Comparison between the Location and the Histomorphological/Immunohistochemical Characteristics of Non-Invasive Neoplasms of the Ampulla of Vater

Yoshiro Yamamoto, M.D.[a, b], Tetsuo Nemoto, M.D., Ph.D.[a,*], Yoichiro Okubo, M.D., Ph.D.[a], Yasuhiro Nihonyanagi, M.D.[a], Takao Ishiwatari, M.D.[a], Kensuke Takuma, M.D., Ph.D.[b], Naobumi Tochigi, M.D., Ph.D.[a], Naoki Okano, M.D., Ph.D.[b], Megumi Wakayama, M.D., Ph.D.[a], Yoshinori Igarashi, M.D., Ph.D.[b], and Kazutoshi Shibuya, M.D., Ph.D.[a]

[a]Department of Surgical Pathology, Toho University School of Medicine, Ota-ku, Tokyo 143-8541, Japan

[b]Division of Gastroenterology and Hepatology, Department of Internal Medicine, Toho University Omori Medical Center, Ota-ku, Tokyo 143-8541, Japan.

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*Correspondence: Tetsuo Nemoto, Department of Surgical Pathology, Toho University School of Medicine, 6-11-1, Omori-Nishi, Ota-ku, Tokyo 143-8541, Japan. Tel: +81-3-3762-4151, Fax: +81-3-3767-1567, E-mail: tetsuo.nemoto@med.toho-u.ac.jp

Non-invasive ampullary neoplasm

[Abstract]

In order to determine useful factors when selecting an appropriate procedure for non-invasive ampullary neoplasia, we investigated the relationship between the location and the histomorphological/immunohistochemical characteristics of 56 non-invasive ampullary neoplasms obtained by endoscopic papillectomy (EP).

All subjects were classified according to histomorphology and location of neoplasms, and we evaluated the characteristics of each classified group using complemental immunohistochemical procedures. The CK20-positive rates of each location type were also evaluated.

Subjects presented with 52 intestinal-type adenomas (low:high grade, 32:20) and 4 non-invasive pancreatobiliary papillary neoplasms (low:high grade, 1:3). Twenty-seven periampullary (peri-AMP)-type tumors and 23 extended-type tumors comprised the intestinal type, and the intra-ampullary (intra-AMP)-type was composed of four pancreatobiliary and two intestinal histomorphological types. The CK20-positive rates of these three location types differed significantly (peri-AMP type: 50.6 ± 21.0%; extended type: 35.4 ± 18.6%; intra-AMP type: 6.9 ± 6.3%). The CK20-positive rate for intestinal-type tumors of the intra-AMP location type was lower than that of the peri-AMP location type. Intestinal-type tumors without CDX2 expression included extended and intra-AMP types, which are tumors that may show positive vertical margins when EP is performed.

Non-invasive ampullary neoplasm

In this study, we found that an understanding of pancreatobiliary-type histology is an important aspect for the investigation of tumors involving the common channel of the ampulla. Furthermore, immunostaining of CDX2 and CK20 provides beneficial information if considering whether to perform an EP.

Non-invasive ampullary neoplasm

[1. Introduction]

Since endoscopic papillectomy (EP) has become a common procedure involving minimally invasive treatment of epithelial neoplasia in the ampulla of Vater, pathologists have evaluated the early stages of the disease. Determinations concerning the location of the disease and whether it involves the common channel of the ampulla should be important when selecting an appropriate management procedure, particularly in cases showing a significant burden for a patient when deciding whether to have major surgery because of the extensive invasiveness of the operation.

The complicated anatomical structure of the ampulla of Vater (Figure 1A) consists of the duodenum (ampullo-duodenum), a common channel, the distal inferior bile duct (ampullo-biliary duct), and the distal main pancreatic duct (ampullo-pancreatic duct) [1]. Histologically, the ampullo-duodenum is covered by a small intestinal epithelium, and the common channel, the ampullo-biliary duct, and the ampullo-pancreatic duct are covered by pancreatobiliary duct epithelium. It has been largely accepted that two types of tumors, the intestinal type (IT) and pancreatobiliary type (PBT), are derived from their epithelial counterparts [2,3].

Regarding the tumor location, ampullary neoplasms have been classified on the basis of the following three distinct growth patterns: the periampullary type, intra-ampullary type, and the mixed type [4-6].

