Title : Objective detection for biliary tract carcinoma using autofluorescence imaging

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Abstract

Purpose Bile duct cancer is a refractory cancer. Progress in diagnostic imaging has greatly enhanced diagnostic capability as well as treatment outcomes. However, evaluation of pathological changes associated with biliary cancer is often dependent on immediate analysis of cryosections during surgery. We evaluated the pathological changes associated with biliary cancer by autofluorescence imaging (AFI) of the excised organ using a stereoscopic microscope.

Methods A total of 18 patients with biliary disease (1 with gallbladder cancer) and 2 with invasive carcinoma of the pancreas (1) and biliary tract (1) were examined at Kurume University Hospital from April 2010 to December 2011. The relationship between AFI and pathological tissue structure was investigated to determine the range of tumor extension in the mucous membranes of tumors observed by AFI.

Results Papillary tumors were observed with the naked eye; tumors with a nodular pattern were displayed in magenta by AFI, and clearly contrasted

with non-cancerous areas. These tumors conformed to our histopathological analysis of pathological tissues. On the other hand, flat tumors were unclear and did not conform to the histopathological analysis. Conclusions It was suggested that AFI is an effective method for diagnosing mucous membrane lesions associated with bile duct cancer.

Introduction

Bile duct cancer is associated with poor prognosis. The poor outcome of treatment of bile duct cancer is attributed to the high malignancy of the tumor itself, difficulty in early detection, and extreme difficulty of treatment. In recent years, the advent of multidetector-row CT and other imaging modalities has greatly enhanced diagnostic imaging techniques (1). Although direct biliary tract imaging using techniques such as endoscopic retrograde cholangiopancreatography, percutaneous transhepatic cholangiography, endoscopic ultrasonography, intraductal ultrasonography, and cholangioscopy are used for diagnosing cancer in

areas surrounding tumors, there are many cases where diagnosis remains difficult and thus, clinicians still rely on the pathological analysis of frozen sections during surgery. We evaluated the spread of pathological lesions associated with biliary cancer by autofluorescence imaging (AFI). Fluorescence observation enables detection of early neoplastic lesions, which have traditionally been difficult to diagnose using an endoscope, based on changes in the color or intensity of fluorescence. Therefore, detection of the range of tumor extension becomes possible even for cases in which the tumors are difficult to detect with the naked eye.

It has been reported that autofluorescence in cancerous sections compared with that in normal tissues was reduced, as well as clinical application has been difficult (2). During the 1990s, an autofluorescence bronchoscope was developed, which was determined to be effective in the localization and diagnosis of lung cancer (3). In addition, it was reported that AFI was effective in areas of the digestive tract (4-7). However, there have been no reports of AFI for bile duct cancer (8.9) and thus, its efficacy in this regard

is uncertain.

We evaluated the effectiveness of surgical removal of invasive carcinomas of the pancreas and biliary tract, such as bile duct and pancreatic cancers, by AFI. Using AFI and visually confirming the tumor area by evaluating the strength of autofluorescence of cancerous and non-cancerous parts of cancer tissues, we determined whether the area in question had the same characteristics as those of areas determined to be histopathologically malignant.

Patients and Methods

Observation of autofluorescence of the biliary mucosa using an AFI microscope and tissue specimens from 18 patients with biliary disease (1 case of gallbladder cancer) and 2 with invasive carcinoma of the pancreas (1) and biliary tract (1) was performed at Kurume University Hospital from April 2010 to December 2011. A total of 15 males and 5 females with an average age of 71.6 years (range: 53–82) were included in the study. A perihilar (5/7) distribution of flat tumors visible to the naked eye and a

distal (5/7) distribution of papillary tumors were observed. The histological grade of papillary tumors visible to the naked eye was easy to differentiate, but differentiation of histological type was poor for flat and nodular tumors (Table 1).

AFI was used to investigate the mucosal membranes of extracted organs that had been cleansed of bile with normal saline without applying any pigment.

