Utility of intraoperative cytology of resection margins in biliary tract and pancreas tumors

（胆道および膵臓腫瘍の切除断端における術中迅速細胞診断の有用性）

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Abstract

Background: Intraoperative diagnosis of resection margins in the biliary tract and pancreas tumors is important in deciding extent of resection; however, diagnosis based solely on frozen sections is sometimes difficult. Therefore, we investigated the usefulness of intraoperative cytology (IC) combined with frozen section (FS) histology. We present the results with a discussion of the value of this combination and its associated problems.

Methods: We examined 80 bile duct resection margin specimens from 42 patients, and 34 pancreatic resection margin specimens from 29 patients, who underwent intraoperative diagnosis of resection margins during surgery for biliary tract or pancreatic tumors between October 2012 and January 2014. IC was performed on imprint specimens prepared from surfaces of margins being examined. The results were compared with FS and final histology of operative materials.

Results: In IC, excluding cases with insufficient material, the results for bile duct margins; sensitivity was 96.7%, specificity 100% and accuracy 98.7%. The results for pancreatic margins; sensitivity was 100%, specificity 92.9% and accuracy 93.3%. In FS, the results for bile duct margins; sensitivity was 96.8%, specificity 100% and accuracy 98.8%. The results for pancreatic margins; sensitivity was 66.7%, specificity 100% and accuracy 97.1%.

Conclusion: IC is quick, highly accurate and very easy to perform. The present study even included a specimen for which only IC led to an accurate diagnosis. IC used in combination with FS can achieve intraoperative diagnosis with high overall accuracy.

Key words: biliary tract tumor; pancreas tumor; resection margin; intraoperative cytology; frozen section

Introduction

Intraoperative diagnosis of the biliary tract and pancreas is frequently used for the evaluation of resection margins, and is important in determining the extent of surgical resection.[1, 2] Despite remarkable advances in endoscopy and diagnostic imaging technologies in recent years, difficulties remain in accurately ascertaining the extent of tumor before surgery.[3, 4] There is therefore a need for a highly accurate method of intraoperative diagnosis in order to achieve curative resection with no residual tumor.[1-3]

Although most tumors of the biliary tract and pancreas are glandular lesions, they can include benign cells with marked atypia and very well differentiated adenocarcinomas. This presents problems for diagnosis, whether the lesion is benign or malignant, even in permanent sections. Furthermore, when evaluating resection margins using frozen sections, tissues are often crushed or degenerated during surgical procedures. Artifacts can also arise through freezing, making it even more difficult to differentiate between benign and malignant characteristics.[1, 5]

In contrast, cytologic preparations suffer much less crushing and creation of artifact than frozen sections, and also provide the optimum form for observation of individual cells, making cell identification comparatively easy. With intraoperative cytology (IC), sample preparation is also easy and the staining time is short, allowing cell information to be rapidly obtained. This technique is thus a key method in intraoperative diagnosis.[6, 7]

IC was first described by Dudgeon et al. in 1927;[8] since then, its utility in various organs has been reported.[6, 7, 9-13] It is not uncommon for the operative method to be decided solely on the basis of IC results. When used in combination with frozen section (FS) histology diagnosis, IC is known as a useful complementary diagnostic method, since the advantages of cytologic preparations make up for the disadvantages inherent in FS

diagnosis.[11]

However, until now there have been no detailed reports on the use of IC in resection margins in the biliary tract and pancreas tumors. We therefore used IC in combination with FS diagnosis and present here the results, together with a discussion of the significance of this approach and the points for clinical application.

Materials and Methods

We examined 80 bile duct resection margin specimens from 42 patients (Table 1) and 34 pancreatic resection margin specimens from 29 patients (Table 2). These patients had undergone intraoperative diagnosis to evaluate resection margins during surgery performed at Hirosaki University Hospital under the diagnosis of biliary tract or pancreatic tumor between October 2012 and January 2014. The patients with biliary tract tumor consisted of 26 men (61.9%) and 16 women (38.1%), with a mean age of 70.2 years (age range, 51-81 years). The patients with pancreatic tumor consisted of 14 men (48.3%) and 15 women (51.7%), with a mean age of 67.6 years (age range, 40-80 years). All the patients provided their informed consent to participate in the study. This study was approved by The Committee of Medical Ethics of Hirosaki University Graduate School of Medicine, Hirosaki, Japan.

BD SurePath[TM] Precoat Slides Japan (Becton, Dickinson & Company, Franklin Lakes, NJ, USA) were used for all imprint preparations. Imprint preparations for IC of bile duct margins were accomplished by raising the bile duct, directing the surface of the margin of interest downwards and softly placing the surface onto a glass slide. For pancreatic margins, the surface of the margin of interest was held facing upwards, and an imprint was made by lightly touching a glass slide to the surface. Samples underwent wet

fixation for 1 min in 95% ethanol, followed by convenient and rapid hematoxylin and eosin (H&E) staining (Table 3) and cytological examination with a light microscope. At the same time, frozen sections were prepared and histological examination was performed. Each microscopic evaluation was done by cytotechnologists or pathologists independently not to be influenced by other's diagnoses. The results of these two diagnostic methods were then compared with final histology on paraffin sections of operative materials.

The sensitivity, specificity, positive predictive value, negative predictive value, accuracy and their 95% confidence intervals of IC (excluding specimens with insufficient material) were calculated using 2 × 2 contingency tables, with final histology as the gold standard of comparison. Results were defined as true positive or true negative if positive or negative in both IC and final histology. Results that were positive in IC and negative in final histology were defined as false positives, and results that were negative in IC and positive in final histology were defined as false negatives.

Results

Of the 80 bile duct margin specimens, there were 29 positives, 48 negatives, and three specimens with insufficient material in IC. Excluding these three specimens, there was concordance between IC and FS diagnosis in 75 specimens and discordance in two (Table 4). One of the two discordant specimens was negative in IC (Fig. 1a), positive in FS (Fig. 1b) and positive in final histology (Fig. 1c). The other specimen was positive in IC (Fig. 2a), negative in FS (Fig. 2b) and positive in final histology (Fig. 2c).

Of the 34 pancreatic margin specimens, there were four positives, 26 negatives, and four specimens with insufficient material in IC. Excluding the four specimens with

insufficient material, there was concordance between IC and FS diagnosis in 28 specimens and discordance in two (Table 5). Both discordant specimens were positive in IC (Figs. 3a, 4a) but were evaluated to be negative in FS diagnosis although atypical cells were observed (Figs. 3b, 4b). Both discordant specimens were negative in final histology, but findings of pancreatic intraepithelial neoplasia (PanIN)-2 were seen in one of the two specimens (Fig. 4c).

Concerning the correlation between IC and final histology, the results for bile duct margins were 29 true positives, no false positives, 47 true negatives and one false negative (Fig. 1a). The sensitivity was 96.7% (95% confidence interval [CI], 83.3%-99.4%), the specificity was 100% (95% CI, 92.4%-100%), the positive predictive value was 100% (95% CI, 88.3%-100%), the negative predictive value was 97.9% (95% CI, 89.1%-99.6%) and the accuracy was 98.7% (95% CI, 93.0%-99.8%) (Table 6). In the analyses of pancreatic margins there were two true positives, two false positives (Figs. 3a, 4a), 26 true negatives and no false negatives. The sensitivity was 100% (95% CI, 34.2%-100%), the specificity was 92.9% (95% CI, 77.4%-98.0%), the positive predictive value was 50% (95% CI, 15.0%-85.0%), the negative predictive value was 100% (95% CI, 87.1%-100%) and the accuracy was 93.3% (95% CI, 78.7%-98.2%) (Table 7).