The tumor location and histomorphological types are thought to be closely associated; however, there have been some discrepant reports, such as PBT tumors that have arisen at the ampullo-duodenum, which should comprise intestinal epithelium [7,8]. This controversy might be largely due to the paucity of reports showing non-invasive neoplasms that have arisen at the expected area, and the neoplasia might be regarded as a lesion retaining the original characteristics of an early stage, which is a hypothesis that has not been sufficiently examined. In order to provide insights into the selection of appropriate procedures for the treatment of non-invasive ampullary neoplasms, we evaluated and examined specimens obtained by EP.

[2. Materials and Methods]

2.1. Patients

Fifty-eight endoscopically resected cases of adenomas or intraepithelial neoplasia of the ampulla of Vater were selected from the records of tumors of the ampulla of Vater at the Toho University Omori Medical Center, Tokyo, Japan, between October 2002 and December 2012. Two cases were excluded in this study because the size of the tumor was very small (< 1 mm) or the tumor was fragmented by artifacts. Cases included 27 men and 29 women with tumors, and the average age of patients was 68.1 years (range: 31–90 years).

2.2. Histomorphology

Non-invasive ampullary neoplasm

All specimens were fixed with 10% buffered formalin and embedded in paraffin using a standard procedure. For each specimen, 3 μm-thick sections were prepared and used for hematoxylin and eosin (HE) staining and immunohistochemical staining.

All tumors were classified histologically as premalignant lesions using only the HE-stained specimens according to criteria published by the World Health Organization (WHO) classification of tumors of the ampullary region [9]. The tumors were classified into the following 4 groups: “Intestinal type adenoma”, “Noninvasive pancreatobiliary papillary neoplasm with low-grade dysplasia (low-grade intraepithelial neoplasia)”, “Noninvasive pancreatobiliary papillary neoplasm with high-grade dysplasia (high-grade intraepithelial neoplasia)”, and “Flat intraepithelial neoplasia, high grade”.

We regarded the intestinal-type adenoma as the IT tumor, and the non-invasive pancreatobiliary papillary neoplasm as the PBT tumor. Tumors with a mixed pattern were classified according to their predominant component as belonging to the IT or PBT group.

Additionally, tumors were classified as low grade or high grade according to criteria published by the WHO [9].

2.3. Location of the tumor

Non-invasive ampullary neoplasms were classified into three distinct subtypes based on their location (Fig. 1B and C). The following three definitions were employed: 1) the periampullary (peri-AMP) type, in which 75% or more of the tumor exists in the duodenum;

Non-invasive ampullary neoplasm

2) the intra-ampullary (intra-AMP) type, in which 75% or more of the tumor exists within the ampulla (intra-ampulla); and 3) the extended type, in which the tumor straddles the ampullo-duodenum and the common channel, and does not satisfy definitions for either the peri-AMP or intra-AMP type. The boundary of the ampullo-duodenum and common channel mucosa is an extension line of the sphincter of Oddi.

2.4. Immunohistochemistry

Immunohistochemical staining of the 3-μm sections was performed using a Histofine Simple Stain kit (Nichirei Corporation, Tokyo, Japan). The primary antibodies and their working dilutions are shown in Table 1. For the retrieval of CD10, CDX2, MUC1, MUC2, MUC5AC and MUC6, heat treatment in a warm bath at 98°C for 40 minutes in Histofine antigen retrieval solution (Nichirei Corporation) diluted 10 times (pH 9.0) was performed. CK7, CK18, CK19 and CK20 were processed with 0.1% trypsin. Endogenous peroxidase in the section was blocked by incubating sections in 0.1% methanol containing hydroperoxidase, 3.3’-diaminobenzidin was used as the final chromogen, and the nuclei were counterstained with a hematoxylin solution.

Cases showing greater than 10% tumor-cell positivity were regarded as positive, in accordance with Zhou [10].

2.5. Measurement of the CK20-positive rate

A digital image of the entire tumor on a slide with CK20 staining was generated using a

10X objective. With the aid of image analysis software (Image J 1.36b, National Institutes of Health, Bethesda, Maryland, USA), all of these images were measured by recording (A) the area of the whole tumor including the lumina, (B) the area of the lumina, and (C) the area containing CK20-positive cells. The CK20-positive rate was calculated as (C) divided by the value of (B) from (A).

