

**Chemopreventative effect of an inducible nitric oxide synthase inhibitor,
ONO-1714, on inflammation-associated biliary carcinogenesis in hamsters**

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Key words: chemoprevention, biliary carcinoma, nitric oxide, inducible nitric oxide
synthase (iNOS), iNOS inhibitor, cholangitis, bilioenterostomy, hamster

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Abstract

The present study was designed to investigate whether an inducible nitric oxide synthase (iNOS)-specific inhibitor, ONO-1714, could prevent inflammation-associated biliary carcinogenesis in bilioenterostomized hamsters. Syrian golden hamsters underwent choledochojejunostomy and then received subcutaneous injections of the chemical carcinogen *N*-nitrosobis(2-oxopropyl)amine (BOP) every 2 weeks at a dose of 10mg/kg body weight, starting 4 weeks after surgery and continuing for 18 weeks. The hamsters were divided into 2 groups according to their oral intake of either a standard pelleted diet containing ONO-1714 at 100 ppm for 18 weeks (ONO group, n=15) or an ordinary diet alone (control group, n=15). The animals were killed 22 weeks after surgery, and the development of biliary tumors was examined histologically. The presence and degree of cholangitis, cell kinetic status of the biliary epithelium, and iNOS expression were evaluated. Intrahepatic biliary adenomas developed in all control animals, whereas they developed in only 7 (47%) hamsters treated with ONO-1714 ($p<0.05$). Intrahepatic biliary carcinomas were present in 13 (87%) hamsters in the control group and only 6 (40%) hamsters in the ONO groups ($p<0.05$). Histological and immunohistochemical examinations demonstrated a significant decrease in the degree

of cholangitis, biliary epithelial cell kinetics, and the expression of iNOS in the biliary epithelium in the ONO group in comparison to the control ($p < 0.05$). These results indicate that ONO-1714 represses BOP-induced biliary carcinogenesis in bilioenterostomized hamsters and inhibits iNOS expression in the biliary epithelium. ONO-1714 may therefore be a promising agent for the prevention of biliary carcinoma in various inflammation-associated biliary disorders.

Introduction

Bilioenterostomy is commonly used in the field of hepatobiliary and pancreatic surgery. Reflux cholangitis (1-3), biliary stones (3, 4), and liver abscesses (2) are well-known complications after bilioenterostomy. In addition, recent studies have revealed that biliary carcinomas can occur as a delayed complication of bilioenterostomy for benign disease (5-7). We previously demonstrated that persistent reflux cholangitis after bilioenterostomy accelerates biliary carcinogenesis through the activation of biliary epithelial cell kinetics in hamsters (8, 9). In bilioenterostomized hamsters, biliary carcinoma develops 12 weeks after surgery with the use of a chemical carcinogen or shows spontaneous occurrence 60 weeks after bilioenterostomy (8-10).

Nitric oxide (NO) is produced endogenously by a family of NO synthases (NOSs) and exhibits a wide range of physiological and pathophysiological actions (11, 12). Neuronal NOS (nNOS) and endothelial NOS (eNOS) mediate the constitutive synthesis of NO from L-arginine and show little association with the development of inflammation or carcinogenesis. However, inducible NOS (iNOS), a distinct calcium-independent isoform of NOS (130kDa protein), plays an essential role in the

inflammatory response and injury repair. An aberrant or excessive expression of iNOS has been implicated in the pathogenesis of many diseases, including various human cancers such as breast, stomach, ovary, cervix, brain, lung, colon, prostate, and esophageal (13-17). An overexpression of iNOS is significantly associated with chronic inflammation (18-20); furthermore, iNOS overexpression leads to an excessive production of NO, which is thought to accelerate carcinogenesis in several organs of both humans (21) and laboratory animals (22-24). A recent study has demonstrated that iNOS inhibition prevents NO production and shows anti-carcinogenic effects on chemically induced or genetic mutational carcinogenesis (22). In an *in vitro* study utilizing gallbladder epithelial cells isolated from the hamster, we demonstrated that the stimulation of biliary epithelial cells with inflammatory cytokines promotes NO production and subsequent DNA damage, while iNOS inhibition reduces both NO production and NO-mediated DNA damage (25).

In the current study, we investigated whether an iNOS-specific inhibitor, ONO-1714 ((1S, 5S, 6R, 7R)-7-chloro-3-imino-5-methyl-2-azabicyclo[4.1.0] heptane), could prevent biliary carcinogenesis in bilioenterostomized hamsters. Syrian golden hamsters were used as an animal model because the anatomical structure of the pancreaticobiliary ductal system, bile acid composition, and pancreatic juice

components in this species are similar to those observed in humans (26-28).

Materials and Methods

Animals

Seven-week-old female Syrian golden hamsters (SLC, Shizuoka, Japan) were used. They were housed one per plastic cage on sawdust bedding, kept at $24\pm 2^{\circ}\text{C}$ and $50\pm 20\%$ humidity with a 12-hour light-dark cycle, fed a CE-2 pelleted diet (Clea Japan, Tokyo, Japan), and provided drinking water *ad libitum*. The animals were checked daily and weighed weekly throughout the experiments. All experiments were conducted according to the Guidelines for Animal Experimentation of Nagasaki University.

Surgical techniques

Choledochojejunostomy using a Roux-en Y procedure was performed on all hamsters. A schematic drawing of the completed choledochojejunostomy surgical procedure is illustrated in **Figure 1**. Briefly, following anesthesia with sodium pentobarbital (50mg/kg body weight), an upper abdominal midline incision was made and the distal end of the common bile duct was doubly ligated with 6-0 nylon and

divided. The gallbladder was then removed following the ligation of the cystic duct. The jejunum was doubly ligated with 6-0 nylon and cut 7-cm distal to the pyloric ring of the stomach. About 4 cm of the anal side of the jejunum was used for the Roux-en Y anastomosis, and an intestinal anastomosis was made in a side-to-side manner with 7-0 nylon. The elevated jejunum was transfixed approximately 10 mm distal to the jejunal stump by using a 20G needle with an elastic sheath which was left in place. The tied common bile duct was then threaded into the elastic sheath, so that the bile duct passed through into the needle hole. The common bile duct was then cut about halfway towards the tied end for bile drainage, taken back into the jejunum, and fixed to the jejunal wall (8, 9, 29).