Discussion

The objective of this study was to investigate the usefulness of IC as an adjunct intraoperative diagnostic technique for resection margins of the biliary tract and pancreas tumors, and to present a discussion of the significance of this approach and the important points for its clinical application. Tables 6 and 7 show the results of our comparison of IC and final histology for bile duct margins and pancreatic margins, respectively. There have been no previous reports of a similar comparison in biliary tract

or pancreas tumors. However, previous studies focusing on other organs have generally reported a high accuracy rate (≥90%) of IC,[10,11,13,14] with which our results compare favorably. Our results were also notable for the fact that one specimen diagnosed as false negative by FS diagnosis was accurately diagnosed only by IC. In this specimen, a very small number of adenocarcinoma cells were clearly visible in IC (Fig. 2a). Although a very small number of atypical cells were also seen in the FS diagnosis, ultimately, adenocarcinoma could not be diagnosed and a false negative result was given in FS diagnosis (Fig. 2b). Therefore, additional intraoperative resection failed to be performed, and final histology was positive for invasive carcinoma in the margin (Fig. 2c). The FS diagnosis gave a false negative because only a very small number of atypical cells were seen, and these were considered to be an artifact of freezing. Artifacts must always be taken into account in FS diagnosis; however, this sometimes confuses the diagnosis. In contrast, IC is largely unaffected by artifacts, which makes cell identification easy because the original cell morphology is maintained.[11, 13] The use of IC in combination with FS diagnosis has high utility, as evidenced by cases such as the one described above, in which IC compensated for the disadvantages of FS diagnosis and was able to provide a correct diagnosis on its own.

On the other hand, IC may also produce false negatives and false positives. It is extremely vital that the reasons for these discrepancies are understood and strategies are developed so that the precision of IC can be improved.

In the present study, there was one false negative in the analysis of a bile duct margin. In this specimen, no clear adenocarcinoma cells were seen in IC (Fig. 1a), whereas carcinoma in situ was seen in FS and final histology (Fig. 1b, c). This discrepancy was probably due to the fact that there was very little carcinoma in situ in the FS, and imprinting of the target cells for IC was considered to be unsuccessful. If precise IC is to

be achieved, it is extremely vital to secure a sufficient amount of cells. When preparing imprint samples for the present study, we endeavored to obtain sufficient cells using BD SurePath™ Precoat Slides Japan, which have excellent cell adhesion properties. For most specimens, the amount of cells secured on the glass slides was excellent, but in a small number of cases, such as the false negative under discussion, sufficient target cells could be not secured. In the preparation of imprint samples, it is important to be aware of this problem as a limitation of IC.

There were two false positives in the analysis of pancreatic margins. In one case, although adenocarcinoma was clearly evident in IC (Fig. 3a) and atypical cells were seen in FS, the degree of cellular atypia and the relationship with the surrounding tissues suggested a negative result (Fig. 3b). Final histology was also negative; consequently, the result proved to be a false positive in IC. When considering that additional intraoperative resection was not performed and the margin was negative in the analysis of the operative material, it is possible that the atypical cells observed in intraoperative diagnosis could have been the result of contamination from the carcinoma lesion during the operative procedure rather than arising from the margin itself. In IC, there was sufficient atypia in the cell morphology and in the structure of cell clusters to justify the diagnosis of adenocarcinoma. In general, evaluation based on features of tissue structure, such as the relationship between the pancreatic duct and interstitial tissue, is difficult in cytology samples[13] and can lead to false positives if the possibility of contamination is not considered. While very rare, such cases are obviously encountered, and they highlight the need for caution when making diagnosis based on IC alone.

In the other false positive case, well differentiated adenocarcinoma was diagnosed in IC (Fig. 4a); however, the FS diagnosis was negative (Fig. 4b) despite the presence of

atypical cells, and final histology concluded that the lesion was PanIN-2 (Fig. 4c). The diagnosis of the PanIN-2 lesion as adenocarcinoma in IC was an overdiagnosis.

PanIN is a classification system applied to pancreatic intraepithelial lesions as precursor lesions of pancreatic adenocarcinoma, and is divided into PanIN-1A, -1B, -2 and -3 based on the degree of histological atypia.[15, 16] There have not been many reports describing the cytologic features of PanIN.[17-21] Hara and Suda[18] have discussed the differential diagnosis between PanIN-3 and invasive ductal adenocarcinoma of the pancreas based on pancreatic duct scraping cytology, and Nakaizumi et al.[20] have described carcinoma in situ and marked atypical cells based on pancreatic juice cytology. However, it appears that the cytologic features of all PanIN grades have not been addressed in detail in the literature. Jarboe and Layfield[21] reported cases of overdiagnosis of histologically intermediate- to high-grade PanIN as adenocarcinoma in fine-needle aspiration (FNA) cytology of the pancreas, and pointed out that PanIN is a significant source of false positives. Similarly, in our case, the atypical cells were characterized by marked irregularity of nuclear membranes, enlargement of nucleoli and nuclei, high nuclear/cytoplasmic ratio and presentation as a slightly overlapping cell cluster (Fig. 4a). These observations led to a cytologic diagnosis of well differentiated adenocarcinoma; however this proved to be an overdiagnosis of PanIN-2 cells.

On the other hand, well differentiated adenocarcinoma of the pancreas often shows weak cell atypia and is sometimes underdiagnosed, causing accurate diagnosis to be problematic in cytology.[22] Although both Lin and Staerkel[23] and Hysell et al.[24] used fine-needle aspiration biopsy (FNAB) of the pancreas, Lin and Staerkel[23] found that anisonucleosis (greater than four-fold size variation), nuclear membrane irregularity, nuclear crowding/overlapping/three-dimensionality, and nuclear enlargement were the four important cytologic features of well differentiated adenocarcinoma. Hysell et al.[24]

found that nuclear enlargement and cellular crowding are frequently observed in both inflammatory pancreatic diseases and pancreatic adenocarcinomas, while nuclear membrane irregularities and nuclear anisonucleosis (four-fold variation in nuclear size within the same epithelial group) are specific cytologic features of malignancy. Therefore, nuclear membrane irregularity in particular is a key feature in diagnosing well differentiated adenocarcinoma; nevertheless, anisonucleosis must be considered at the same time. In our case, the obvious nuclear membrane irregularity led to an overdiagnosis of well differentiated adenocarcinoma. However, in retrospect, this diagnosis was not justified because the anisonucleosis was not particularly marked. In order to reduce underdiagnosis and overdiagnosis in IC as much as possible, knowledge of the cytologic features of PanIN is essential, and the accumulation of data from imprint specimens is also important.

As the above discussion indicates, it is essential to identify the causes of false negatives and false positives in IC and to take them into account in order to provide highly accurate intraoperative diagnosis. It is also necessary to fully understand and address the general problems and important points in sample preparation and cell determination.