Reflection － AutoFluorescence Subtract image

Our method captures the reflective image with a green illumination source and immediately after autofluorescence is produced by a blue excitation light. Subtracting the brightness of autofluorescence from the reflective image after extracting the green fluorescence creates a difference image. This enables the extraction of tissue structures with large differences in brightness between the reflective and autofluorescence images. If the cancerous tissue structure has the type of fluorescence characteristics as stated previously (2), it is considered that cancerous areas can be extracted

and identified using this method. In the current study, cancerous areas were extracted using a simulated green color and difference images were colored magenta (sections with lower autofluorescence).

Stereoscopic AFI microscope

Visible areas were illuminated by two different LED light sources from both sides using a stereoscopic microscope with the base of a light microscope and a high numerical aperture lens (MVX10: Olympus Medical Systems, Tokyo, Japan). The first light source used green LED light for the reflective image, and was set at a reflective wavelength of 550 nm. The other light source used blue LED light for autofluorescence excitation, and was set at a reflective wavelength of 450 nm. In addition, a 500-nm long pass filter was inserted into the stereoscopic microscope. Differences between autofluorescence and reflective light were captured in an image-processed picture by alternating the light source, with autofluorescence and reflective light captured separately. Collagen, flavin, nicotinamide adenine dinucleotide, tyrosine, and tryptophan are substances in the body

that autofluoresce, and each possesses individual fluorescence characteristics and different dispersion rates depending on their location in each organ (10-12). This study assumed that collagen and flavin, which have a maximum reflective wavelength of 450 nm, are major sources of fluorescence by setting the reflective wavelength at 450 nm (13).

Results

The invasion pattern of the tumors identified in the 18 patients with bile duct cancer, which were visible to the naked eye, was papillary in 7, nodular in 3, and flat in 7. There were 15 cases of invasion and 2 of swelling. In addition, the biliary cancer was identified as the nodular type with swelling. The cancerous parts of the papillary and nodular tumors visible to the naked eye were extracted and displayed in magenta. Furthermore, AFI was able to detect cancerous areas of tumors that could not be detected by the naked eye. The cancerous areas observed by AFI clearly contrasted with the non-cancerous areas. Moreover, the extracted lesions matched with the areas histopathologically determined to be

malignant (Fig. 1). On the other hand, for flat tumors, the cancerous areas were unclear on AFI and did not match with the areas histopathologically determined to be malignant (Fig.2). The single case of biliary cancer was a papillary tumor visible to the naked eye. The border between cancerous and non-cancerous areas was clear and matched with the area histopathologically determined to be malignant.

Discussion

Normal mucosal membranes were displayed in green and tumor areas in magenta during AFI. Papillary and nodular tumors visible to the naked eye were clearly distinguished by the contrast between cancerous and non-cancerous areas, but flat tumors could not be clearly distinguished. The reason for the differences in tumor range among bile duct cancer sections of tumors visible to the naked eye is attributed to changes in the brightness of autofluorescence arising from changes in development patterns. The principal lesion in papillary and nodular tumors with swelling is mainly located in epithelial areas and develops over surface

layers regardless of interstices (Fig.1C). Therefore, several cancerous tissues are exposed in the observable inner cavity of the biliary duct. This in turn weakens autofluorescence and thus makes the contrast easily observable. As reported previously, the tendency of papillary and nodular types to progress over surface layers (14) is one reason for the observed differences. Other reasons include the effect of cancerous tissues replacing the collagen on the underside of the mucosal membrane and thus weakening autofluorescence, and the dispersion and absorption of blue excitation light due to mucosal hypertrophy (15-17) (Fig.3A). On the other hand, with progression, nodular tumors associated with swelling and flat tumors are characterized by many interstices visible in the inner wall of the bile duct (Fig.2C). Progression of flat tumors, in particular, is associated with multiple interstices in the inner areas of the duct wall. Since only few exposed cancerous cells are present in the inner cavity of the bile duct and collagen and flavin are abundant in interstices, autofluorescence would be strong (Fig.3B). Effects of hemoglobin because of hemorrhage