Chemoprevention protocol

All hamsters were given subcutaneous injections of the chemical carcinogen *N*-nitrosobis(2-oxopropyl)amine (BOP) (Nakarai Tesque, Kyoto, Japan) every 2 weeks at a dose of 10 mg/kg body weight in 0.9% saline. BOP administration was started 4 weeks after surgery and continued thereafter for 18 weeks. The animals were randomly divided into two groups according to the different regimens, i.e., 15 hamsters were provided with a standard pelleted diet containing ONO-1714 (donated by Ono

pharmaceutical Co., Ltd Osaka, Japan) at 100 ppm, starting 4 weeks after surgery and continuing for 18weeks (ONO group), while 15 hamsters were fed the ordinary diet alone (control group). Twenty-two weeks after the operation, all hamsters were killed in order to perform histological examinations.

Histological studies

The liver and the extrahepatic bile duct were removed at autopsy. After fixation in 10% neutral formalin, the specimens were cut into 5 blocks, so that 4 sections contained the liver and one contained the hepatic duct. The specimens were subsequently embedded in paraffin. The histological sections were stained with hematoxylin and eosin (H&E) and examined by a pathologist who was blinded to the treatment allocation of the sections. The number of histologically verified biliary adenomas and carcinomas were counted in the 5 tissue sections in each animal. Adenoma and carcinoma were defined as lesions that showed signs of expansive growth, in accordance with the WHO classification of tumors in hamsters (30, 31). In contrast to adenomas, carcinomas displayed signs of malignancy, such as nuclear atypia, mitotic activities, a disruption of epithelial cell polarity, and invasion.

Inflammatory changes

In order to evaluate the relationship between cholangitis and biliary carcinogenesis, the grade of cholangitis was scored according to the infiltration of inflammatory cells and the fibrous change of Glisson as follows: grade 0, no cholangitis; grade 1, mild invasion of inflammatory cells around the bile duct without fibrous change of Glisson; grade 2, moderate invasion of inflammatory cells around the bile duct and/or partial fibrous change of Glisson; and grade 3, severe invasion of inflammatory cells around the bile duct and/or diffuse fibrous change of Glisson (29).

Immunohistochemical staining for iNOS

Immunohistochemical staining for iNOS was performed using the streptavidin-biotin-peroxidase method (32) on formalin-fixed, paraffin-embedded tissue sections. The sections were cut at 4- μ m, deparaffinized and dehydrated through xylene and graded alcohols. After antigen retrieval, endogenous peroxidase was blocked (15 min) with 3% (v/v) hydrogen peroxide and washed in phosphate-buffered saline (PBS). The tissue sections were incubated for one hour at room temperature with a primary rabbit anti-iNOS antibody kit (Rabbit polyclonal antibody to iNOS, prediluted (ab15326); Abcam Co., Ltd., Tokyo, Japan). The slides were then incubated with

primary antiserum diluted in buffer and subsequently with diluted biotinylated secondary antibody solution. After washing, the slides were incubated with avidin-biotin-horseradish peroxidase conjugate (VECTASTATIN® *Elite* ABC Reagent; Vector Laboratories Inc., CA, USA) for 30 minutes, according to the Vectastain protocol. iNOS was visualized with the chromogen 3,3'-diaminobenzidine (DAB). The slides were counterstained with hematoxylin, dehydrated in a graded alcohol series, cleared in xylene, and coverslipped. The expression grade of iNOS was scored using the percentage of positively stained biliary epithelial cells as follows: class 0, less than 30%; class 1, 30~70%; and class 2, more than 70% (33).

Cell kinetic studies

Proliferating cell nuclear antigen (PCNA) was used as a marker of biliary epithelial cell kinetics. Tissue sections were cut at 4- μ m, mounted on glass slides coated with 5-aminoprophyltriethoxy saline, and dewaxed in xylene. The sections were treated with microwave heating for 5 minutes in PBS at 500W. After the blocking of endogenous peroxidase, the sections were incubated with mouse monoclonal antibodies against PCNA (clone-PC 10; DAKO, Kyoto, Japan) at a dilution of 1:100. The cell nuclei were counter-stained with hematoxylin. The proportion of labeled nuclei

(labeling index; LI) was determined by counting the labeled nuclei in >1000 non-neoplastic epithelial cells of the intrahepatic bile ducts.

Adverse effect of ONO-1714 on the vital state

The animals were checked daily and weighed weekly throughout the experiments. Their food consumption was also noted. In addition, both gross and histological examinations were performed on the lungs, heart, kidney, and digestive tract.

Statistical analyses

The incidence of tumor development and the grade of cholangitis were analyzed using the chi square exact test. The Mann-Whitney U test was also used for statistical analyses to compare the number of tumors per animal, PCNA-LI, and the iNOS expression between groups. A logistic regression analysis was used to clarify the correlation between the grade of cholangitis and the occurrence of biliary tumors. A *P* value of less than 0.05 was regarded as statistically significant.

Results

Occurrence of biliary tumors

Intrahepatic biliary adenomas (**Figure 2**) and carcinomas (**Figure 3**) were observed in hamsters from both groups (**Table 1**). The occurrence rates of adenoma were 100% and 47% in the hamsters from the control and ONO groups, respectively. The difference in the incidence of adenoma between the two groups was significant ($p<0.05$). Adenomas displayed a multicentric occurrence, and the average number of adenomas per animal was 15.0 and 5.0 in the control and ONO groups, respectively ($p<0.05$).

Intrahepatic biliary carcinomas developed in 13 (87%) hamsters in the control group, and the average number of carcinomas per animal was 3.3. In the ONO group, only 6 (40%) hamsters developed intrahepatic biliary carcinoma, and the average number of carcinomas per animal was 1.1. In addition, statistically significant differences were observed in both the incidence of carcinoma and the average number of carcinomas per animal between the two groups ($p<0.05$).

Cholangitis, biliary epithelial cell kinetics and biliary carcinogenesis

Cholangitis was observed in most hamsters of both groups (**Table 2**). However, the severity of cholangitis was significantly different; namely, the average cholangitis score was 1.7 in the control group and 1.1 in the ONO group ($p < 0.05$). There was a tendency for the grade of cholangitis to be more severe in the large bile ducts in comparison to the small ducts or ductules in both groups of hamsters.

The PCNA-LI of the biliary epithelium in the control group was 27.0 %, which was significantly higher than that in the ONO group (19.3%, $p < 0.05$).

Figure 4 shows the relationship between the cholangitis score and biliary tumorigenesis. All hamsters with severe cholangitis of grades 2 or 3 developed biliary adenoma and carcinoma. In contrast, hamsters with grade 0 or 1 cholangitis, especially hamsters in the ONO group, rarely developed biliary carcinoma. The occurrence of adenoma and carcinoma was thus correlated with the severity of cholangitis in the ONO group ($p < 0.05$).

iNOS expression

Upon immunohistochemical staining, iNOS expression was identified in the cytoplasm of the biliary epithelial cells (**Figure 5**) where the biliary mucosa showed

various degrees of inflammatory changes. Although iNOS expression was seen in hamsters of both groups, it was weaker in the ONO group (**Table 2**). The average score of iNOS expression was 0.7 in the ONO group and 1.4 in the control group ($p < 0.05$). In the ONO group, a significant correlation between the iNOS expression score and the occurrence of biliary carcinoma was evident ($p < 0.05$).