Bile duct epithelium is known to readily exfoliate, and must therefore be handled with great care when preparing imprint samples to avoid cell contamination from other than the margin surface. Therefore, the bile duct stumps should simply be touched lightly to glass slides without any pressing. For the pancreas stumps, having learned from our experience that it is difficult to obtain sufficient pancreatic duct epithelial cells by simply imprinting the pancreatic margin because the main pancreatic duct is surrounded by the pancreatic parenchyma, we sought to obtain the epithelial cells from imprinting the main pancreatic duct by holding the pancreas and slightly bending the margin surface so as to expose the main pancreatic duct. We never used gauze to remove blood on the

imprinting surface since it might damage the interesting cells. Adequate slides without obstructive blood could be prepared by making plural imprinting slides. There were three bile duct margin specimens and four pancreatic margin specimens from which insufficient cellular materials were obtained, but this was largely because the resected tissue had been cauterized. IC was ineffective in these cases.

The staining used for IC in the present study was H&E staining, which is convenient and rapid.[9, 10] Other options include Diff-Quik staining,[11, 25, 26] the Ultrafast Papanicolaou stain[27, 28] and the modified Gill-Shorr stain.[29] The advantages of H&E staining are that cells do not readily undergo exfoliation despite the short wet fixation time, staining only takes about 1 min, cell findings equal to those of the Papanicolaou stain are achieved, and comparison with frozen sections is easy because both samples use an identical stain.

Recently, several authors have noted that ductal margin status has no influence on long-term prognosis in bile duct margin positive cases, among which carcinoma in situ positive cases have a significantly better prognosis than invasive carcinoma positive cases.[30-33] Despite the importance mentioned above of distinguishing between carcinoma in situ and invasive carcinoma in bile duct margins, it is currently very difficult to ascertain the relationship with the surrounding tissue and interstitium in cytology specimens[13] and thus difficult to differentiate between the two diagnoses. It is necessary to accumulate more data on cytologic features in order to differentiate between carcinoma in situ and invasive carcinoma, based on differences of cellular atypia and appearances of cell clusters.

Finally, while adenocarcinoma with weak cellular atypia can be found in biliary tract and pancreas tumors, marked atypical epithelium is known to appear in the region in

reaction to duct obstruction, catheter manipulation and stent placement.[5] It is vital to perform comprehensive cytology diagnosis after making a comparison with clearly benign epithelial cells in the same specimen, observing the background, individual cell findings and the structure of cell clusters, and checking preoperative clinical information.

Conclusions

IC for evaluating resection margins in the biliary tract and pancreas has a high accuracy rate and agrees with final histology in most specimens. The present study even included a specimen for which only IC led to an accurate diagnosis. IC is therefore a highly useful method of intraoperative diagnosis. Additionally, IC is a simple and rapid method that allows excellent preservation of cellular details devoid of freezing artifacts and compensates for the disadvantages of FS diagnosis. The use of both methods together provides a large amount of diagnostic information and contributes overall to highly precise intraoperative diagnosis. IC cannot always be used alone for definitive diagnosis, as it sometimes gives false negatives and false positives; however, its use together with FS diagnosis is quite helpful. There is a need to conduct further research on this topic by continuing to accumulate data from additional cases and to seek ways to overcome the problems in sample preparation and cell determination.

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Table 1. Summary of pathological diagnoses and operative methods in biliary tract tumors

	Number of patients
Pathological diagnosis	
Intrahepatic cholangiocarcinoma	1
Extrahepatic bile duct carcinoma	
Proximal	6
Distal	16
Gallbladder carcinoma	3
Ampullary carcinoma	1
Others	15
Total	42
Operative method	
Right hepatic lobectomy with extrahepatic bile duct resection	4
Pancreaticoduodenectomy	29
Others	9
Total	42

Table 2. Summary of pathological diagnoses and operative methods in pancreatic tumors

	Number of patients
Pathological diagnosis	
Ductal adenocarcinoma (invasive)	16
Intraductal papillary mucinous neoplasms	8
Serous cystic neoplasms	2
Others	3
Total	29
Operative method	
Pancreaticoduodenectomy	17
Distal pancreatectomy	11
Others	1
Total	29

Table 3. Rapid hematoxylin and eosin staining protocol

1. 95% ethanol 1 min (fixation).

2. Tap water 10 dips.

3. Hematoxylin 3G preheated to 50°C for 10 s.

4. Tap water 10 dips.

5. Tap water preheated to 50°C for 20 s.

6. Eosin 3 dips.

7. 100% ethanol 10 dips, 3 changes.

8. Xylene 10 dips, 3 changes.

9. Mount with Malinol.

Table 4. Comparison of intraoperative cytologic diagnoses and frozen section diagnoses in bile duct resection margins

Intraoperative Cytology	Frozen Section		
	Negative	Positive	Total
Negative	47	1	48
Positive	1	28	29
Insufficient	2	1	3

Intraoperative Cytology[a] versus Frozen Section	Number of specimens
Concordance	75
Discordance	2

[a]Excludes three specimens with insufficient material.

Table 5. Comparison of intraoperative cytologic diagnoses and frozen section diagnoses in pancreatic resection margins

Intraoperative Cytology	Frozen Section		
	Negative	Positive	Total
Negative	26	0	26
Positive	2	2	4
Insufficient	4	0	4
Intraoperative Cytology[a] versus Frozen Section	Number of specimens		
Concordance	28		
Discordance	2		

[a]Excludes four specimens with insufficient material.

Table 6. Cytologic-histologic correlation in satisfactory imprint cytology of bile duct resection margins

Intraoperative Cytology Diagnosis	Histology (Final)	
	Negative	Positive
Negative	47 (TN)	1 (FN)
Positive	0 (FP)	29 (TP)
Cytology Correlation	Value, %	95% CI, %
Sensitivity	96.7	83.3-99.4
Specificity	100	92.4-100
PPV	100	88.3-100
NPV	97.9	89.1-99.6
Accuracy	98.7	93.0-99.8

FN, false-negative; FP, false-positive; TN, true-negative; TP, true-positive; PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval.

Table 7. Cytologic-histologic correlation in satisfactory imprint cytology of pancreatic resection margins

Intraoperative Cytology Diagnosis	Histology (Final)	
	Negative	Positive
Negative	26 (TN)	0 (FN)
Positive	2 (FP)	2 (TP)
Cytology Correlation	Value, %	95% CI, %
Sensitivity	100	34.2-100
Specificity	92.9	77.4-98.0
PPV	50.0	15.0-85.0
NPV	100	87.1-100
Accuracy	93.3	78.7-98.2

