Chemopreventive effects of a selective cyclooxygenase-2 inhibitor (Etodolac) on chemically induced intraductal papillary carcinoma of the pancreas in hamsters

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ABSTRACT

The present study was designed to determine whether Etodolac, a selective cyclooxygenase-2 inhibitor, prevents chemically induced intraductal papillary carcinoma (IPC) in the main pancreatic duct of hamsters. Hamsters were subjected to cholecystoduodenostomy with dissection of the distal end of the common duct. Four weeks after surgery, the surviving hamsters received subcutaneous injections of N-nitrosobis(2-oxopropyl)amine (BOP) 4 times at a dose of 10 mg/kg body weight, every 2 weeks. The animals were divided into 3 groups according to the simultaneous oral intake of a standard pelleted diet containing Etodolac at 0% (group CE, n=30), 0.01% (group ET, n=21), and 0.04% (group ET4, n=25), respectively. Hamsters were killed for pathological examination at 36 weeks after the operation. The incidence of induced pancreatic carcinoma was 93%, 81%, and 72% in groups CE, ET, and ET4, respectively. The pancreatic carcinomas were histologically classified into 4 types, i.e., tubular, papillary, and cyst adenocarcinoma, and IPC. The incidence of IPC and the number of IPCs per animal were significantly lower in groups ET4 (36% and 0.48) and ET (48% and 0.62) when compared to group CE (67% and 1.30). The proliferating cell nuclear antigen labeling indices in the noncancerous epithelial cells of the main pancreatic duct were 2.8% and 6.8% in groups ET4 and ET, respectively, and were significantly lower than that in group CE (10.8%). In conclusion,

Etodolac inhibited BOP-induced IPC in hamsters. Suppression of epithelial cell proliferation of the main pancreatic duct was considered as a possible mechanism of cancer prevention in this hamster model.

INTRODUCTION

Intraductal papillary mucinous neoplasm (IPMN) of the pancreas, which is characterized by the papillary proliferation of neoplastic epithelium with mucin-hypersecretion (1), has been a well-established clinical and pathological entity since Ohashi et al. first described it in 1982 (2). The recorded incidence of this unique pancreatic disorder has been gradually increasing in accordance with the recent advances in diagnostic imaging modalities. An ordinary pancreatic ductal carcinoma derives from the pancreatic ductules, while IPMN arises from the main pancreatic duct or its major branches (1,3), being classified grossly into 2 types, i.e., main-duct IPMN and branch-duct IPMN (4). In addition, IPMNs show a wide spectrum of histological differentiation, ranging from benign to malignant with the hyperplasia-adenoma-carcinoma sequence (5-7), and can often be multifocal. Therefore, the appropriate management for IPMN remains unclear. Some patients with IPMN indeed require aggressive surgery such as total pancreatectomy, whereas some patients can be managed with limited pancreatic resections as well as careful observation without surgery. The advantage of a surgical approach must be balanced against the surgical risk and the postoperative poor quality of life with pancreatic endocrine and exocrine deficiency, especially in elderly patients. In the management of IPMN, therefore, a potent chemopreventive agent would be beneficial for patients.

Nonsteroidal anti-inflammatory drugs, including selective cyclooxygenase-2 (COX-2) inhibitors, have been expected to function as chemopreventive drugs against several carcinomas (8,9). Kokawa et al. (5), however, have recently demonstrated the incidence of COX-2 expression as 58% and 70% in intraductal papillary mucinous adenoma (IPMA) and carcinoma (IPMC) of the pancreas, respectively. It has been described that the incidence of COX-2 expression arises in accordance with the progression of the adenoma-carcinoma sequence in IPMNs (6,7). These findings suggest that the suppression of COX-2 expression can prevent the development and progression of IPMN. In the present study, we investigated the effects of Etodolac, a selective COX-2 inhibitor, on the prevention of IPMNs of the pancreas by using a hamster model in which intraductal papillary carcinoma (IPC) could be induced in the main pancreatic duct or its major branches (10). We used Syrian hamsters as the animal model because the anatomical structure of their pancreaticobiliary ductal system, bile acid composition, and pancreatic juice components in this species are similar to those of humans (11,12).