2.6. Statistical analysis

Statistical analyses were performed using the Mann-Whitney *U* test, the chi-square test, ANOVA (Bonferroni), and the paired *t*-test as provided by IBM SPSS Statistics software (version 20, Armonk, New York, USA). All tests were 2-sided, and statistical significance was set at $P < 0.05$.

2.7. Ethics

This study was approved by the Ethics Committee of the Toho University Omori Medical Center, Tokyo, Japan (No. 24-162).

[3. Results]

3.1. Tumor size and histomorphological classification

The mean size of tumors was 17.7 mm and ranged from 9.0 to 37.0 mm. The histological examination revealed 52 IT adenomas comprising 32 low-grade (Fig. 2A and B) and 20 high-grade cases (Fig. 2C). The remaining 4 cases involved non-invasive pancreatobiliary

papillary neoplasms, comprising a low-grade (Fig. 2D and E) and 3 high-grade (Fig 2F) cases. No cases were found with flat intraepithelial neoplasia, signet ring cell carcinoma, or mucinous carcinoma. The mean size of low-grade and high-grade tumors was 16.6 ± 5.97 and 19.2 ± 7.11 mm, respectively. There were no significant differences between histological grade and tumor size (Mann-Whitney *U* test, $P = 0.180$, Fig. 3).

3.2. Tumor location and histomorphological or clinicopathological / immunohistochemical characteristics

Table 2 shows a comparison of tumor location and various histomorphological or clinicopathological and immunohistochemical characteristics. The average tumor size of the peri-AMP, extended, and intra-AMP types was 18.7 ± 6.1, 16.1 ± 6.5, and 18.8 ± 6.5 (mm, mean ± S.D.), respectively. All location types of the 27 peri-AMP and 23 extended cases were consistent with IT. Among the 6 cases of the intra-AMP type, 4 (66.7%) involved PBT tumors and 2 (33.3%) involved IT tumors. Eleven of the 27 peri-AMP-type cases (40.7%), 8 of the 23 extended-type cases (34.8%), and 4 of the 6 intra-AMP-type cases (66.7%) showed high-grade dysplasia. An extended-type case and an intra-AMP-type case had an additional surgical excision among 11 cases in which tumor extension involved vertical margins obtained by EP. None of the peri-AMP-type cases involved these margins (compared with the extended type and intra-AMP type, chi-square test, $P < 0.001$ and $P < 0.001$, respectively). Patients in an extended-type case and an intra-AMP-type case died due

to recurrence of the tumor without additional surgery because of the high risk largely attributed to the advanced age of each patient. Records did not show any patient who had additional surgeries in response to complications due to EP.

Most of the peri-AMP- and extended-type tumors showed positive reactivity for CK20, CDX2, and MUC2, showing rates of 100%, 100%, and 96.3% in the 27 cases of peri-AMP-type tumors, and 95.7%, 82.6%, and 87.0% in the 23 cases of extended-type tumors, respectively. CK20-positive cases of the intra-AMP type were significantly fewer (3 in 6 cases) compared with those of the peri-AMP and extended types (chi-square test, P = 0.002 and 0.026, respectively). All of the IT tumors that were CDX2-negative were either extended or intra-AMP types.

3.3. Correlation analysis of histomorphological classification and immunohistochemical characteristics

Table 3 shows a comparison of the histomorphological classification and immunohistochemical characteristics. CK20, CD10, CDX2, and MUC2 were expressed with a greater positive incidence in histomorphological IT tumors (51 of 52 (98.1%), 41 of 52 (78.8%), 48 of 52 (92.3%), and 48 of 52 (92.3%), respectively). CK20, CD10, and CDX2 showed a significant difference between IT and PBT tumors (chi-square test, $P < 0.001$, 0.004 and < 0.001, respectively). CK7, CK19, MUC5AC, and MUC6 were expressed more positively in histomorphological PBT tumors (3 of 4 (75.0%), 4 of 4 (100%), 4 of 4 (100%),

and 3 of 4 (75.0%), respectively), whereas there was no significant difference between IT and PBT tumors.