FN, false-negative; FP, false-positive; TN, true-negative; TP, true-positive; PPV, positive predictive value; NPV, negative predictive value; CI, confidence interval.
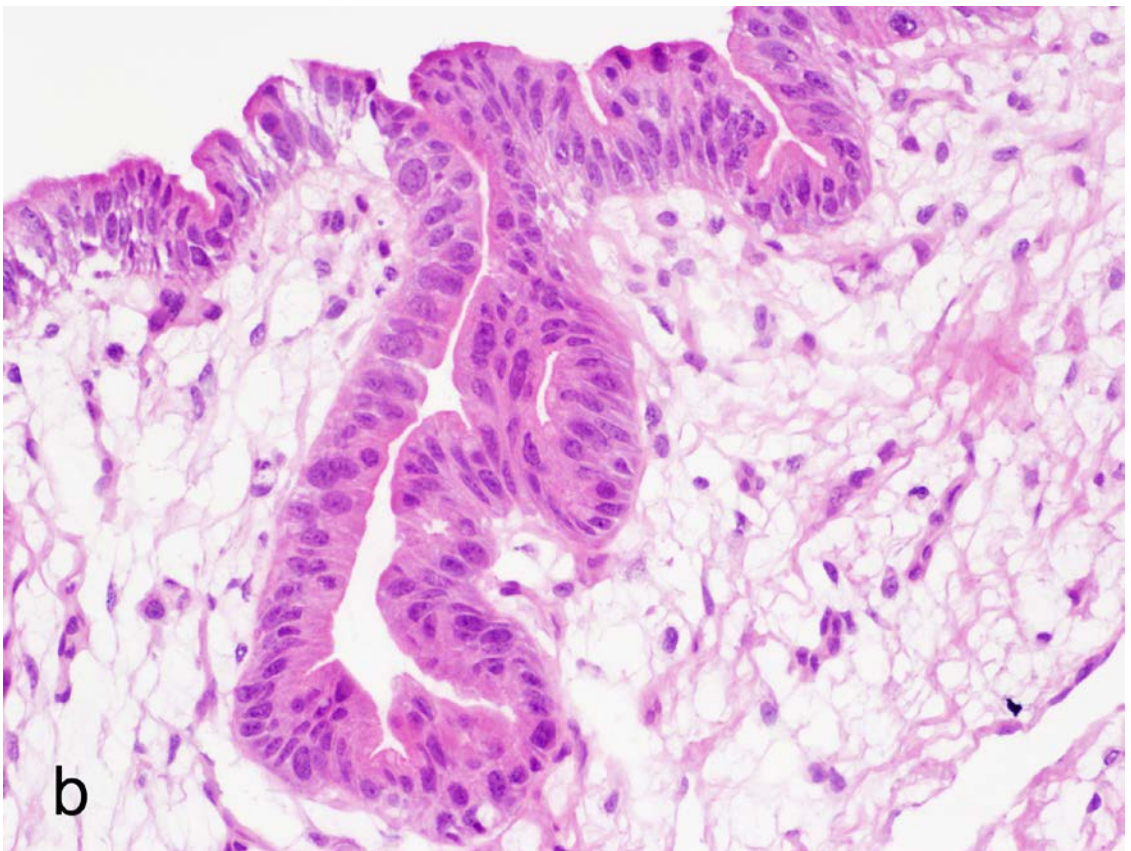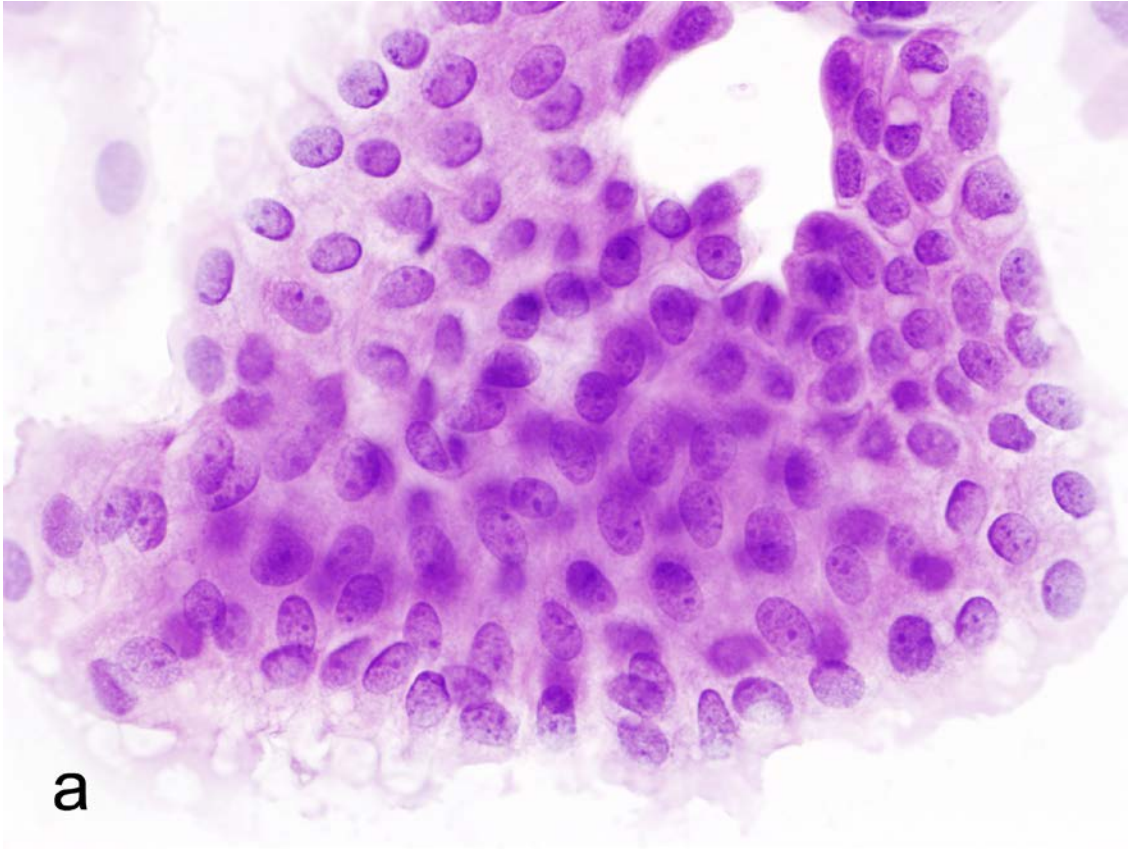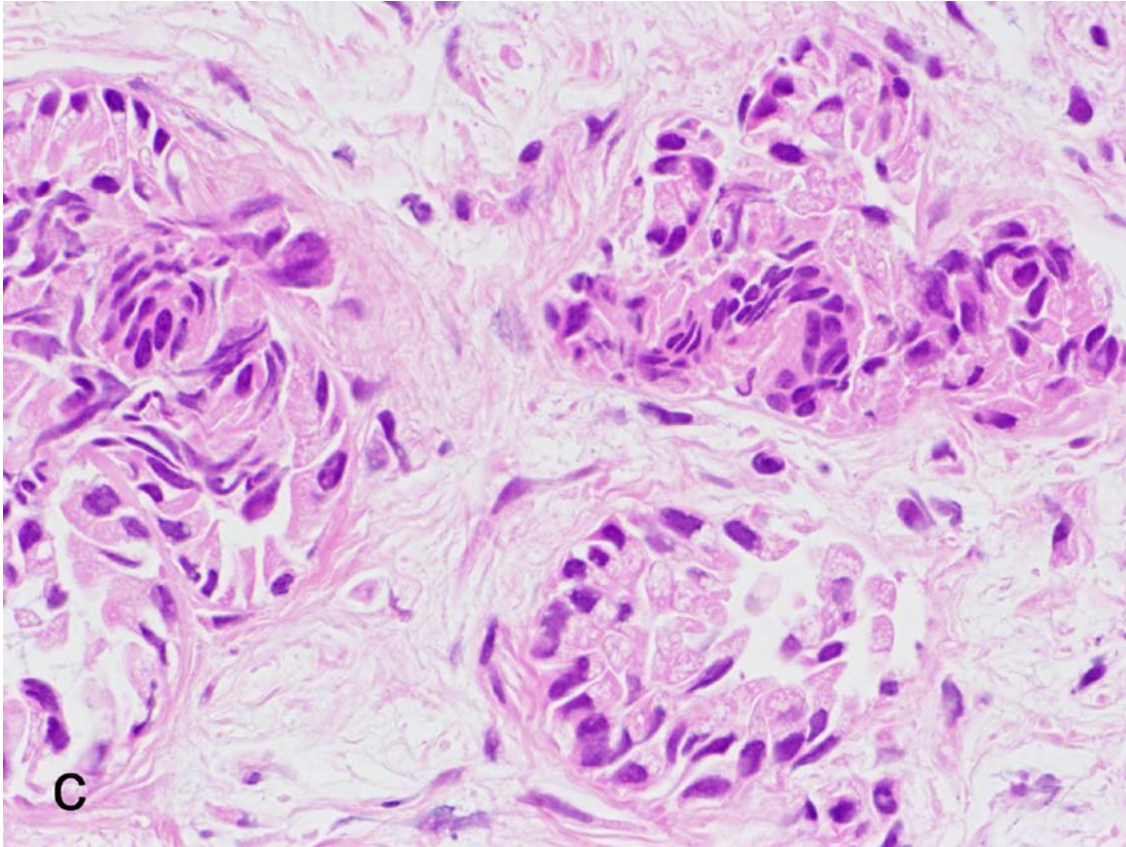
Fig. 1. False negative by intraoperative cytology (IC) of bile duct margin.