MATERIALS AND METHODS

Animals

A total of 76, 7-week-old female Syrian golden hamsters (Shizuoka Laboratory Animal

Center, Shizuoka, Japan) were used. The average weight of hamsters at the time of initiation of the experiments was 100 g. Animals were housed one per cage with sawdust bedding under standard laboratory conditions in the Laboratory Animal Center for Biochemical Research at Nagasaki University Graduate School of Biomedical Sciences. The animals were checked daily and weighed weekly throughout the experimental period. All experiments were performed following the Guidelines for Animal Experimentation of Nagasaki University Graduate School of Biomedical Sciences.

Surgical techniques

Following intraperitoneal administration of sodium pentobarbital (50 mg/kg body weight), hamsters were subjected to cholecystoduodenostomy with dissection of the distal end of the common duct, so that bile would regurgitate into the pancreatic ducts and activate the epithelial cell kinetics of the main pancreatic duct (10).

Experimental protocol

All hamsters received four biweekly subcutaneous injections of N-nitrosobis(2-oxopropyl)amine (BOP) (Nakarai Chemical Co., Kyoto, Japan), started 4 weeks after surgery, at a dose of 10 mg/kg body weight. The animals were divided into 3 groups according to the simultaneous oral intake of a CE-2 pelleted diet (Clea Japan, Tokyo, Japan), which contained 0% (group CE), 0.01% (group ET), and 0.04% Etodolac (group ET4),

respectively. The dosage of 0.01% Etodolac in the CE-2 pelleted diet was determined by calculating the daily amount of diet ingestion of hamsters and considering the clinical daily-dose of Etodolac in humans. The body weight and amount of diet ingestion in each animal was checked weekly throughout the experiment. Hamsters were killed for pathological investigations at 36 weeks after surgery. At autopsy, pancreatic tissue samples were taken from the distal portion of the splenic lobe of the pancreas, frozen immediately in liquid nitrogen, and stored at -80^{o}C in a sterile 1.5-ml Eppendolf tube for the analysis of prostaglandin products. The residual pancreas was embedded in paraffin and processed routinely for hematoxylin and eosin staining and then examined by a pathologist who was blinded to the treatment allocation of the study. For the investigation of adverse effects of Etodolac such as gastric ulceration and myocardial injury, the stomach and the heart were investigated by means of macroscopic and microscopic examination. Tumor lesions induced in the pancreas were classified on the basis of the WHO classification of tumors of the hamster (13).

Prostaglandin measurement

To assess the suppressive effects of Etodolac on COX-2 activity, the prostaglandin E_2 (PGE_2) products in the pancreatic tissues were measured. The frozen tissue obtained from the splenic lobe of the pancreas was homogenized in saline containing 10 mg/l indomethacin, and ethanol was added to achieve the final proportion of 20%. After centrifugation, the supernatant

was removed and agitated in the octadecylsilyl silica (FUJIGEL HANBAI Co. Ltd., Tokyo, Japan) suspension to absorb PGE_2. Deproteinization and delipidization were performed, and prostaglandins were eluted by ethyl acetate. The dried residue containing prostaglandins was dissolved in eluent 1 (acetonitrile: chloroform: acetic acid, 10:90:0.5) and applied to a silica open minicolumn Bond Elut Si (Varian, Inc. Scientific Instruments, CA, USA). The column was washed with 10 ml eluent 1, after which PGE_2 was eluted first with 5 ml eluent 2 (acetonitrile: chloroform: acetic acid, 20:80:0.5) and then 5 ml eluent 3 (acetonitrile: chloroform: acetic acid, 50:50:0.5). We assayed PGE_2 by a radioimmunoassay technique using a [^{125} I] Prostaglandin E_2 RIA kit NEK-020 (PerkinElmer Life And Analytical Sciences, Inc., MA, USA).

Cell kinetic studies

Proliferating cell nuclear antigen (PCNA) was used for evaluation of the epithelial cell kinetic activity of the main pancreatic duct. Pancreatic tissue sections obtained from all hamsters were cut at 4 μm, mounted on glass slides coated with 5-aminoprophyltriethoxy saline, and dewaxed in xylene. The sections were treated with microwave heating for 5 min in phosphate-buffered saline at 500W. After 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 counterstained with hematoxylin. The proportion of labeled nuclei (labeling index; LI) was determined by counting the labeled nuclei in >1,000

normal epithelial cells of the main pancreatic duct. We examined 19, 16, and 26 regions of normal epithelium in the main pancreatic duct in groups CE, ET, and ET4 respectively.