3.4. Correlation analysis of CK20-positive rate and tumor location.

We examined the CK20-positive rate of the three tumor location types and obtained values of 50.6 ± 21.0% for the peri-AMP type, 35.4 ± 18.6% for the extended type, and 6.9 ± 6.3% for the intra-AMP type. Significant differences were found between the peri-AMP and extended types (Bonferroni, $P = 0.025$), the peri-AMP and intra-AMP types (Bonferroni, $P < 0.001$), and the extended and intra-AMP types (Bonferroni, $P = 0.007$) (Fig. 4).

CK20-positive rates of the ampullo-duodenum and ampullo-common channel in extended-type tumors were 39.3 ± 21.4% and 37.5 ± 29.8%, respectively. There was no significant difference in rate between these two sites (paired *t*-test, $P = 0.672$; Fig. 5).

[4. Discussion]

To elucidate the relationship between location and phenotypic characteristics of epithelial neoplasia developed in the ampullary region, detailed histological and immunohistochemical examinations were carried out using tumors from 56 specimens obtained by EP, all of which comprised epithelial neoplasia in the early clinical stage.

The most important finding of our immunohistological analysis is that a significant difference was found in CK20-positive rate between peri-AMP, extended, and intra-AMP

types. In particular, the fact that the CK20-positive rates of 2 cases of IT classified as the intra-AMP type were lower than those of the IT type in other locations suggests they might be isolated from IT tumors developed at the ampullo-duodenal region. This may be explained by the hypothesis that IT tumor that developed from the common channel might have originated from epithelial cells with intestinal metaplasia or pluripotential cells resident in this region. However, the lower positive rate recorded in our study does not explain that the tumor originated from aberrant duodenal epithelium, since tumor arising from mature duodenal epithelial cells must exhibit a higher CK20-positive rate. This explanation may be partially supported by the report of Matsubayashi et al. that the K-*ras* mutation status in ampullary neoplasms is associated with tumors within the mucus metaplastic change [7]. However, further molecular analysis might reveal the relationship between oncogenic mutation and tumor characteristics, such as the difference of CK20 expressions at each location.

Additionally, although a significant difference in CK20-positive rate was also observed between extended and peri-AMP tumors, our detailed observations did not reveal any significant differences in morphology between these 2 types. To clarify whether the CK20-positive rate is affected by the regional microenvironment, we compared the density of CK20-positive cells in the area of each extended-type tumor between areas involving the ampullo-duodenal region and common channel. No significant difference was found for the

distribution of CK20-positive cells, which implies that CK20-positive cells are distributed uniformly in the tumors. Therefore, the expression of CK20 in tumor cells may not be affected by the difference of microenvironments where tumor cells proliferate, and this suggests that the CK20-positive rate is determined by a characteristic of the origin of the tumor.

Another important issue that emerges from the present study is that IT tumors without CDX2 expression were found as tumors with a common channel involvement and were classified as extended or intra-AMP types, whereas a large body of IT tumors expressed CDX2, which has been generally accepted as one of the markers of intestinal differentiation [11,12].

Our results concerning the relevance of histomorphological classification and immunohistochemical characteristics agreed with those of previous reports investigating invasive carcinomas and the usefulness of CK20 for the identification of IT tumors [10,13,14,15]. Our findings confirm the high incidence (98.1%) of CK20 expression in IT tumors. However, more than half of the tumors were positive for CK7, MUC5AC, and MUC6, which are ubiquitously expressed in epithelia of bile or the pancreatic tract, gastric foveolar epithelium, and the proper gastric and/or Brunner's gland, respectively. Moreover, the fact that 1 in 4 PBT tumors focally showed positive reactivity for CK20 might also suggest the phenotypic heterogeneity of epithelial neoplasia in this region. This has usually

been discussed in relation to “hybrid mucosa” (intermingling of goblet cells and foveolar-like epithelial cells) [16]. The phenotypic heterogeneity demonstrated in our subjects may be because the tumors originated from stem cells distributed over the small intestinal mucosa including the ampulla of Vater. It is known that stem cells have the potency to differentiate into both intestinal and pancreatobiliary cells that can be identified by expression of the Sox9 gene, and give rise to the biliary tree [17,18]. On the other hand, tumors histomorphologically classified as PBT were merely found as the intra-AMP type. This suggests that it is not likely that PBT tumors originated from epithelium of the ampullo-duodenum, although “distended glands” have been proposed as the origin of PBT tumors arising in this region by Suda et al. [8,19]. The presence of “distended glands” may be significant, but our results suggest that epithelia of the gland seldom induce PBT tumors.