and inflammation are also to be considered during AFI. Blue excitation light and green illumination light are easily affected by hemoglobin. It has been reported that in cases of hemorrhage or inflammation, these are strongly absorbed by hemoglobin, reducing autofluorescence and green light reflection, and thus making it difficult to distinguish between tumors (12). In the case of biliary cancer, there are several cases in which procedures to alleviate jaundice are performed prior to surgery for concomitant obstructive icterus and angiocolitis. In this study, jaundice was treated using a drainage tube, but cancerous areas without inflammation or any tube were visibly confirmed in the biliary mucosal membrane by AFI. Blue excitation light and green illumination light are strongly absorbed by hemoglobin. Autofluorescence and green reflective light were weakened, resulting in smaller differences in brightness and a dark green hue that was visibly confirmed. In conclusion, AFI is a technique for indirectly observing neoplastic lesions, and it is affected by hemoglobin in hypertrophic mucosa and inflamed tissues. Therefore, careful attention is

required to avoid false positives. Additionally, there are issues involving bile associated with the mucosal membrane, as in AFI this is displayed as a black hue that may mask the lesion area or its scattered image may be depicted on the reflective image. However, we were previously able to observe the range of cancer spread in the layers of the surface area of biliary duct cancer using a combination of AFI and peroral choledochoscopy (POCS) (7, 8). Furthermore, we were able to obtain an image by incorporating AFI into a stereoscopic microscope. The positive result identified by stereoscopic AFI microscope matched the cancerous area. Thus, it is possible that AFI will be an effective means of determining the pre-operational path of surgical incision for bile duct removal. In addition, compared with endoscopy, it shows higher sensitivity to autofluorescence and a wider range of image resolutions, and may aid in detecting a wider range of bile duct cancers. In cases involving the removal of bile duct tumors, cancer remnants on portions of the bile duct and the delaminated area are considered to impact prognosis (18, 19). Careful

attention is required while determing the path of surgical incision for bile duct removal. In this study, papillary tumors visible to the naked eye and nodular tumors were observed by AFI. Therefore, there is a possibility that AFI significantly contributes to determining the range of tumors progressing through surface layers, which are difficult to detect through direct contrast, as well as intraductal papillary neoplasms of the bile duct (20).

Conclusions

Recent advances in the development of devices such as the thin cholangioscope and NBI endoscope have been remarkable (21-24). In the near future, POCS, in combination with AFI, may significantly contribute to pre-surgical diagnosis of biliary cancer.

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Legends

Fig.1 Non-invasive type （Case12） .

a Macroscopic finding ; Papillary tumor visible to the naked eye.

b AFI ; Detected tumor displayed in magenta and matching with the histopathologically-identified malignant area.

c Microscopic finding; Tumor localized in the epithelial tissues and spread over the surface (loupe image).

Fig.2 Invasive type（ Case1 ）.

a Macroscopic finding ; Flat tumor visible to the naked eye.

b AFI ; Range of tumor extent unclear and not matching with the histopathologically-identified malignant area.

c Microscopic finding; Tumor spread deep within the bile duct wall and accompanied by multiple interstices.

Fig.3

a Non-invasive type Cancer cells spread over the surface and displacing the lower mucosal membrane (×20, H&E).

b Invasive type Cancer cells invading deeply accompanying with multiple interstices, and few cancer cells exposed in the inner cavity of the bile duct (×20, H&E).