(a) In IC, cells showing mild enlargement of nuclei and nucleoli appeared in a cluster of regular arrangement. The cells were deemed to be benign epithelial cells, and the margin was diagnosed as negative. This false negative was due to sampling error (hematoxylin and eosin [H&E]-stained imprint cytology, original magnification ×400).

(b) In frozen section (FS) diagnosis, atypical cells showing enlarged nuclei and increased nuclear chromatin were seen to have proliferated in low papillary structures. The cells were deemed to be adenocarcinoma (carcinoma in situ), and the margin was diagnosed as positive (H&E-stained frozen sections, original magnification ×200).

(c) In final histology, carcinoma in situ was seen, and the margin was diagnosed as positive (H&E-stained permanent sections, original magnification ×400).
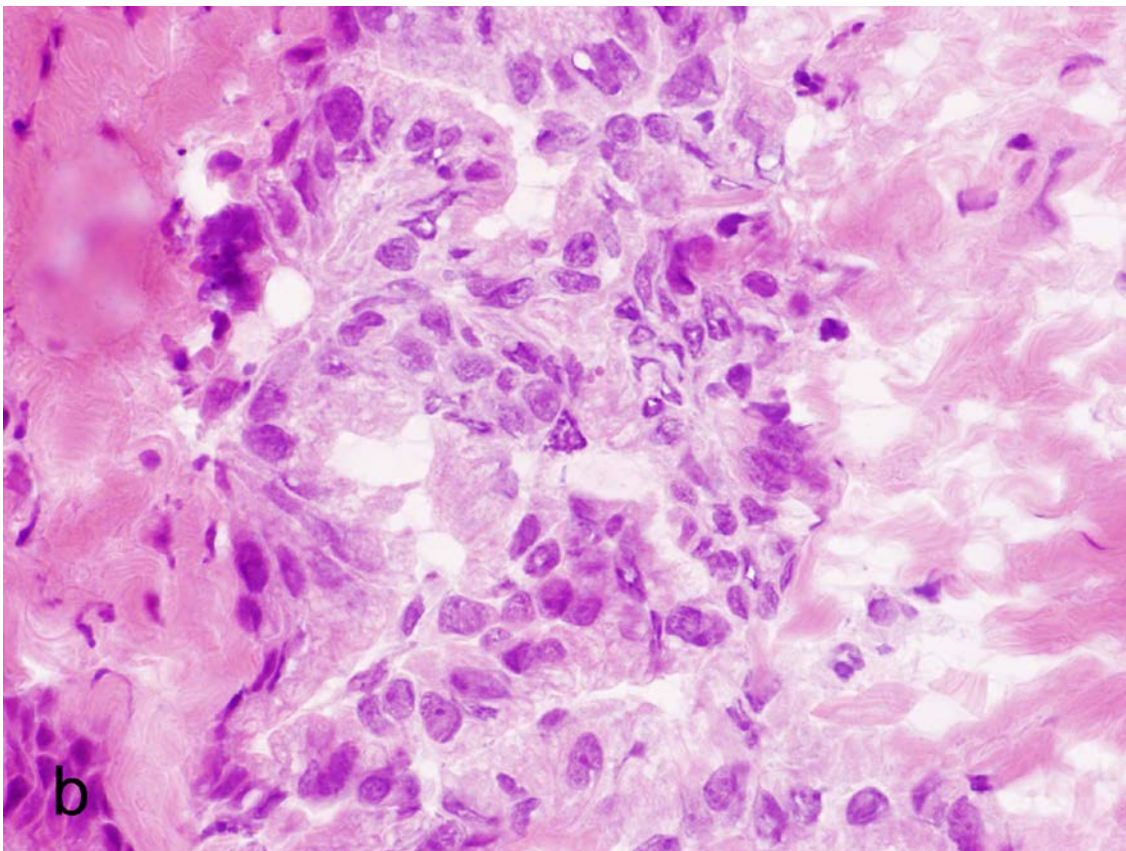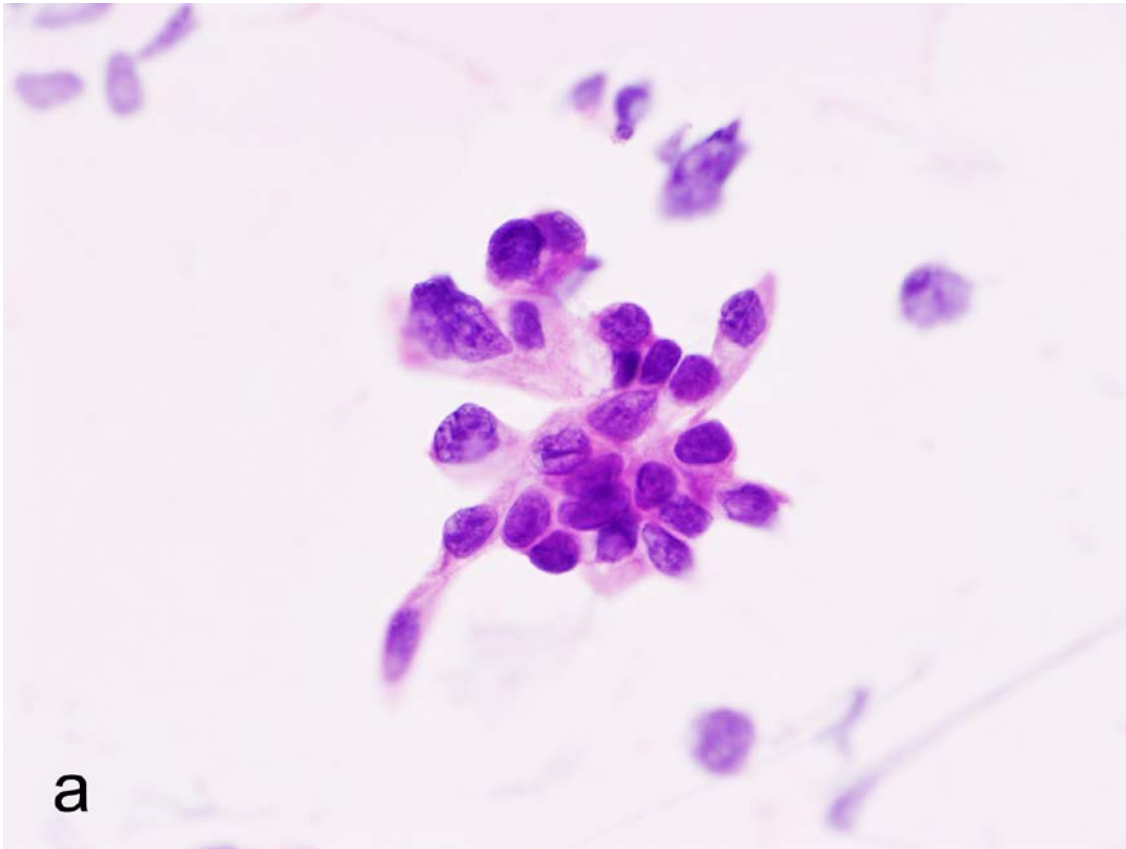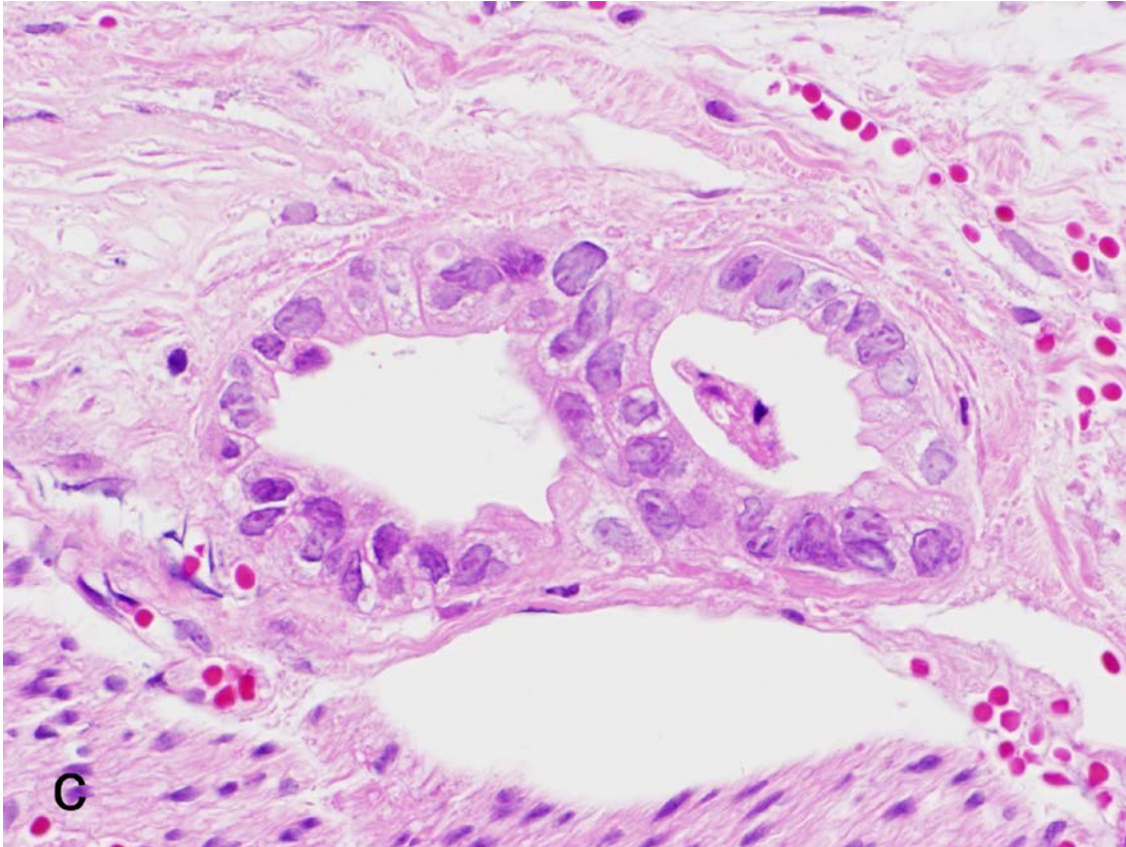
Fig. 2. True positive by intraoperative cytology (IC) and false negative by frozen section (FS) diagnosis of bile duct margin.