Statistical analysis

The Mann-Whitney U-test was used for statistical analysis. Differences of $p<0.05$ were considered to be statistically significant.

RESULTS

The number of hamsters examined was 30, 21, and 25 in groups CE, ET, and ET4, respectively. The difference in the number of hamsters in each group was mainly due to the operative death within 4 weeks after surgery.

Transition of body weight and amount of diet ingestion

The transition curves of the average body weight and amount of diet ingestion in each hamster group during the experiment are shown in Fig. 1. There were no statistically differences in either body weight or diet ingestion between the 3 groups in any period. In addition, no hamsters showed either gastric ulcer or myocardial injury with regard to either macroscopic or microscopic aspects.

The amount of Etodolac administration in hamsters

The amount of Etodolac administration in hamsters, calculated on the basis of diet ingestion, was 36 to 53 mg/kg body weight/week in group ET and 160 to 192 mg/kg body weight/week in group ET4, respectively (Fig. 2). In group ET, the amount of Etodolac intake was within the human clinical dosage (i.e., 28 to 56 mg/kg body weight/week).

Prostaglandin products

The mean PGE_2 production in the pancreatic tissue was 19.7±13.3 (mean±SD), 15.4±13.3, and 10.2±7.8 pg/wet weight mg in groups CE, ET, and ET4, respectively, with production decreasing in proportion to the mixing dosage of Etodolac with a statistically significant difference between groups CE and ET4 ($p<0.01$) (Fig. 3).

Carcinogenic studies

The incidence, number, and histological findings of pancreatic carcinomas induced in hamsters are summarized in Table I. Pancreatic carcinomas developed in 93%, 81%, and 72% of hamsters in groups CE, ET, and ET4, respectively. The difference was statistically significant between groups CE and ET4 ($p<0.05$). The induced pancreatic tumors were classified grossly into 4 types histologically: tubular, papillary and cystic adenocarcinoma, and IPC arising in the main pancreatic duct. Although some IPCs were recognized in the first-order pancreatic branches, these lesions were not counted in this study since they could not be distinguished from the IPC originating in the main pancreatic duct. The majority of induced pancreatic carcinomas were

tubular adenocarcinomas in each group. However, numerous IPCs were recognized in the main pancreatic duct in hamsters of group CE (Fig. 4). The incidences of IPC were 67%, 48%, and 36% in groups CE, ET, and ET4, respectively, and were significantly lower in group ET4 ($p<0.05$) when compared to group CE. The number of IPCs per animal was 1.30, 0.62, and 0.48 in groups CE, ET, and ET4, respectively, and was significantly fewer in groups ET ($p<0.05$) and ET4 ($p<0.01$) when compared to group CE. Both the incidence and number of tubular adenocarcinomas were not affected by the Etodolac treatment.

Cell kinetic studies

The PCNA-LIs of normal epithelial cells of the main pancreatic duct were 10.8±4.9% (mean±SD), 6.8±3.8%, and 2.8±2.5% in groups CE, ET, and ET4, respectively. All of the differences between groups were statistically significant (Fig. 5).

DISCUSSION

Recent clinical investigations have revealed the presence of COX-2 expression in a variety of cancers of the colon, lung, stomach, and esophagus (14-16). The over expression of COX-2 inhibits the apoptosis of cancer cells (17), resulting in prolonged survival of DNA-damaged cells, increases in metastatic potential (18), or promotion of angiogenesis (19). Therefore, selective