Adverse effects of ONO-1714

Figure 6 shows the transition curves of the body weight of hamsters in each group during the experiment. Throughout the course of the study, the average body weight of hamsters in both groups increased in a similar fashion, and food consumption did not differ between the two groups. No gross or histological changes in the lungs, heart, kidney, and digestive tracts of either group were observed.

Discussion

The present study successfully demonstrated the preventative effect of iNOS inhibitor on BOP-induced biliary carcinogenesis in hamsters undergoing choledochojejunostomy. To the best of our knowledge, this is the first successful *in vivo*

study on the chemoprevention of biliary carcinogenesis by means of an iNOS-specific inhibitor.

In this study, both histological and immunohistochemical examinations revealed the degree of cholangitis, expression of iNOS, and cell kinetic activity of the biliary epithelium to be significantly suppressed with the use of an iNOS inhibitor, ONO-1714, in bilioenterostomized hamsters. ONO-1714 reduces not only iNOS activity but also COX-2 activity that is involved in K-ras activating mutations (22). Moreover, K-ras mutations enhance the iNOS expression mediated by some cytokines (22). These factors may be involved in the anti-cancer mechanisms of ONO-1714 in our hamster model, because it is reasonable to assume that the reduced iNOS expression should result in a decrease in NO production and subsequent NO-mediated genotoxicity. In addition, the reduced biliary epithelial cell kinetics should decrease the susceptibility of biliary epithelial cells to carcinoma because cells in the DNA synthesis phase are more susceptible to the tumorigenic effects of chemical carcinogens (34, 35).

iNOS inhibitors may have adverse effects by causing vasoconstriction and thrombosis in various organs such as the liver, kidney, lung and heart (36). In this study, the body weights and food consumption of the hamsters in the both the ONO group and the control group were almost equal throughout the experiment. In addition, both gross

and histological evaluations revealed no remarkable findings suspicious of any adverse effects of iNOS inhibitor on the lungs, heart, kidney, or digestive tracts. Therefore, the long-term administration of an iNOS inhibitor may be feasible for cancer prevention in clinical practice.

In conclusion, the suppression of the iNOS expression and proliferative activity of biliary epithelial cells, along with the restraint of cholangitis, are possible mechanisms of cancer prevention in this hamster model. Further studies should be conducted to investigate the iNOS activity and the expression of iNOS mRNA in our hamster model using molecular biological technology (such as RT-PCR and Southern blotting assays) as well as to elucidate the role of the iNOS/NO pathway in human carcinogenesis. Although additional study is needed, iNOS inhibitors appear to be potentially promising agents for the prevention of biliary carcinogenesis in several inflammatory cholangiopathies, such as primary sclerosing cholangitis, hepatolithiasis, or recurrent cholangitis complicating biliary-enteric anastomosis.

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Figure Legends

Figure 1. Operating scheme: choledochojejunostomy in the hamster using the Roux-en Y procedure. The jejunal limb was 4-cm long. a: the gallbladder was removed. b: the common bile duct was transected at the distal end. c: side-to-side intestinal anastomosis.

Figure 2. A: An intrahepatic biliary adenoma induced in a hamster, showing an expansive growth with simple glandular proliferation (H&E, x20). B: High magnification of the adenoma in Figure 2A. The individual glands are separated from each other by a broad stroma (H&E, x 100).

Figure 3. A: An intrahepatic biliary carcinoma induced in a hamster (H&E, x40). B: High magnification of the carcinoma in Figure 3A. The tumor shows a mass-forming growth with extensive infiltration to the liver parenchyma (H&E, x 100).

Figure 4. Correlation between the cholangitis score and biliary carcinogenesis in hamsters undergoing bilioenterostomy. Black circle: a hamster with tumor. Clear circle: a hamster without tumor.

Figure 5. Immunohistochemistry for iNOS. The cytoplasm of the biliary epithelial cells was positively stained according to the degree of iNOS expression. A: a class 2 expression of iNOS seen in a hamster undergoing choledochojejunostomy without ONO-1714 treatment. The majority of the cytoplasm in the biliary epithelial cells is positively stained with iNOS (x100). B: a class 0 expression of iNOS seen in a hamster with ONO-1714 treatment. The iNOS expression is seen in a few biliary epithelial cells (x100).

Figure 6. Transition curves of the average body weight of the hamsters in each group during the experiment. Solid line, ONO group; dashed line, control group. The bars demonstrate standard errors.