Fig.1

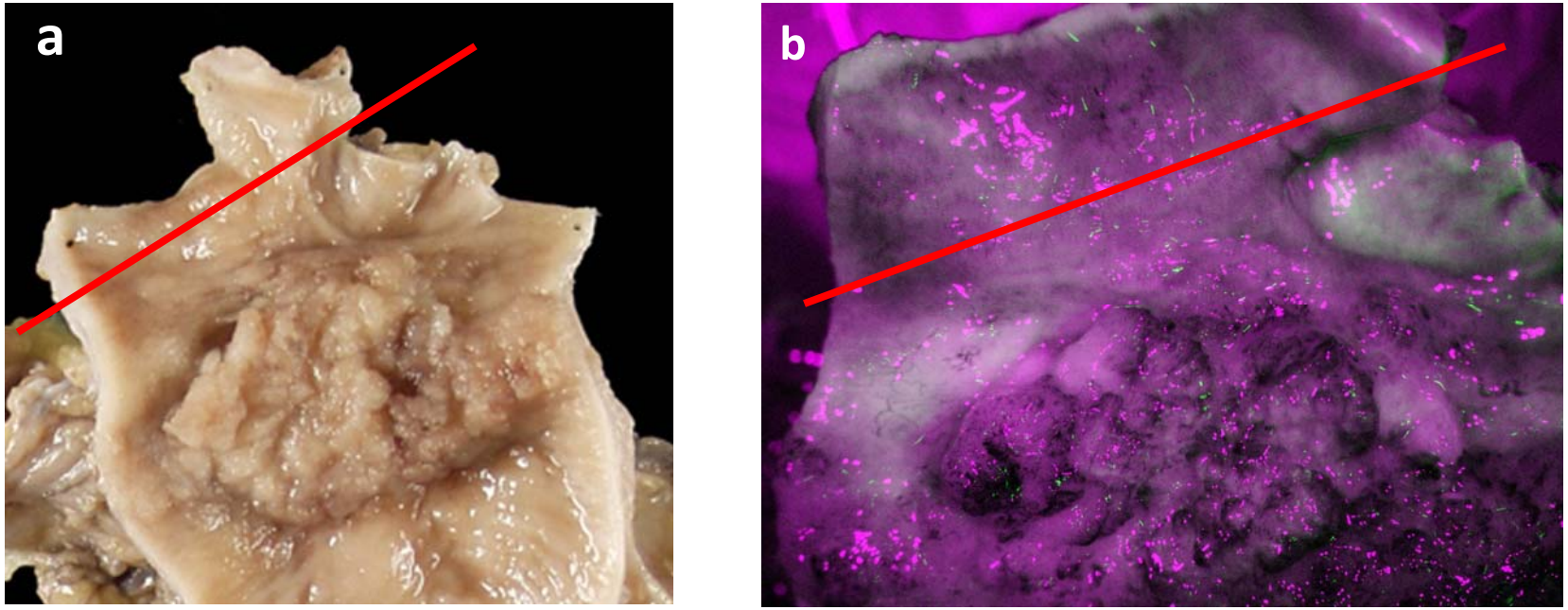