COX-2 inhibitors have been proposed to be an appropriate chemopreventive drug against cancer. In fact, the chemopreventive effects of selective COX-2 inhibitors have been demonstrated in pancreatic cancer cell lines (20,21) and animal models (22,23) because COX-2 expression is also involved in the development and progression of invasive ductal carcinoma of the pancreas (24,25). However, controversy remains regarding the usefulness of chemoprevention against pancreatic cancer (26), and El-Rayes et al. (27) have recently reported that a selective COX-2 inhibitor (celecoxib) alone might be insufficient to reverse the chemoresistance in pancreatic cancer in a clinical study of simultaneous use of gemcitabine. In contrast, Crowell et al. (24) have advocated that COX-2 activation is extremely important in the early stages of human pancreatic carcinogenesis; namely, in PanIN1 and PanIN2 lesions. In addition, the significance of COX-2 expression at an early stage of polyp formation in the intestine has been reported (28). Because IPMNs of the pancreas show the hyperplasia-adenoma-carcinoma sequence (5-7) and have less aggressive behavior compared to ordinary pancreatic ductal carcinoma (29), selective COX-2 inhibitor might be a potential drug for the prevention of IPMNs.

In the present study, the oral administration of Etodolac had no chemopreventive effects on the development of BOP-induced tubular adenocarcinoma of the pancreas, which is the ordinary type of human pancreatic cancer. However, Etodolac inhibited the occurrence of IPCs in the main pancreatic duct even in the hamsters of group ET, in which the Etodolac administration to

hamsters was equal to the human clinical dosage. To the best of our knowledge, this is the first successful *in vivo* study of the chemoprevention of IPCs of the pancreas by means of a selective COX-2 inhibitor. Both the PGE_2 production in the pancreas tissue and PCNA-LIs of noncancerous epithelial cells of the main pancreatic duct were suppressed in proportion to the administrating dosage of Etodolac in this study. It is well known that COX mediates the late limiting step of prostaglandin biosynthesis in the arachidonic acid cascade and that PGE_2 is a reliable biomarker of COX activity (24,30). Thus, the present results indicate that suppression of epithelial cell proliferation of the main pancreatic duct through the reduction of PGE_2 production in the pancreas by Etodolac is a possible mechanism of cancer prevention in our hamster model.

IPCs induced in the main pancreatic duct of our hamster model produced a smaller amount of mucin when compared to IPMNs in humans. However, there were many similarities between IPCs and IPMNs from an oncological perspective, i.e., the tumor development from the main or large pancreatic ducts, intraductal papillary growth with a histological pattern of papillary proliferation of tumor cells, and low-grade malignancy (10,29). In addition, no hamsters showed any adverse effects of Etodolac on the stomach and the heart, even in hamsters of the ET groups, although cardiovascular morbidity remains a clinical concern with long-term use of selective COX-2 inhibitors. These findings may also support the idea that Etodolac could be a safe and useful drug for the prevention of IPMNs in humans. In particular, branch duct IPMNs without

clinico-radiological parameters indicative of possible malignancy in younger patients and/or IPMNs in the elderly with a high risk for surgery are good subjects for cancer chemoprevention. In multifocal branch duct IPMNs, surgical removal of the prominent lesions and a close observation of the remaining lesions in the remnant pancreas with chemoprevention by means of selective COX-2 inhibitors may be a reasonable treatment.

In conclusion, the present study has demonstrated the chemopreventive effects of Etodolac on BOP-induced intraductal papillary carcinoma of the main pancreatic duct in hamsters. Because human IPMNs have many characteristic features suited for cancer chemoprevention, the administration of selective COX-2 inhibitors may be a good adaptation to control IPMNs or to prevent IPMN recurrence after surgery.

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FIGURE LEGENDS

Figure 1 Transition curves of average body weight and the amount of diet ingestion of hamsters in each group during the experiment. Solid line, the average body weight of hamsters; dashed line, average amount of diet ingestion.

Figure 2 The weekly amount of Etodolac administration to hamsters in groups ET and ET4 that was calculated on the basis of diet ingestion containing Etodolac. The gray area shows the range of the usual clinical dosage of Etodolac. In group ET, Etodolac administration levels were within human clinical dosage levels throughout the experiment.

Figure 3 The mean PGE2 production per wet weight of the pancreas tissue in each group. PGE2 production in the pancreas tissue was decreased in proportion to the mixing dosage of Etodolac.

Figure 4 Representative case of IPC of the main pancreatic duct induced in hamsters. IPC shows a marked papillary proliferation growing into the lumen of the main pancreatic duct (H&E × 150).

Figure 5 PCNA-LIs of normal epithelial cells of the main pancreatic duct of hamsters in each group. PCNA-LIs were suppressed in accordance with the mixing dosage of Etodolac with statistical significance.