Although the present study found no significant relation between histological grade and the size of tumors, no peri-AMP-type tumor involved the vertical margins of EP, which included part of the common channel. This suggests that a peri-AMP-type tumor can be regarded as a good indication for the EP procedure, and it is therefore important to distinguish peri-AMP-type tumors from extended and intra-AMP types.

Our findings therefore demonstrate that IT histology and a high CK20-positive rate can be significant factors in determining whether a tumor is not extending the common channel, which may be shown in a forceps biopsy specimen obtained via an endoscope. On the other

hand, a tumor that is either PBT or IT without CDX2 expression suggests involvement with the common channel, whereas a tumor arising in this region essentially has heterogeneous phenotypic characteristics.

Non-invasive ampullary neoplasm

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Non-invasive ampullary neoplasm

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[Figure legends]

Fig. 1 Structure of the ampulla of Vater and classification of tumor locations.

A, Representation of the ampulla of Vater, which is defined as the region surrounded by the dotted line. Ac, ampullo-common channel; Ab, ampullo-biliary duct; Ap, ampullo-pancreatic duct; Ad, ampullo-duodenum; Ph, head of pancreas; Bi, inferior bile duct; D, duodenum. Fig. 1A is extracted from Classification of Biliary Tract Carcinoma, 2nd edition[1], with permission.

B, The boundary of the ampullo-duodenum and the common channel mucosa. An extended line of the sphincter of Oddi represents the boundary. The sphincter of Oddi and the duodenal muscularis mucosae are positive for smooth muscle actin (SMA) immunostaining. Immunostaining using anti-SMA antibody as primary antibody with hematoxylin counterstaining. Original magnification: ×40.

C, Tumor classification according to location. Peri-AMP type: 75% or more of the tumor exists in the duodenum. Intra-ampullary (intra-AMP) type: 75% or more of the tumor exists within the ampulla (intra-ampulla). Extended type: the tumor straddles the duodenum and the intra-ampulla, and does not satisfy definitions for either the peri-AMP or intra-AMP type.

Fig. 2 Histology of tumors of the ampulla of Vater. A, B and C: intestinal-type tumors. The tumor cells have oval and hyperchromatic nuclei with pseudostratification. D, E and F:

pancreatobiliary-type tumors. The epithelial lining consists of cuboidal cells with round nuclei arranged predominantly in a single layer. B and E: histologically low grade. C and F: histologically high grade. Hematoxylin and eosin (A, B, C, D, E and F). The original magnification: ×20 in A and D, and ×400 in B, C, E, and F.

Fig. 3 Comparison of size and location between high and low grade tumors. The mean sizes of low-grade and high-grade tumors were 16.6 ± 5.97 and 19.2 ± 7.11 (mm, mean ± S.D.), respectively. There were no significant differences between histological grade and tumor size (Mann-Whitney *U* test, $P = 0.180$). Bar indicates the mean size.

Fig. 4 Tumor location and the CK20-positive rate. Peri-AMP type: 50.6 ± 21.0%; extended type: 35.4 ± 18.6%; intra-AMP type: 6.9 ± 6.3% (mean ± S.D.) (Bonferroni: peri-AMP vs. extended, $P = 0.025$; peri-AMP vs. intra-AMP, $P < 0.001$; extended vs. intra-AMP, $P = 0.007$). Bar indicates the S.D.

Fig. 5 Comparison of CK20-positive rates between the ampullo-duodenum and ampullo-common channel in 23 extended-type tumors. There was no significant difference between the two groups (paired *t*-test, $P = 0.672$). Bar indicates the S.D.

