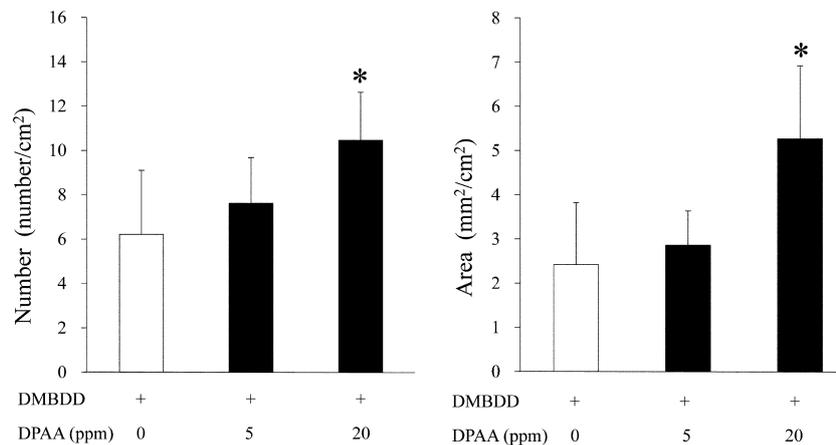
### Histopathological findings

Table 2 summarizes the data regarding the incidence and multiplicity of preneoplastic and neoplastic lesions in the liver, colon, lung, thyroid gland, urinary bladder, and kidney.

Bile duct hyperplasia (mild, moderate, severe) was seen in all of the examined rats. The incidences of moderate and severe bile duct hyperplasia were significantly increased in the 20 ppm DPAA group (29.4%, 70.6%, respectively) compared with the 0 ppm DPAA group (0%, 0%, respectively).



**Fig. 3.** Representative appearance of a cholangioma in the 20 ppm DPAA group. Hematoxylin and eosin staining with low-power magnification (A) and high-power magnification (B). The lesion is well circumscribed and cuboidal cells are proliferating, forming glandular structures without atypia.



**Fig. 4.** Numbers and areas of GST-P-positive foci, preneoplastic lesions in rat livers, in DMBDD-treated rats. Both the total number and area of GST-P-positive foci were significantly increased in the 20 ppm DPAA group compared with the 0 ppm DPAA group. \* Significantly different from the 0 ppm DPAA group at  $p < 0.0001$ .

There was no cholangiofibrosis, cholangiofibroma, or oval cell hyperplasia. The incidence and multiplicity of cholangioma (Fig. 3) were significantly increased in the 20 ppm DPAA group (29.4%,  $0.4 \pm 0.8$ /rat) compared with the 0 ppm DPAA group (0%, 0/rat). There were no significant differences in the incidences or multiplicities of hepatocellular adenoma, hepatocellular carcinoma, or total hepatocellular tumors (adenoma + carcinoma) among the groups.

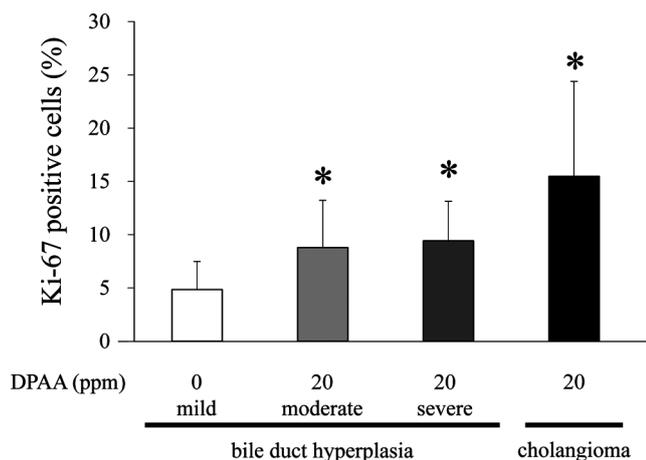
The multiplicity of follicular cell hyperplasia of the thyroid gland was significantly decreased in the 20 ppm DPAA group ( $0.5 \pm 0.6$ /rat) compared with the 0 ppm DPAA group ( $1.6 \pm 1.5$ /rat). There were no significant differences in the incidences of follicular cell hyperplasia, adenoma, carcinoma, or total tumors of the thyroid gland.

The incidence of nephroblastoma was significantly in-

creased in the 20 ppm DPAA group (100%) compared with the 0 ppm DPAA group (73.7%). There were no significant differences in the multiplicities of nephroblastoma, renal cell carcinoma, renal pelvic carcinoma (urothelial carcinoma), or hemangiosarcoma.