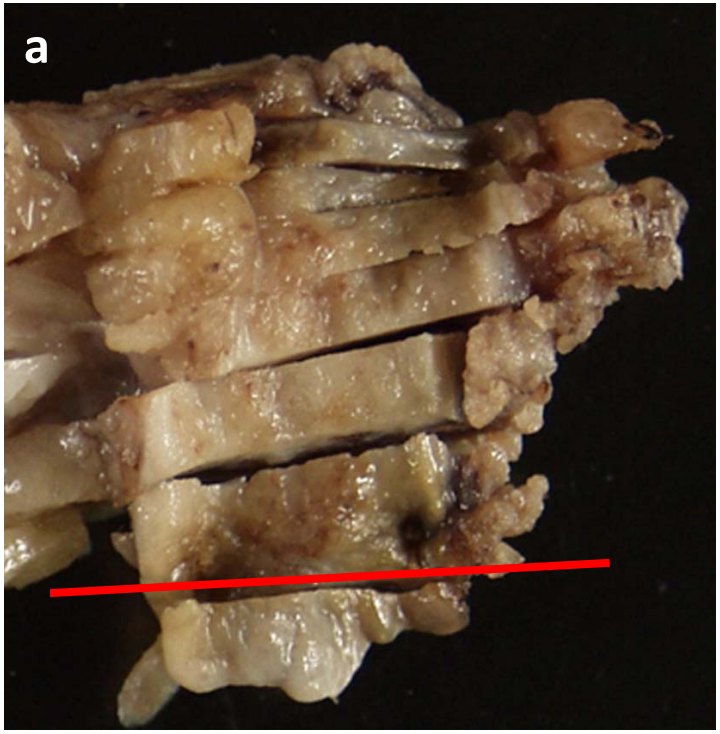
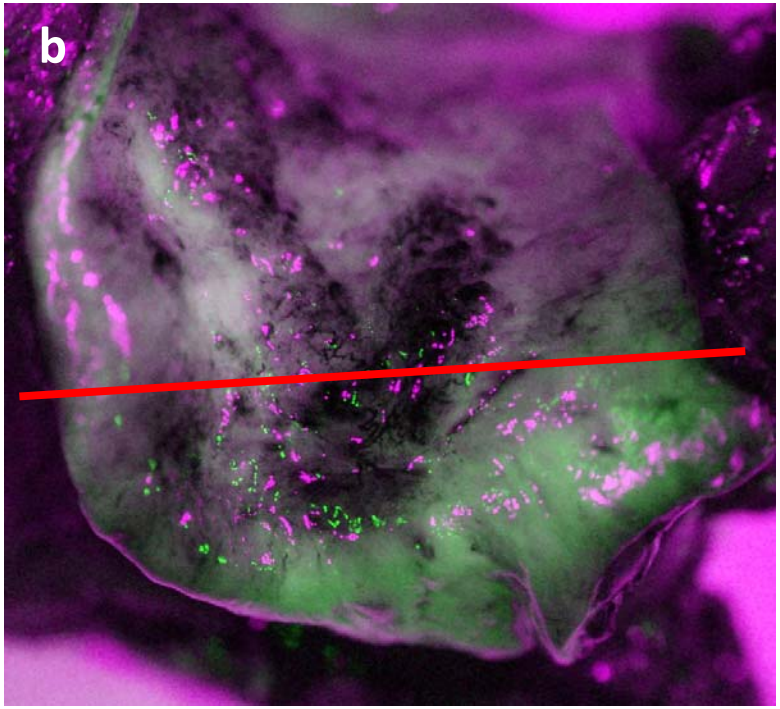