(a) In IC, atypical cells showing increased nuclear chromatin, nuclear irregularities, enlarged nuclei and nucleoli and high nuclear/cytoplasmic (N/C) ratio appeared in a few small clusters. This was deemed to be adenocarcinoma, and the margin was diagnosed as positive (hematoxylin and eosin [H&E]-stained imprint cytology, original magnification ×400).

(b) In FS diagnosis, atypical cells showing enlarged nuclei and nuclear irregularities were observed. The margin was diagnosed as negative, since a freezing artifact was considered to have created the atypia (H&E-stained frozen sections, original magnification ×400).

(c) In final histology, atypical cells were seen in the stroma of the common bile duct margin. The slide was deemed to clearly show an invasive adenocarcinoma, and the margin was finally diagnosed as positive (H&E-stained permanent sections, original magnification ×400).
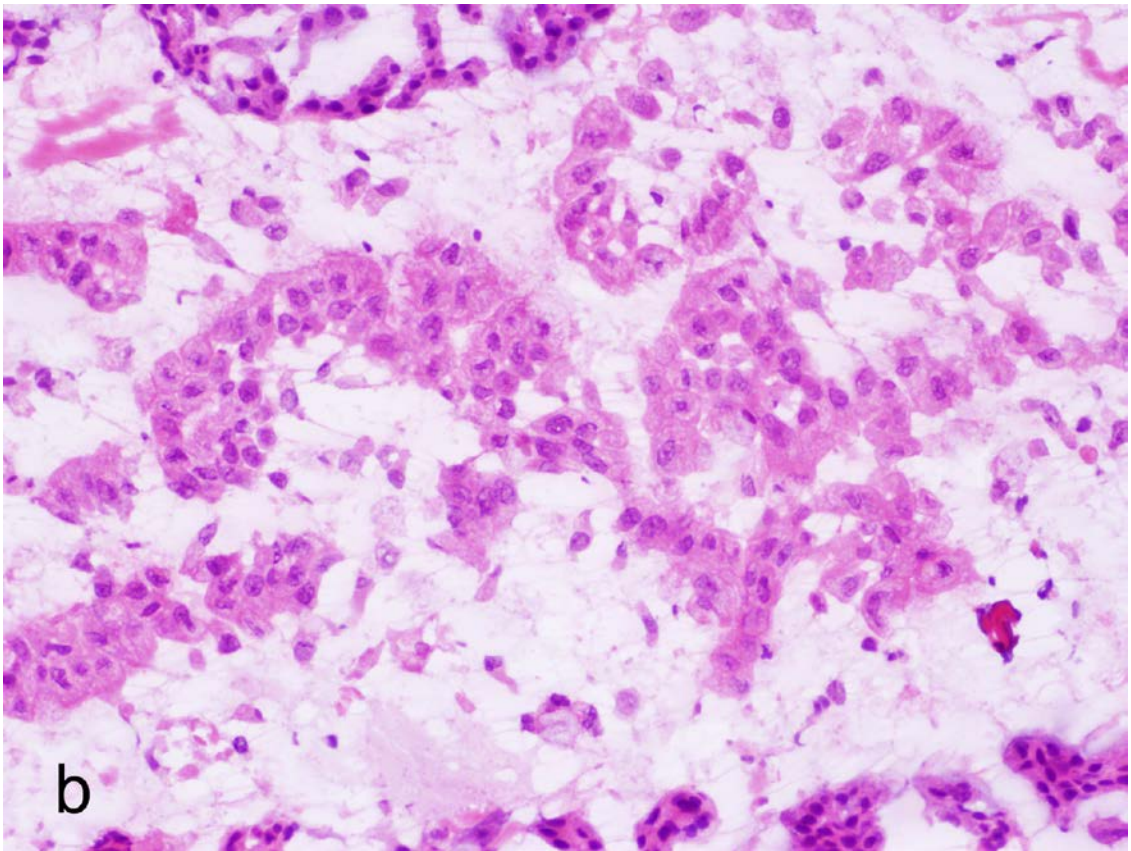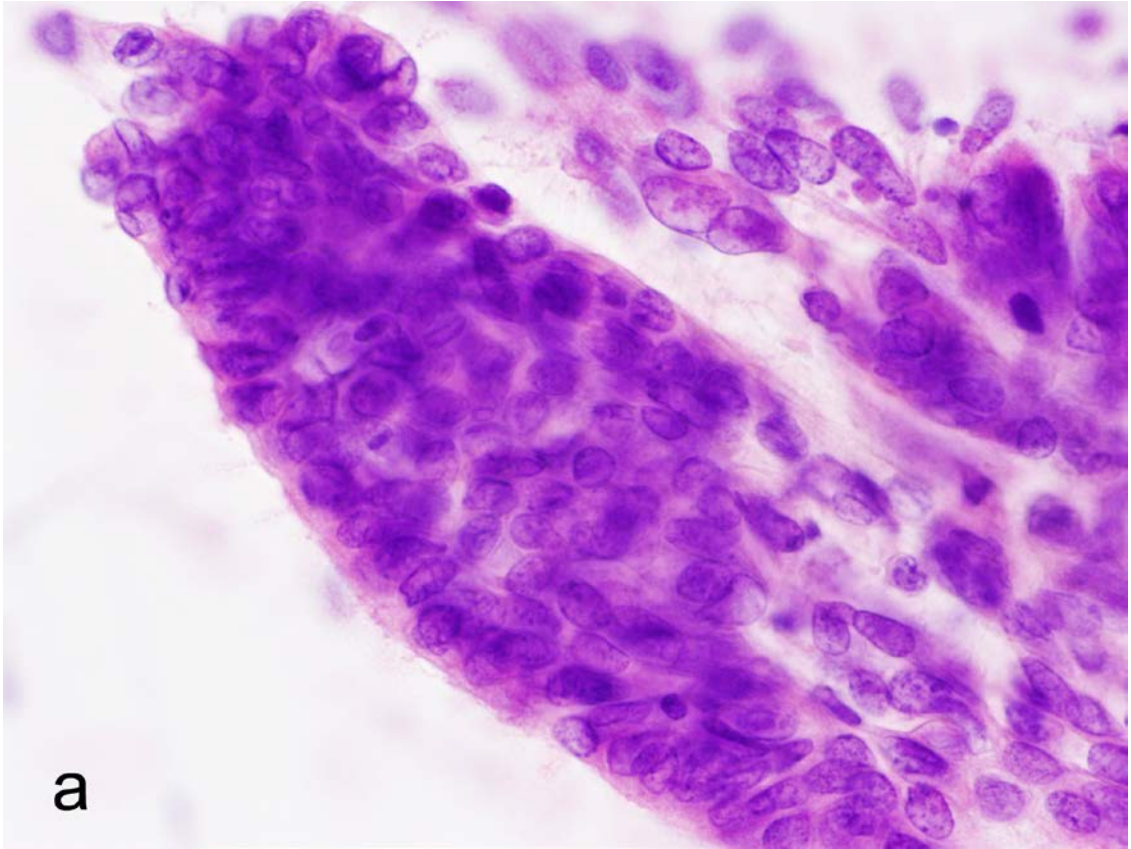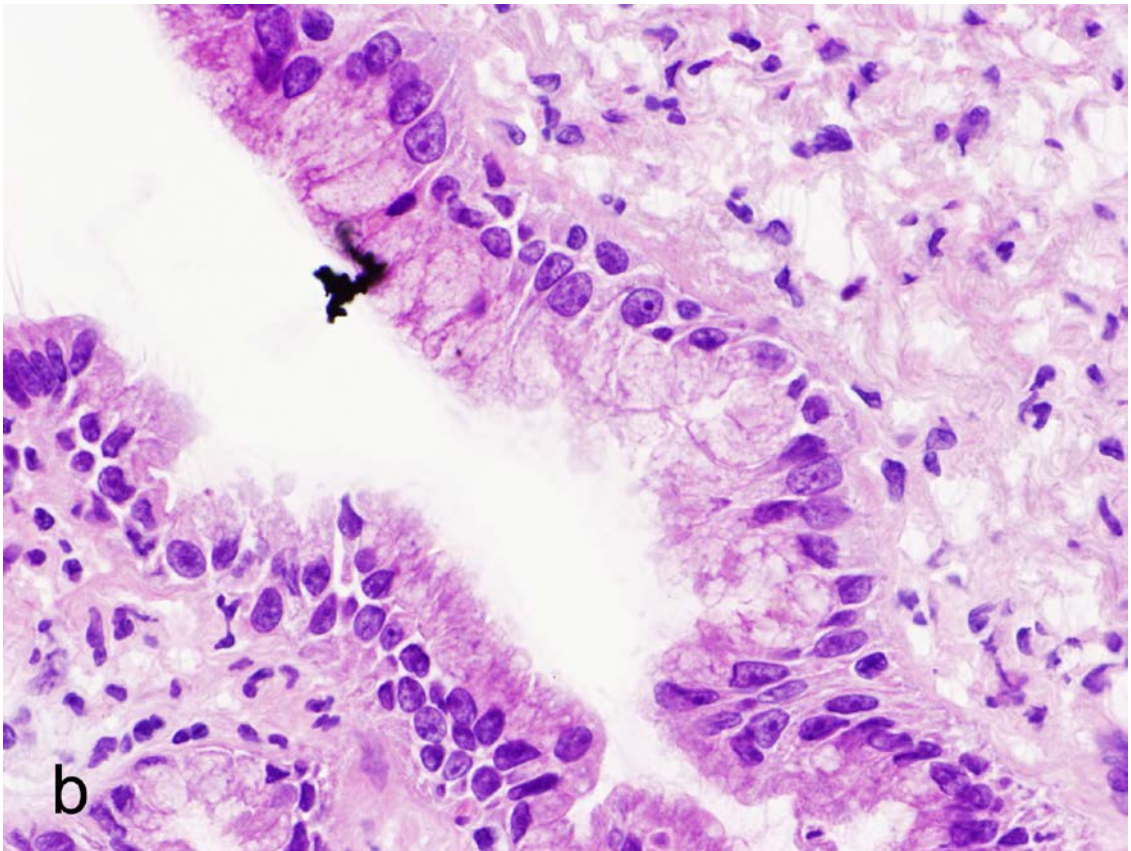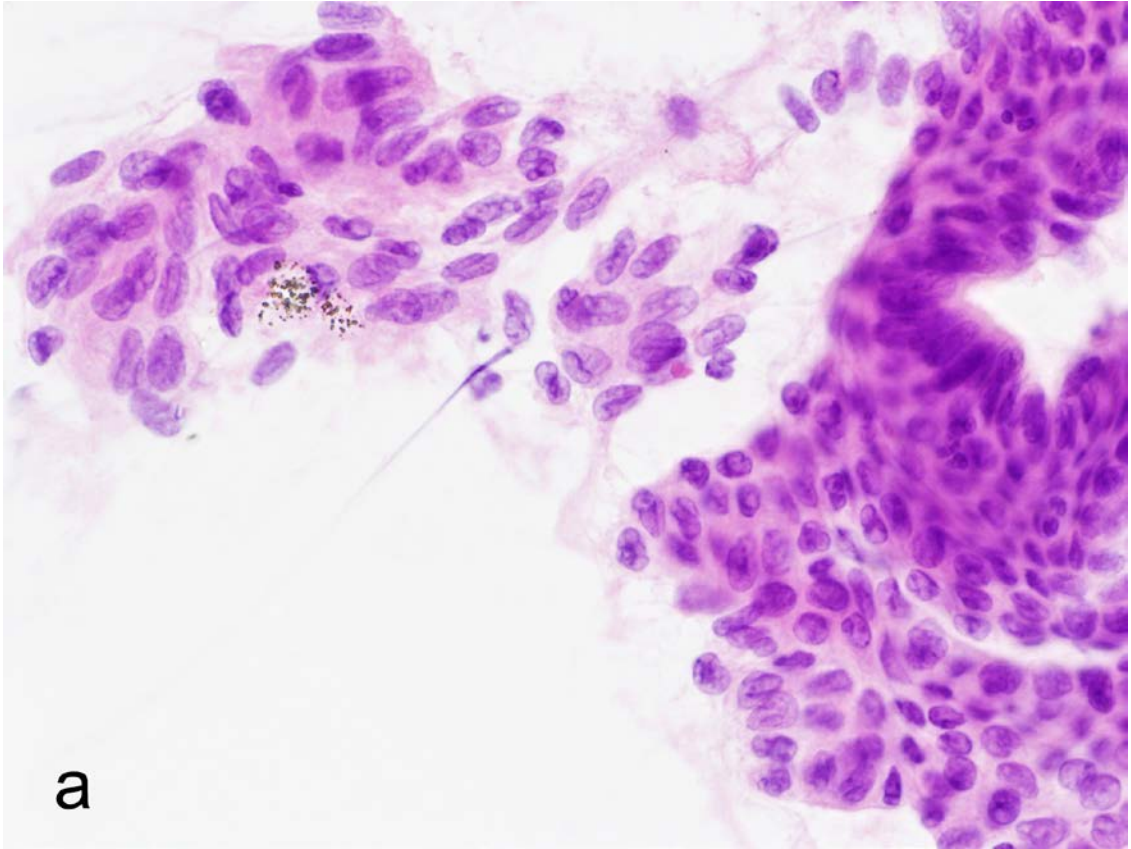
Fig. 3. False positive due to contamination by intraoperative cytology (IC) of pancreatic margin.