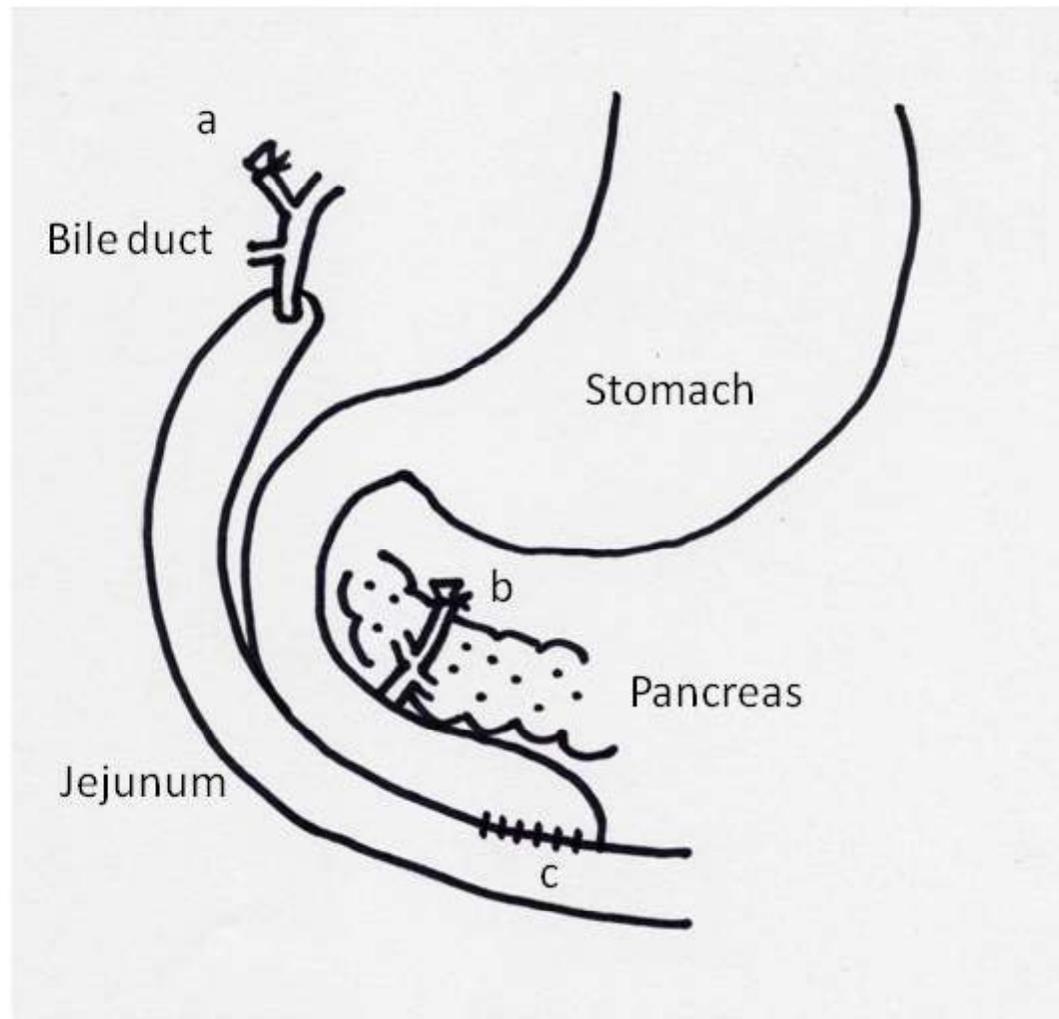


Fig. 1

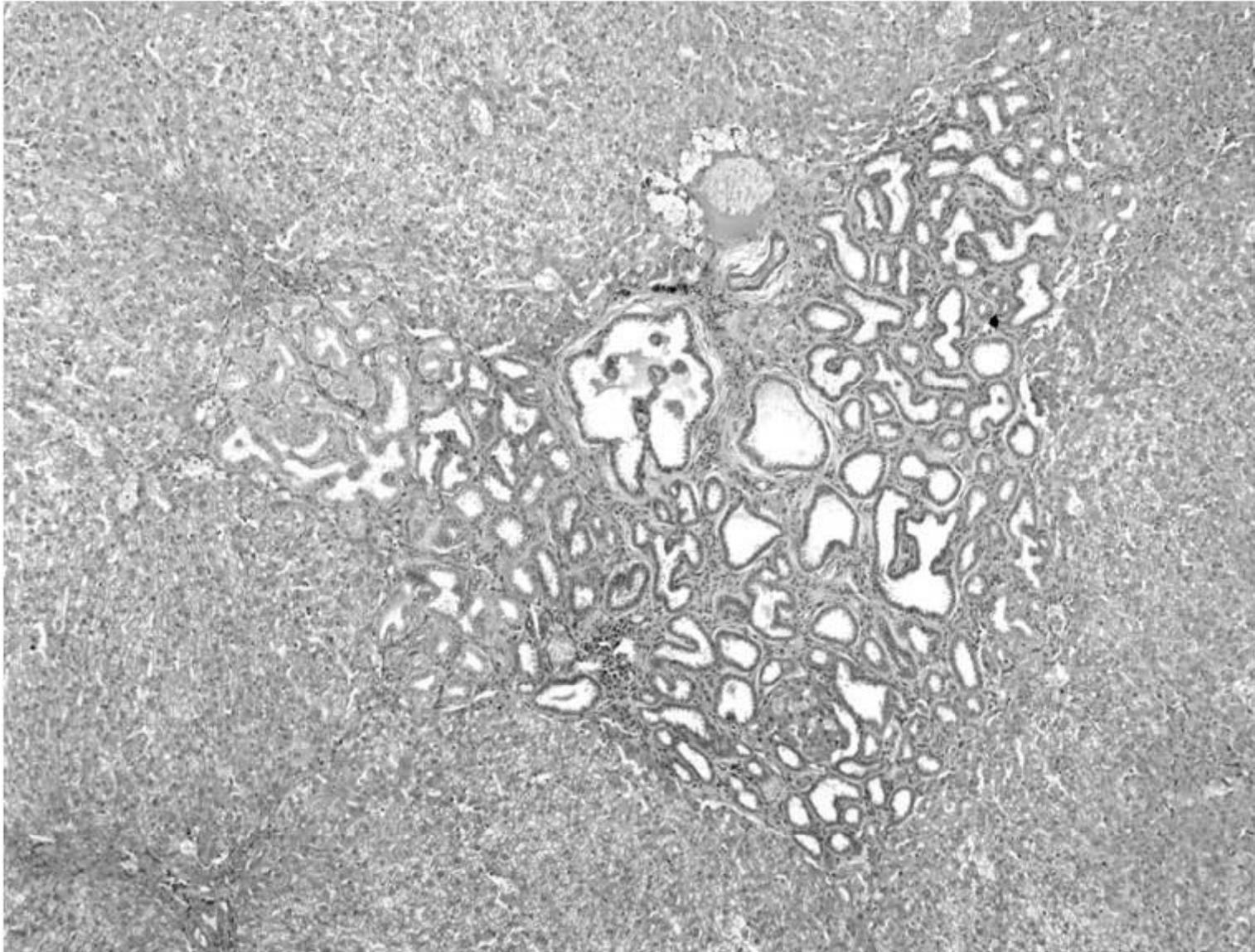


Fig. 2A