Fig.1

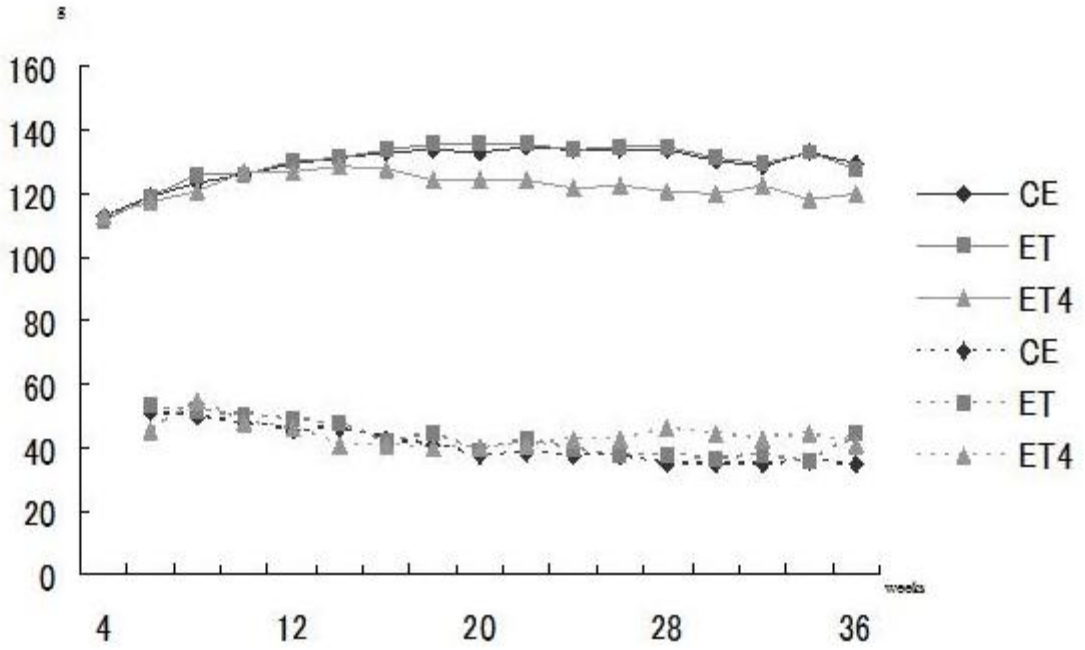
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Fig.2

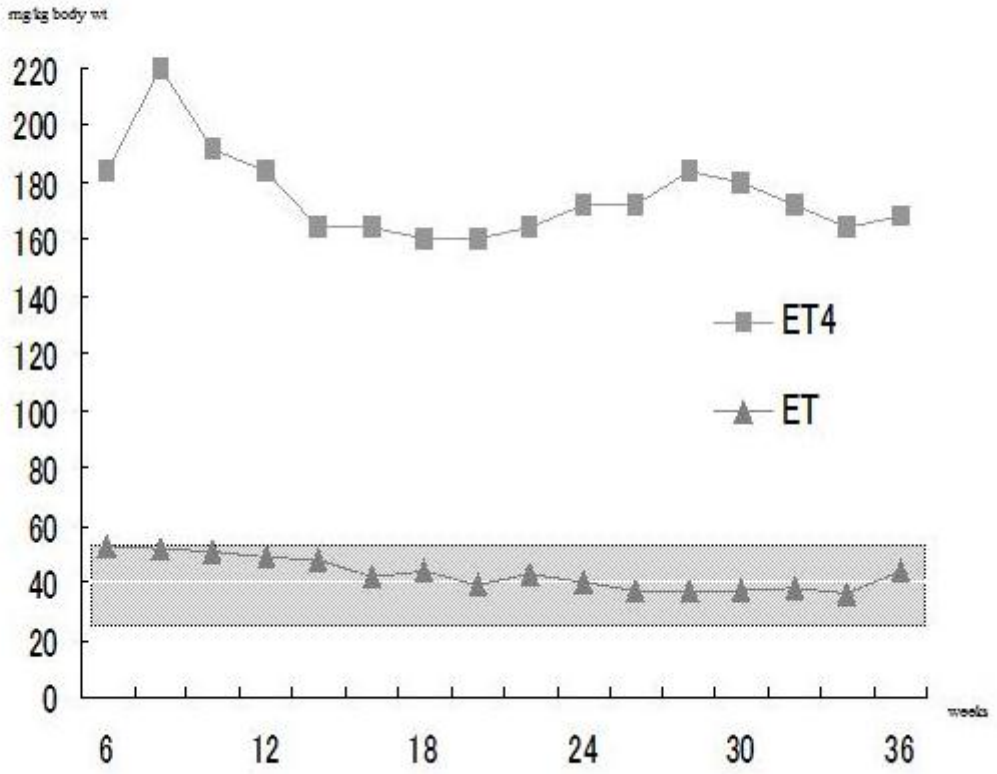
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Fig.3