Non-invasive ampullary neoplasm

Table 1 The list of immunohistochemical antibodies

Antibodies	Clone	Dilution	Source
CK7	OV-TL-12/30	1:200	DAKO Cytomation, Copenhagen, Denmark
CK18	DC10	1:50	DAKO Cytomation, Copenhagen, Denmark
CK19	RCK108	1:100	DAKO Cytomation, Copenhagen, Denmark
CK20	Ks20.8	1:100	DAKO Cytomation, Copenhagen, Denmark
CD10	56C6	1:80	Leica Microsystems, Newcastle upon Tyne, UK
CDX2	AMT28	1:50	Leica Microsystems, Newcastle upon Tyne, UK
MUC1	Ma695	1:100	Leica Microsystems, Newcastle upon Tyne, UK
MUC2	Ccp58	1:100	Leica Microsystems, Newcastle upon Tyne, UK
MUC5AC	CLH2	1:100	Leica Microsystems, Newcastle upon Tyne, UK
MUC6	CLH5	1:100	Leica Microsystems, Newcastle upon Tyne, UK

Table 2 Relationship between tumor location and various clinicopathological characteristics

	Peri-AMP	Extended	Intra-AMP	Statistical analysis[a]
Tumor size (mm), (mean ± S.D.)	18.7 ± 6.1	16.1 ± 6.5	18.8 ± 6.5	
Histological grade				
Low grade	16 (59.3%)	15 (65.2%)	2 (33.3%)	
High grade	11 (40.7%)	8 (34.8%)	4 (66.7%)	
Vertical margin				Peri-AMP vs. Extended; $P < 0.001$, Peri-AMP vs. Intra-AMP; $P < 0.001$
Negative	27 (100%)	15 (65.2%)	3 (50.0%)	
Positive	0	8 (34.8%)	3 (50.0%)	
Histomorphology				Peri-AMP vs. Intra-AMP; $P < 0.001$, Extended vs. Intra-AMP; $P < 0.001$
Intestinal type	27 (100%)	23 (100%)	2 (33.3%)	
Pancreatobiliary type	0	0	4 (66.7%)	
Immunohistochemistry				
CK7	17 (63.0%)	18 (78.3%)	3 (50.0%)	
CK18	21 (77.8%)	15 (65.2%)	1 (16.7%)	Peri-AMP vs. Intra-AMP; $P = 0.017$
CK19	22 (81.5%)	19 (82.6%)	4 (66.7%)	
CK20	27 (100%)	22 (95.7%)	3 (50.0%)	Peri-AMP vs. Intra-AMP; $P = 0.002$, Extended vs. Intra-AMP; $P = 0.026$
CD10	22 (81.5%)	18 (78.3%)	1 (16.7%)	Peri-AMP vs. Intra-AMP; $P = 0.008$, Extended vs. Intra-AMP; $P = 0.019$
CDX2	27 (100%)	19 (82.6%)	2 (33.3%)	Peri-AMP vs. Intra-AMP; $P < 0.001$
MUC1	0	0	1 (16.7%)	
MUC2	26 (96.3%)	20 (87.0%)	4 (66.7%)	
MUC5AC	14 (51.9%)	11 (47.8%)	5 (83.3%)	
MUC6	16 (59.3%)	12 (52.2%)	4 (66.7%)	

Abbreviation: AMP, ampullary

a) Statistical analysis was based on ANOVA (Bonferroni) for continuous variables and chi-square test for categorical variables.

Table 3 Relationship between histomorphological classification and immunohistochemical expression (chi-square test)

	Intestinal type (n = 52)	Pancreatobiliary type (n = 4)	*P* value
CK7	35 (67.3%)	3 (75.0%)	0.811
CK18	36 (69.2%)	1 (25.0%)	0.210
CK19	40 (76.9%)	4 (100%)	0.651
CK20	51 (98.1%)	1 (25.0%)	< 0.001
CD10	41 (78.8%)	0	0.004
CDX2	48 (92.3%)	0	< 0.001
MUC1	0	1 (25.0%)	0.093
MUC2	48 (92.3%)	2 (50.0%)	0.072
MUC5AC	26 (50.0%)	4 (100%)	0.158
MUC6	29 (55.8%)	3 (75.0%)	0.822