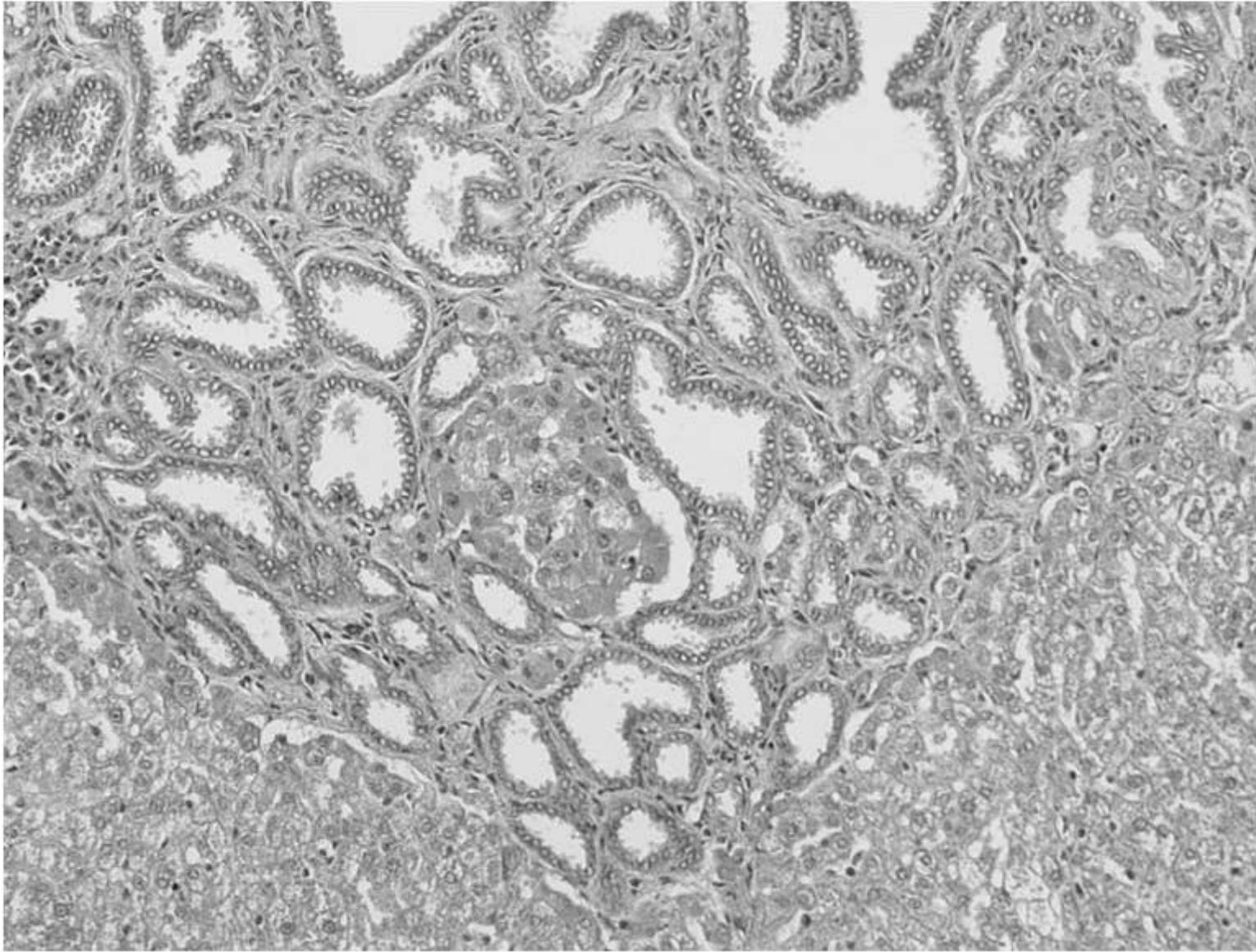


Fig. 2B

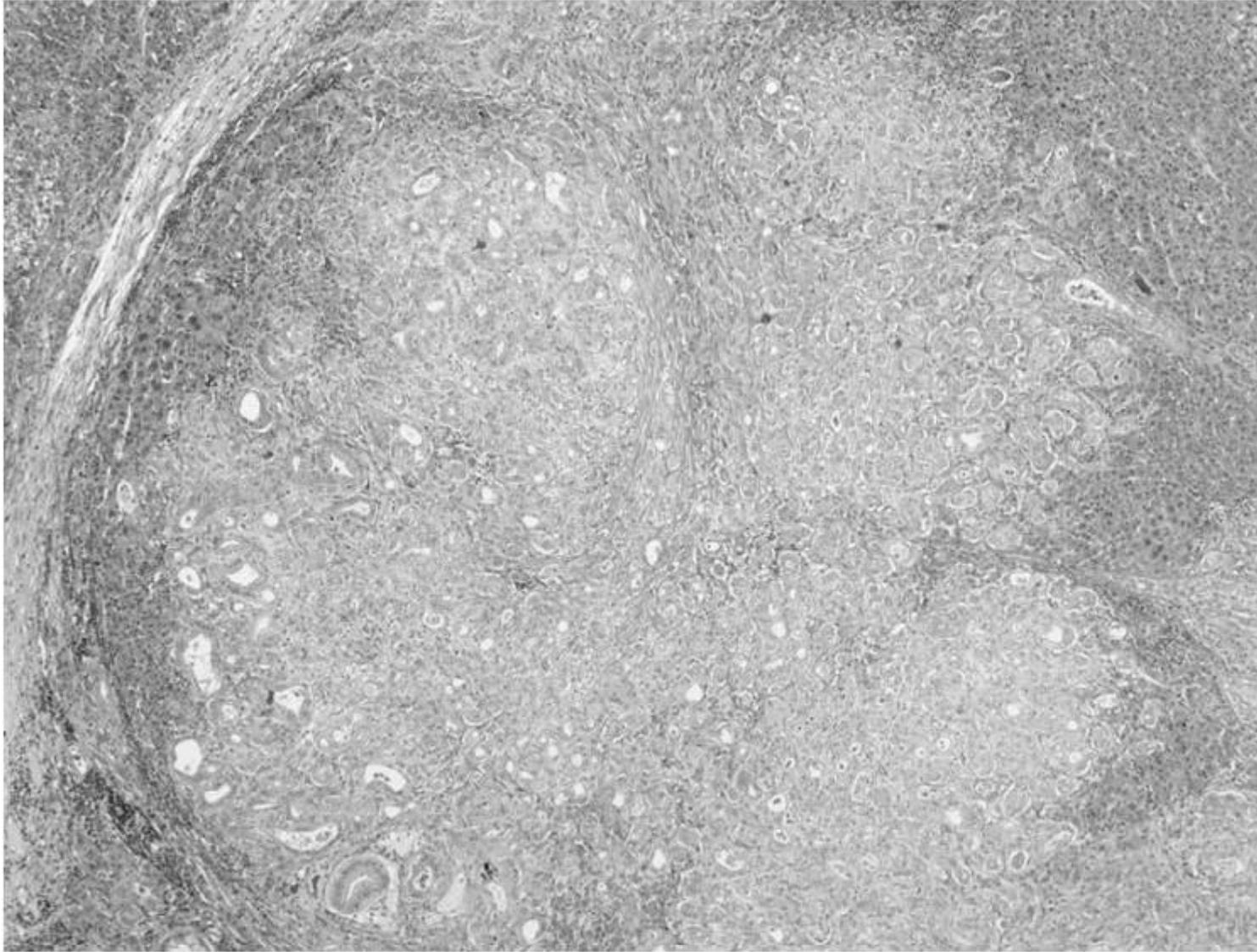


Fig. 3A

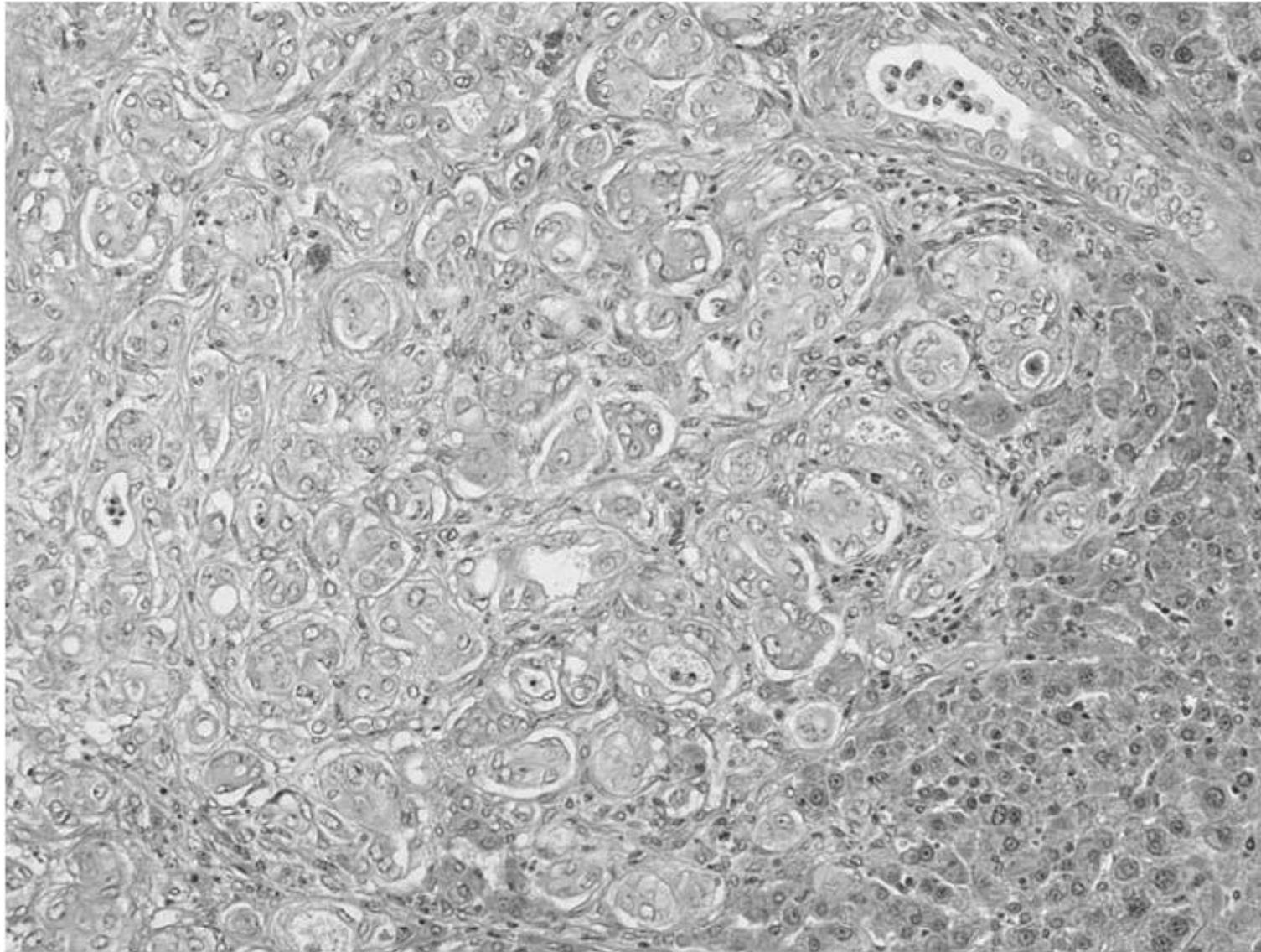