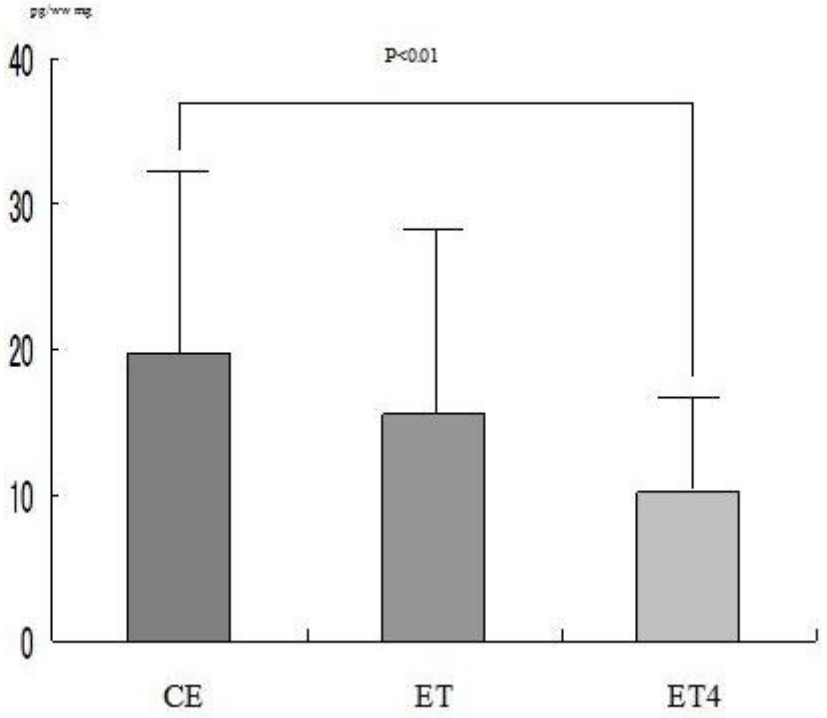

Fig.4

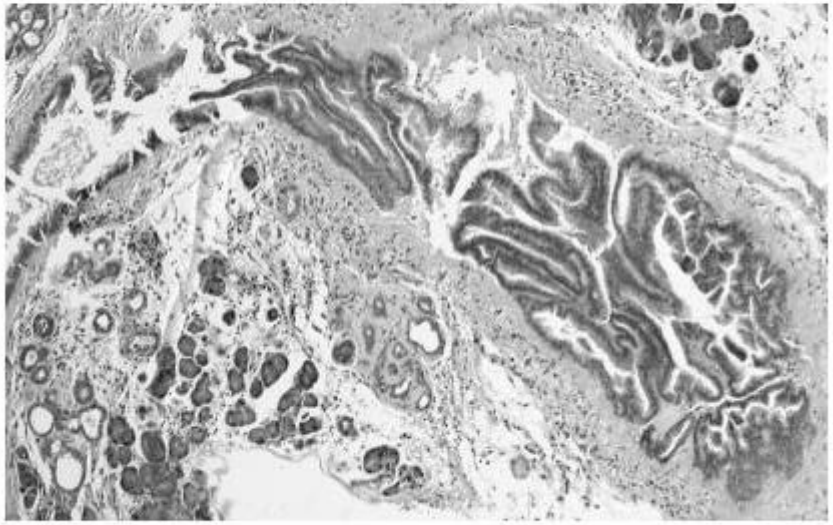

Fig.5