(a) In IC, atypical cells showing increased nuclear chromatin, nuclear irregularities, enlarged nuclei and nucleoli, high nuclear/cytoplasmic (N/C) ratio and anisonucleosis appeared in overlapping clusters with irregular arrangement. This was deemed to be adenocarcinoma, and the margin was diagnosed as positive (hematoxylin and eosin [H&E]-stained imprint cytology, original magnification ×400).

(b) In frozen section (FS) diagnosis, fragmented atypical epithelium with nuclear irregularities was observed. The margin was diagnosed as negative based on the absence of marked atypia sufficient for adenocarcinoma and the relationship with the surrounding tissue (H&E-stained frozen sections, original magnification ×200).
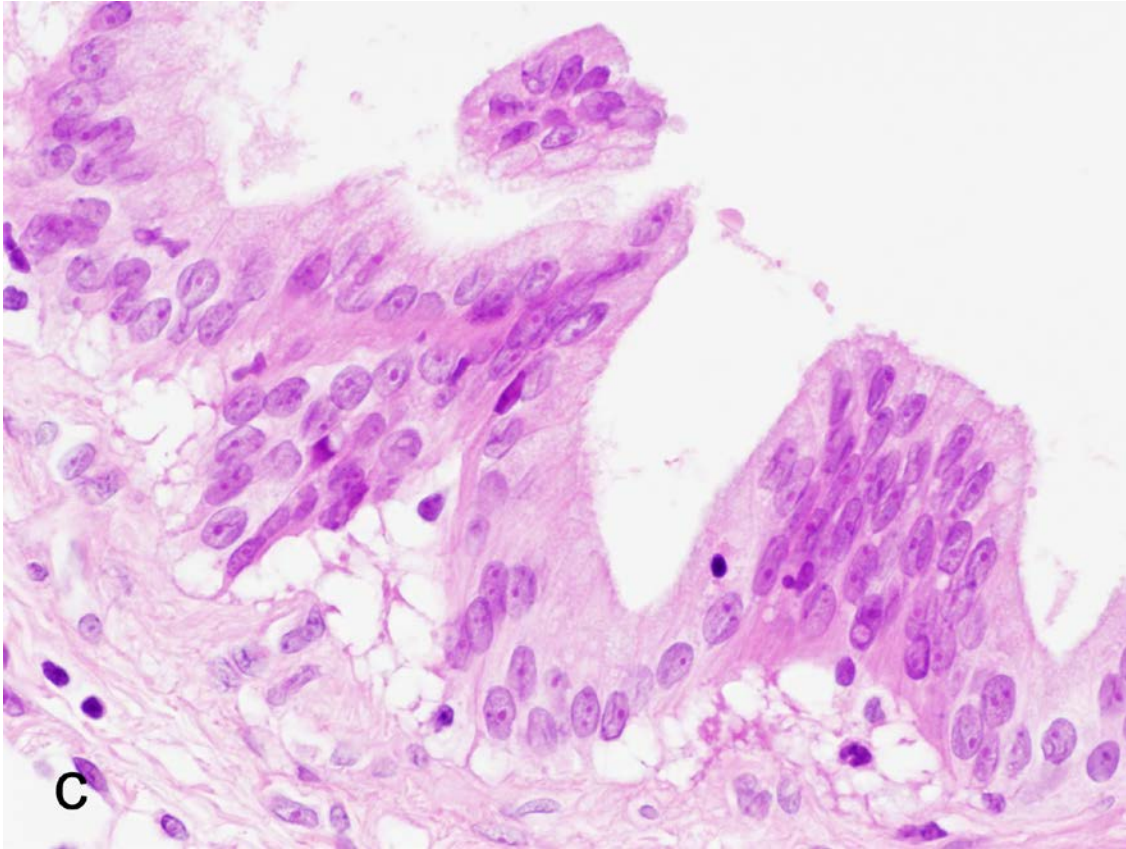
Fig. 4. False positive by intraoperative cytology (IC) of pancreatic margin.

(a) In IC, atypical cells showing marked nuclear membrane irregularities, enlarged nuclei and nucleoli and high nuclear/cytoplasmic (N/C) ratio appeared in slightly overlapping clusters. This was deemed to be a well differentiated adenocarcinoma, and the margin was diagnosed as positive (hematoxylin and eosin [H&E]-stained imprint cytology, original magnification ×400).

(b) In frozen section (FS) diagnosis, atypical cells showing nuclear irregularities and enlarged nuclei were observed in pancreatic duct epithelium. This was not deemed to be sufficient atypia for adenocarcinoma, and the margin was diagnosed as negative (H&E-stained frozen sections, original magnification ×400).

(c) In final histology, the duct was dilated and atypical epithelium presenting papillary structures was seen. The lesion was deemed to correspond to pancreatic intraepithelial neoplasia (PanIN)-2, and the margin was diagnosed as negative (H&E-stained permanent sections, original magnification ×400).