Fig. 3B

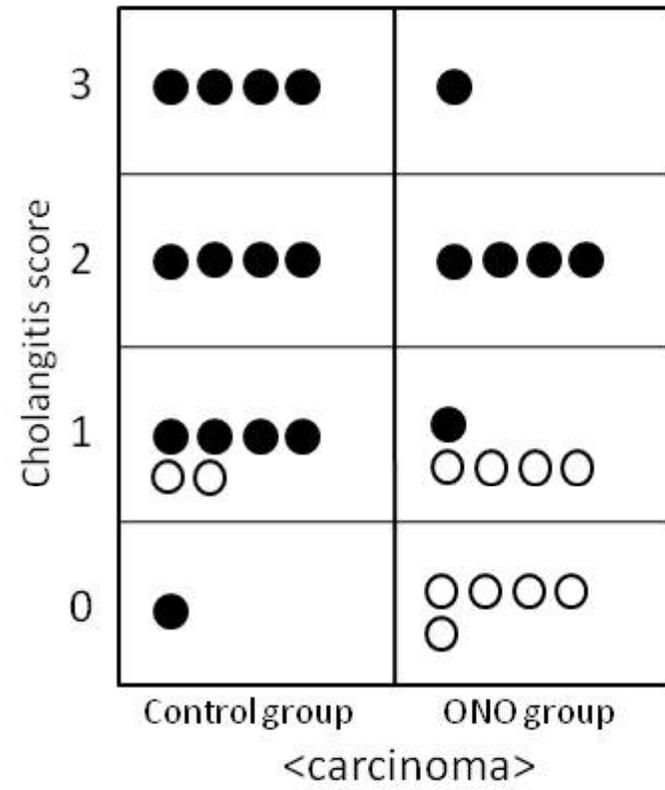
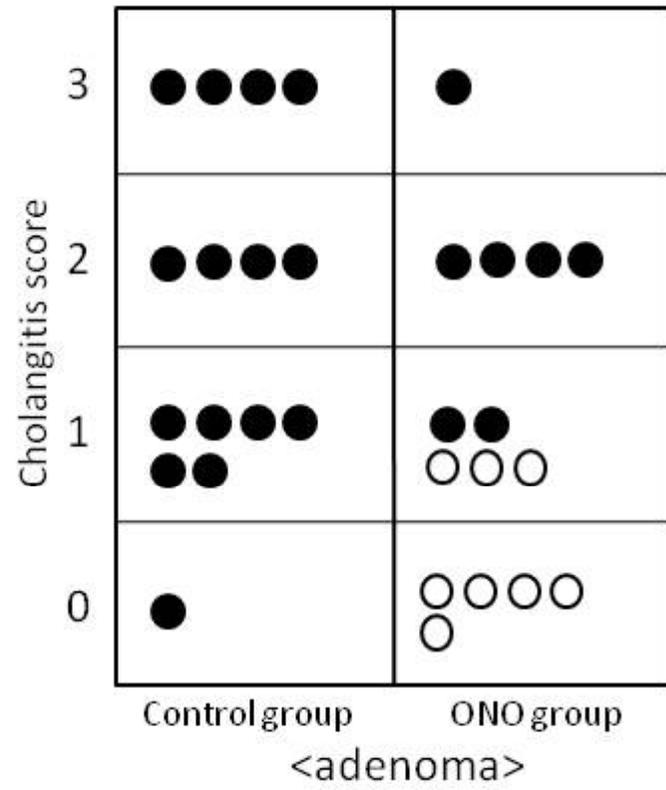