Non-invasive ampullary neoplasm

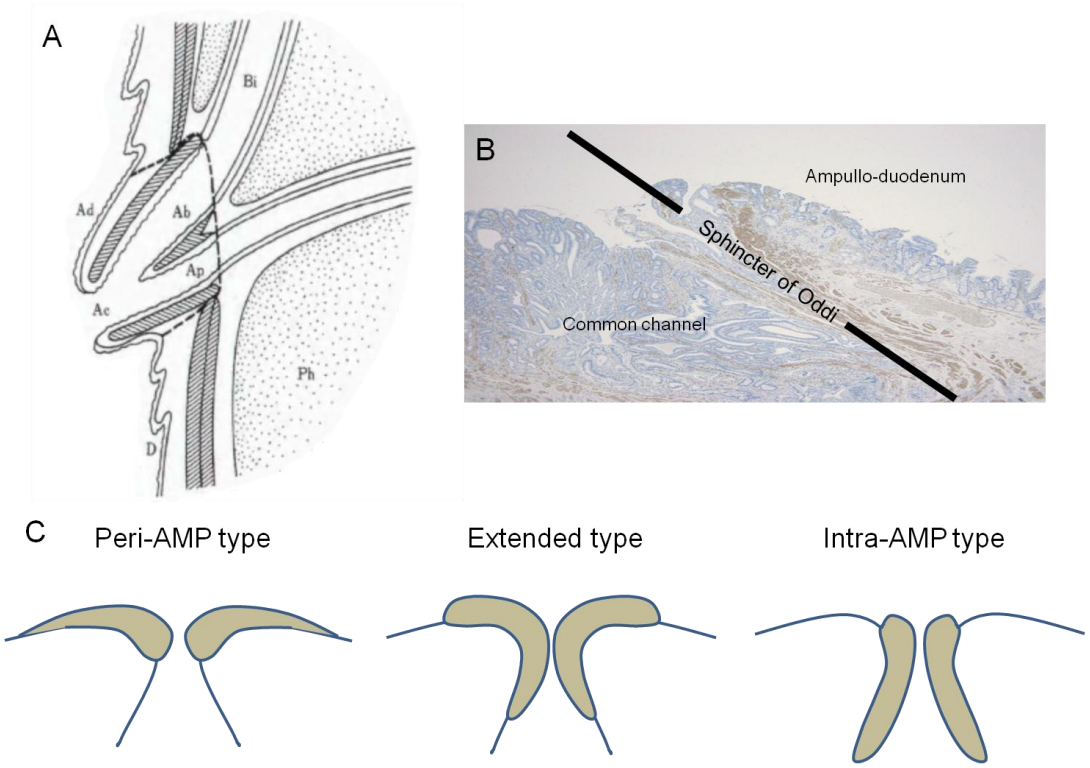

Fig 1

Non-invasive ampullary neoplasm

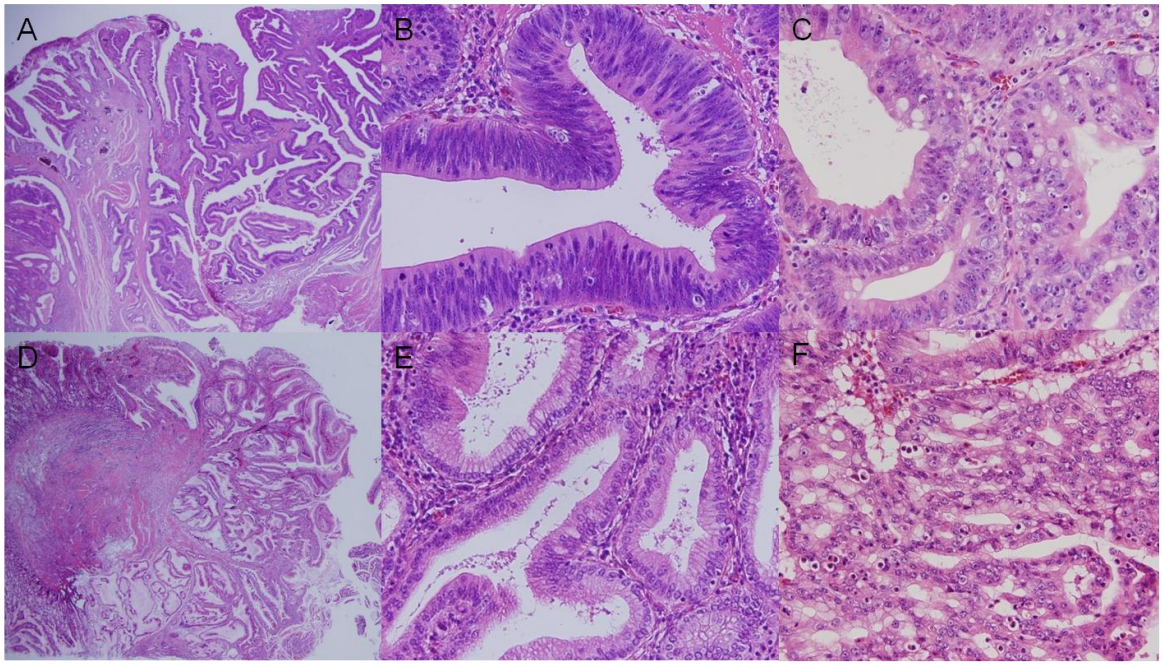