#### *GST-P-positive foci formation in livers*

The numbers and areas of GST-P-positive foci, preneoplastic lesions in rat livers, in the DMBDD-treated rat liver are shown in Fig. 4. Both total number and average area of GST-P-positive foci were significantly increased in the 20 ppm DPAA group ( $10.5 \pm 2.2$ /cm<sup>2</sup>,  $5.3 \pm 1.7$  mm<sup>2</sup>/cm<sup>2</sup>) compared with the 0 ppm DPAA group ( $6.2 \pm 2.9$ /cm<sup>2</sup>,  $2.4 \pm 1.4$  mm<sup>2</sup>/cm<sup>2</sup>).



**Fig. 5.** Ki-67 index in mild, moderate, and severe bile duct hyperplasia and cholangioma in the livers. The Ki-67 index in moderate and severe bile duct hyperplasias and cholangiomas in the 20 ppm DPAA group was significantly increased compared with that in mild bile duct hyperplasia in livers of rats from the 0 ppm DPAA group. \* Significantly different from mild bile duct hyperplasia at  $p < 0.05$ .

#### Quantification of cell proliferation by immunohistochemistry of Ki-67

Fig. 5 shows the Ki-67 data obtained from mild, moderate, and severe bile duct hyperplasia and cholangioma in the livers. The Ki-67 indexes in moderate and severe bile duct hyperplasias and cholangioma ( $8.8 \pm 4.4\%$ ,  $9.4 \pm 3.7\%$ , and  $15.5 \pm 8.9\%$ , respectively) were significantly increased compared with that in mild bile duct hyperplasia in the 0 ppm DPAA group ( $4.8 \pm 2.6\%$ ).

#### Discussion

The present study demonstrated that DPAA exerts promotion effects on hepatocellular and biliary carcinogenesis in rats, as evidenced by the findings showing that 20 ppm DPAA significantly enhanced the incidence and multiplicity of cholangioma and the development of DMBDD-induced GST-P-positive foci, a well-established preneoplastic liver lesions in rats.

The increase in the number and area of GST-P-positive foci in the DMBDD-treated rat liver agree with our previous findings showing that DPAA promotes liver carcinogenesis in a medium-term rat liver carcinogenicity assay<sup>4</sup>. GST-P-positive foci are a surrogate end-point marker for predicting liver carcinogenicity in rats and serve as a sensitive marker for liver carcinogenesis in the rat medium-term multiorgan carcinogenicity model<sup>9–11</sup>. While our findings indicate that DPAA promotes hepatocarcinogenesis in rats, there were no significant changes in the incidences and multiplicities of hepatocellular adenoma or hepatocellular carcinoma in the present study. However, given the increase in preneoplastic lesions in the liver in the 20 ppm DPAA group, it is likely that the incidence and/or multiplicity of hepatocellular tu-

mors would have been different among the 3 groups if the period of the experiment was extended. We are not able to exclude the possibility that factors other than the length of the experiment were associated with the results of the present study.

DPAA activates the aryl hydrocarbon receptor signaling pathway, which promotes oxidative DNA damage and inhibits apoptosis, thereby promoting liver carcinogenesis in rats<sup>4</sup>. There is little information about biliary carcinogenesis caused by arsenic compounds. The mechanisms of toxicity in the bile duct caused by chemicals remains poorly understood<sup>12</sup>. In our previous medium-term rat liver carcinogenesis assay, 6 weeks of treatment with 20 ppm DPAA induced mild bile duct hyperplasia regardless of DEN initiation<sup>4</sup>, and in a more recent study, administration of 20 ppm DPAA alone induced severe bile duct hyperplasia in a 52-week chronic toxicity study in rats, although no bile duct tumors were observed (manuscript in preparation). In the present study, DPAA increased the cell proliferative activity in bile duct hyperplasias and promoted the development of cholangioma. There are some reports indicating that the cytotoxic effects of DPAA are enhanced by the interaction with sulfhydryl compounds, such as glutathione, dimercaptopropane sulfonate, and dithiothreitol<sup>13,14</sup>. Taken together, these results suggest that DPAA might be toxic to the bile duct and that increased cholangiocyte proliferation might contribute to the promoting effects of DPAA on biliary carcinogenesis. Further studies are needed to elucidate the mechanism underlying the promoting effects of DPAA on biliary carcinogenesis in rats.

In conclusion, our results demonstrate that DPAA promotes hepatocellular and biliary carcinogenesis in the liver, but not in other organs, in the rat. Our ongoing 2-year rat carcinogenicity study will clarify whether DPAA is a complete carcinogen in the liver.

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**Disclosure of Potential Conflicts of Interest:** The authors declare that there are no conflicts of interest.

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