Fig. 4

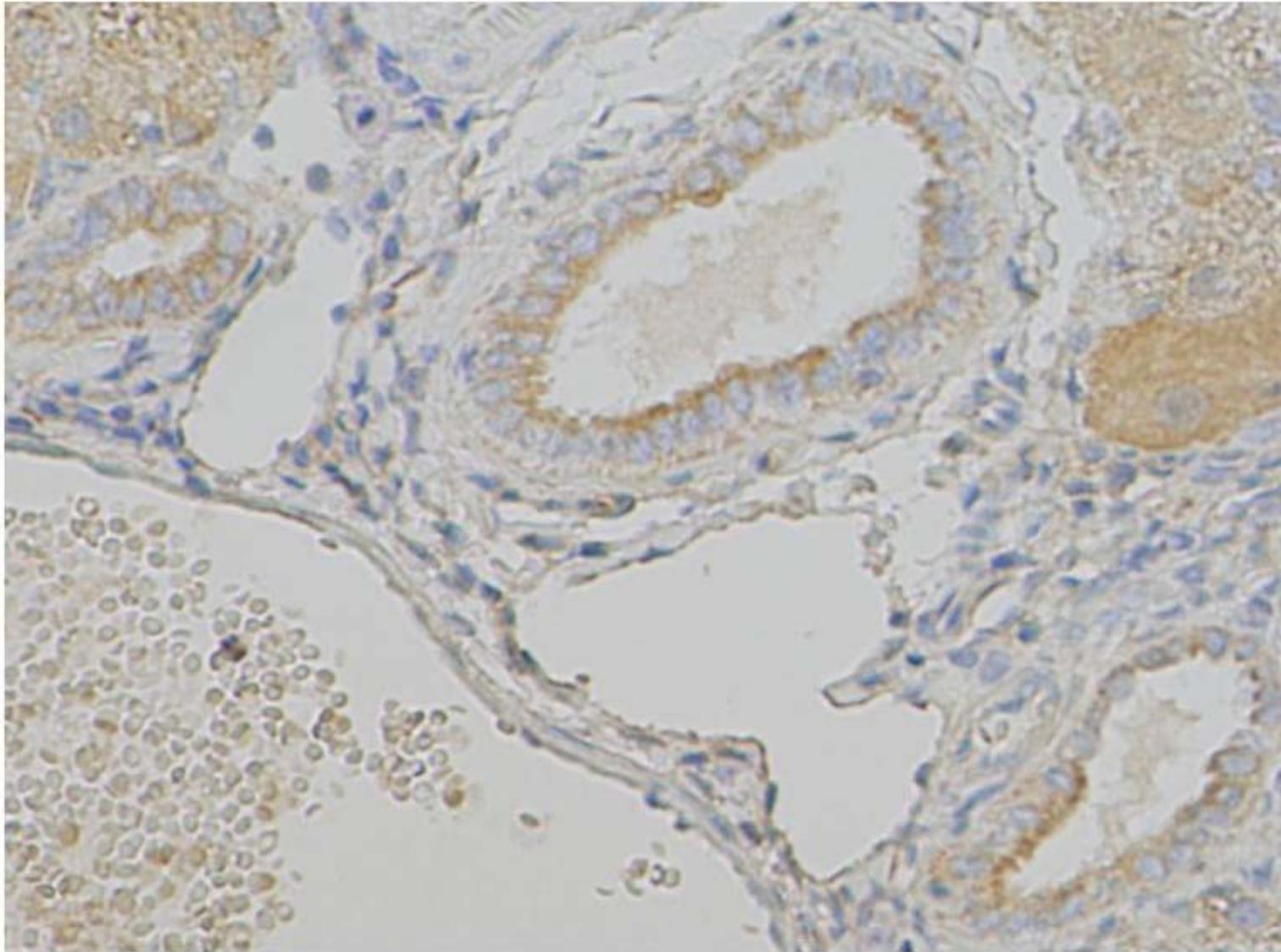


Fig. 5A

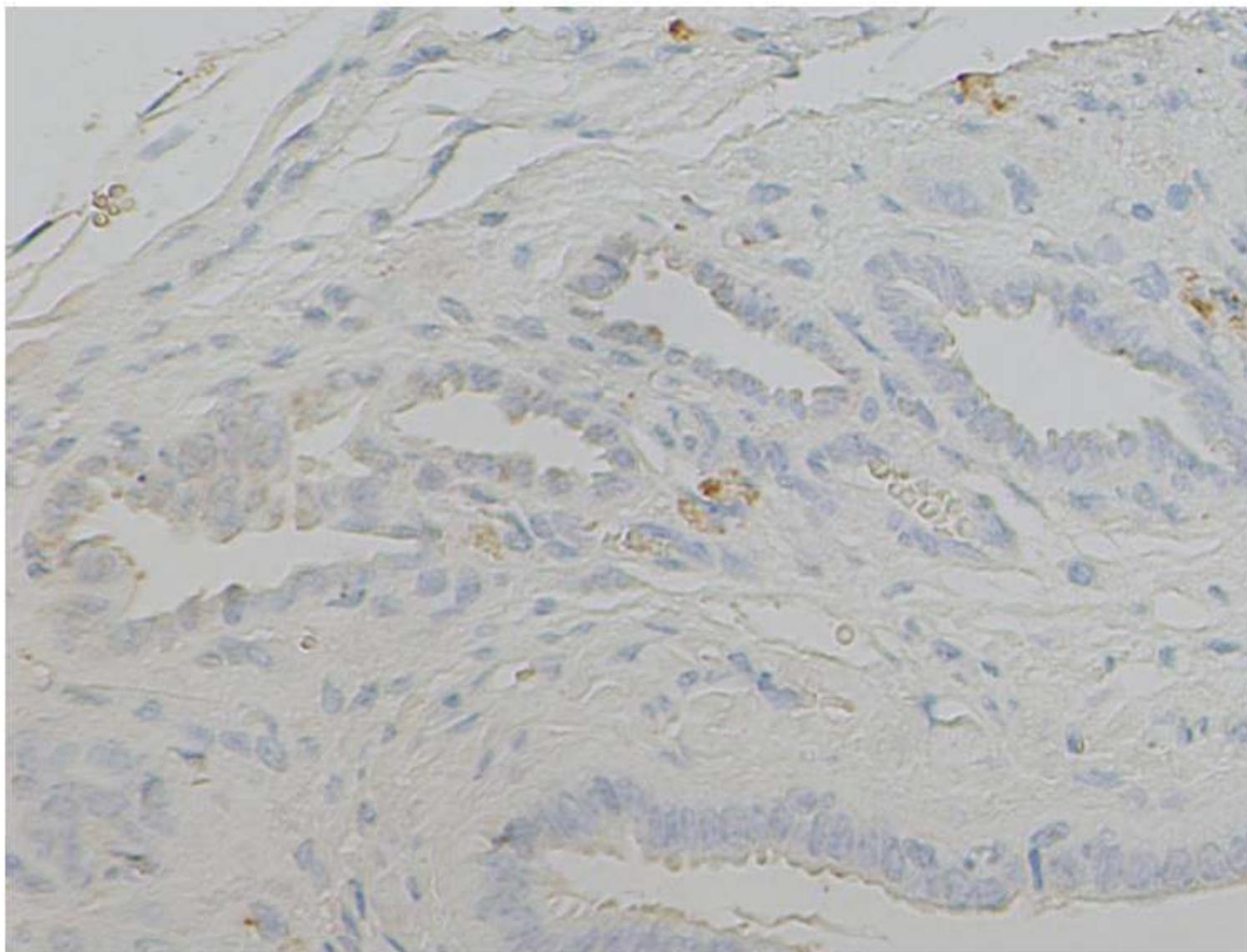


Fig. 5B

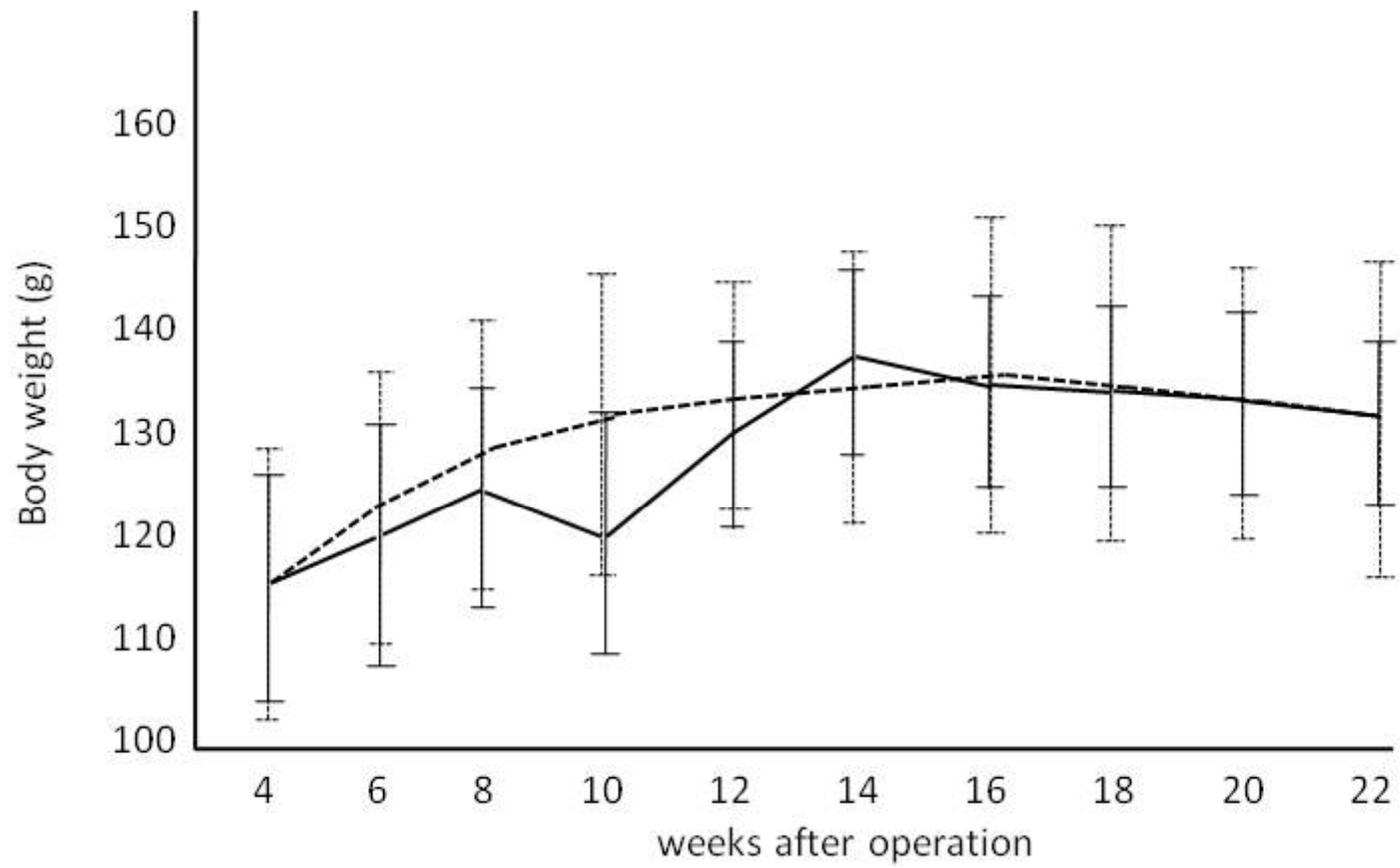


Fig. 6

Table 1. The incidence and number of intrahepatic bile duct tumors developed in hamsters after bilioenterostomy

Groups	No. of hamsters examined	No.(%) of hamsters with		Average no. of tumors per animal*	
		Adenoma	Carcinoma	Adenoma	Carcinoma
Control	15	15 (100)	13 (87)	15.0±5.8	3.3±4.9
ONO	15	7 (47) ^a	6 (40) ^a	5.0±6.7 ^a	1.1±2.1 ^a

*Mean ± SD.

^a Significantly different from the control group (p<0.05).

Table 2. The occurrence of cholangitis and changes in iNOS expression and biliary epithelial cell kinetics in hamsters after bilioenterostomy

Groups	No. of hamsters examined	No.(%) of hamsters with cholangitis	Cholangitis score*	iNOS score*	PCNA-LI*
Control	15	14 (93)	1.7±0.9	1.4±0.7	27.0±11.3
ONO	15	10 (67)	1.1±0.9 ^a	0.7±0.7 ^a	19.3±9.8 ^a

*Mean ± SD.

PCNA-LI, proliferating cell nuclear antigen labeling index

^a Significantly different from the control group (p<0.05).