Fig.2





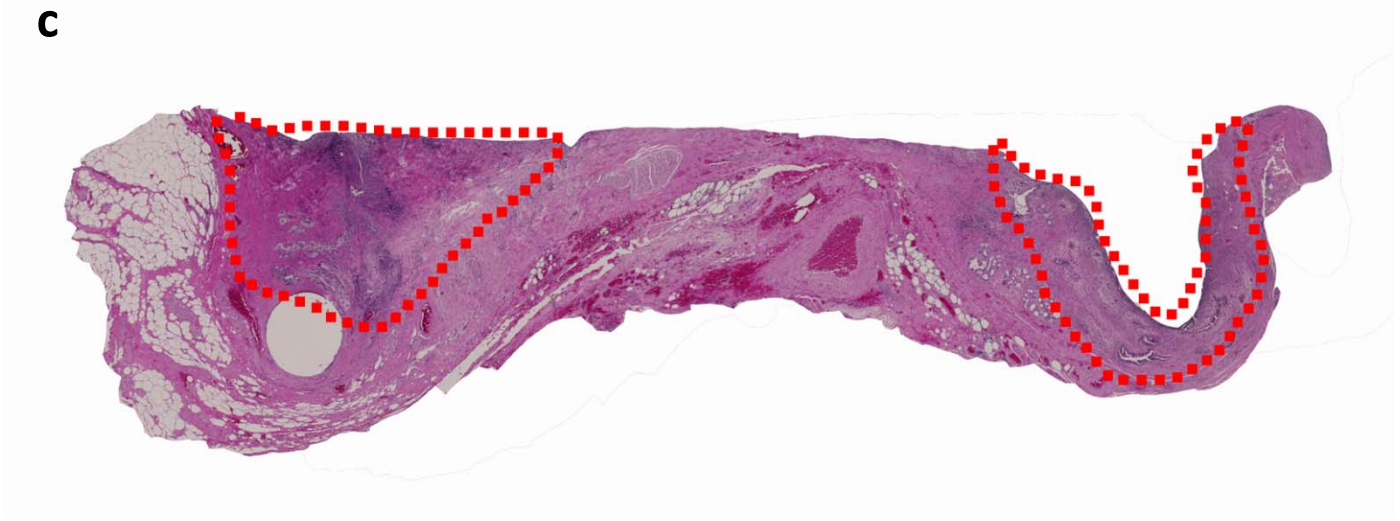
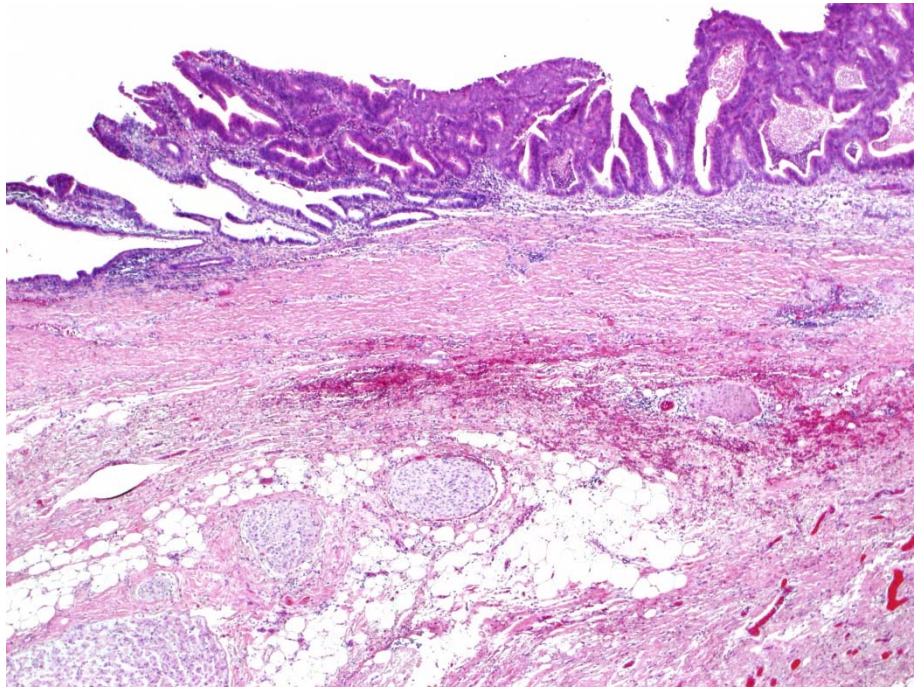


Fig.3

a



b

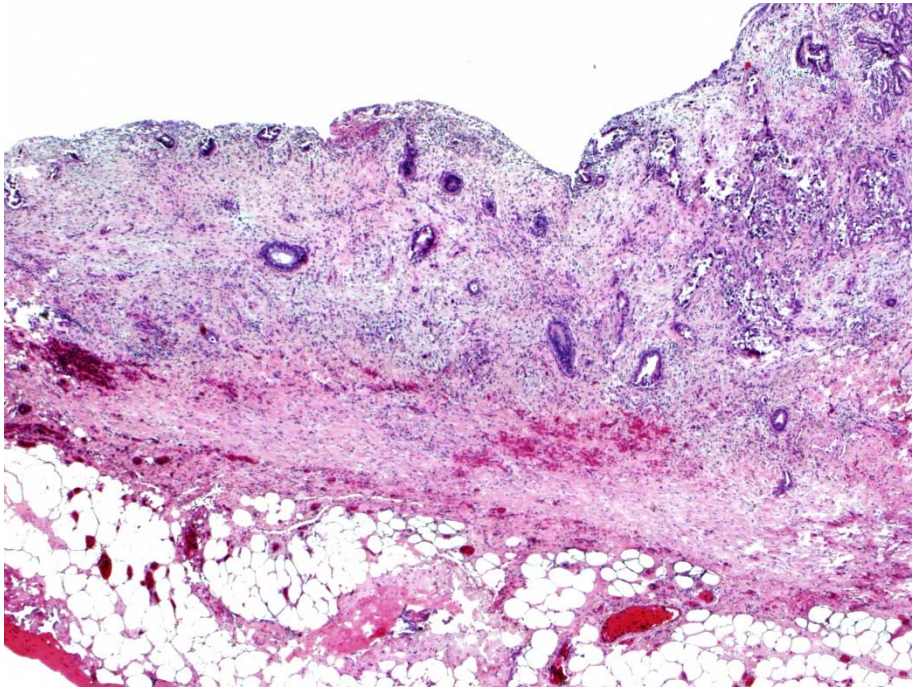


Table 1 Characteristics of patients

Case no.	Age	Gender	Tumor location	Histrogic grade	Growth pattern	Ductal margin status	pTNM(UICC)	Operative procedure
1	73	M	Perihilar	G1	Flat infiltrative	Positive with invasive carcinoma(Both sides)	pT1N0M0,stage Ⅰ	EBDR
2	58	M	Perihilar	G3	Flat infiltrative	Positive with invasive carcinoma(HM)	pT２a N0M0,stage Ⅱ	EBDR
3	62	F	Perihilar	G2	Flat infiltrative	Positive with invasive carcinoma(HM)	pT2bN0M0,stage Ⅱ	PD
4	77	M	Perihilar	G1	Flat infiltrative	Positive with invasive carcinoma(HM)	pT1N0M0,stage Ⅰ	EBDR
5	81	M	Distal	G3	Flat infiltrative	Negative	pT３N0M0,stage ⅡA	PD
6	75	M	Perihilar	G2	Flat infiltrative	Negative	pT２ｂN0M0,stage Ⅱ	PD
7	82	M	Distal	G2	Flat infiltrative	Negative	pT３N0M0,stage Ⅱ	PD
8	71	M	Distal	G2	Nodular infiltrative	Negative	pT1N1M0,stage ⅡB	PD
9	79	M	Distal	G2	Nodular infiltrative	Negative	pT1N0M0,stage Ⅰ	PD
10	78	F	Distal	G1	Nodular infiltrative	Negative	pT1N1M0,stage ⅡB	EBDR
11	53	M	Perihilar	G1	Papillary infiltrative	Positive with invasive carcinoma(DM)	pT1N0M0,stage Ⅰ	PD
12	74	M	Perihilar	G1	Papillary infiltrative	Positive with invasive carcinoma(HM)	pT２aN１M0,stageⅢB	PD
13	63	M	Distal	G1	Papillary infiltrative	Negative	pT1N0M0,stage Ⅰ	PD
14	64	M	Distal	G1	Papillary infiltrative	Negative	pT３N0M0,stage Ⅱ	PD
15	70	M	Distal	G1	Papillary infiltrative	Negative	pT1N1M0,stageⅢB	PD
16	82	F	Distal	G1	Papillary expansive	Negative	pT３N0M0,stage Ⅱ	PD
17	68	M	Distal	G1	Papillary expansive	Negative	pT1N0M0,stage Ⅰ	Cholecystrectomy
18	74	F	GB	G1	Papillary expansive	Negative	pT１N0M0,stageⅠ	PD
19	74	M	Distal	G1(pancreatic carcinoma)	-	Negative	pT３N0M0,stage ⅡA	PD
20	75	F	Distal	G3(pancreatic carcinoma)	-	Negative	pT３N1M0,stage ⅡB	PD

M male, F female, G1 well differentiated, G2 moderately differentiated, G3 poorly differentiated, HM hepatic side margin, DM duodenum side margin, EBDR extrahepatic bile duct resection, PD pancreatoduodenectomy