Fig 2

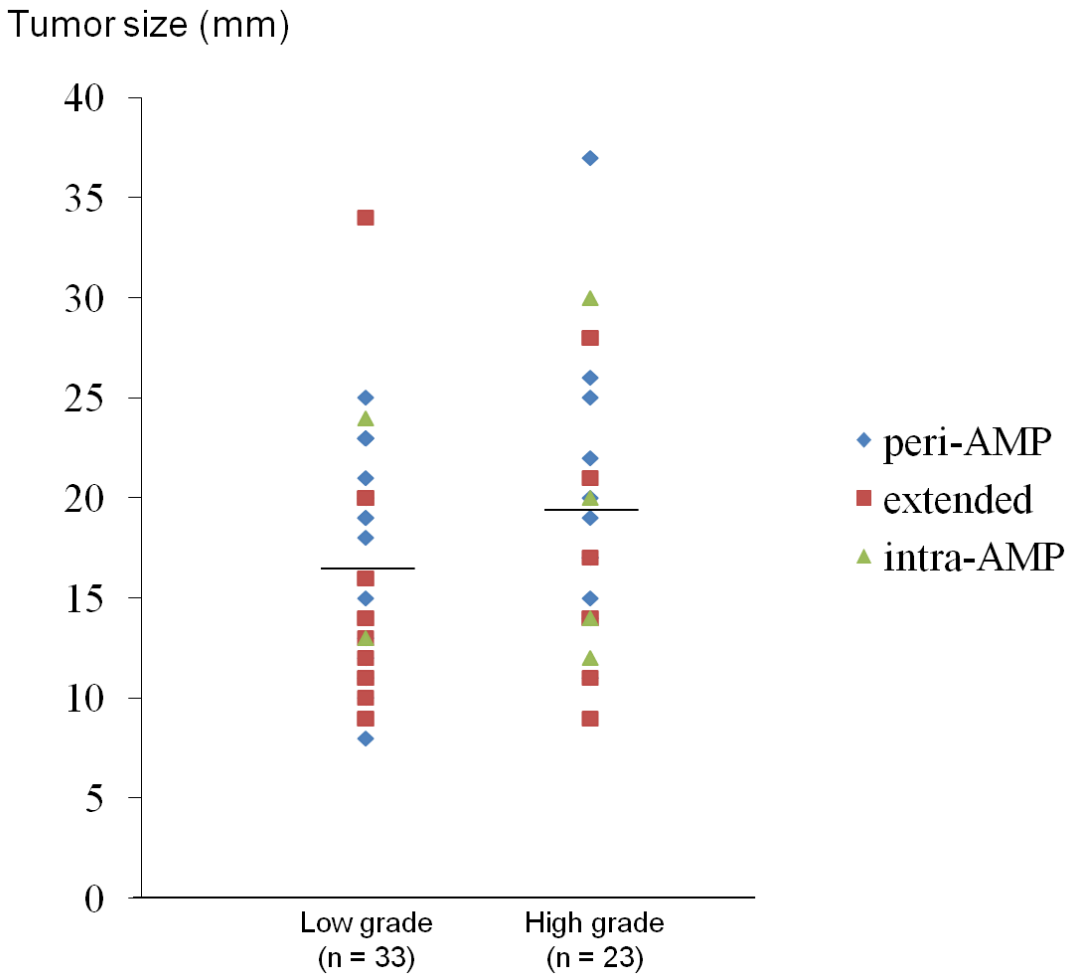
Tumor size (mm)
40
35
30
25
20
15
10
5
0
Low grade
(n = 33)
High grade
(n = 23)
peri-AMP
extended
intra-AMP

Fig 3

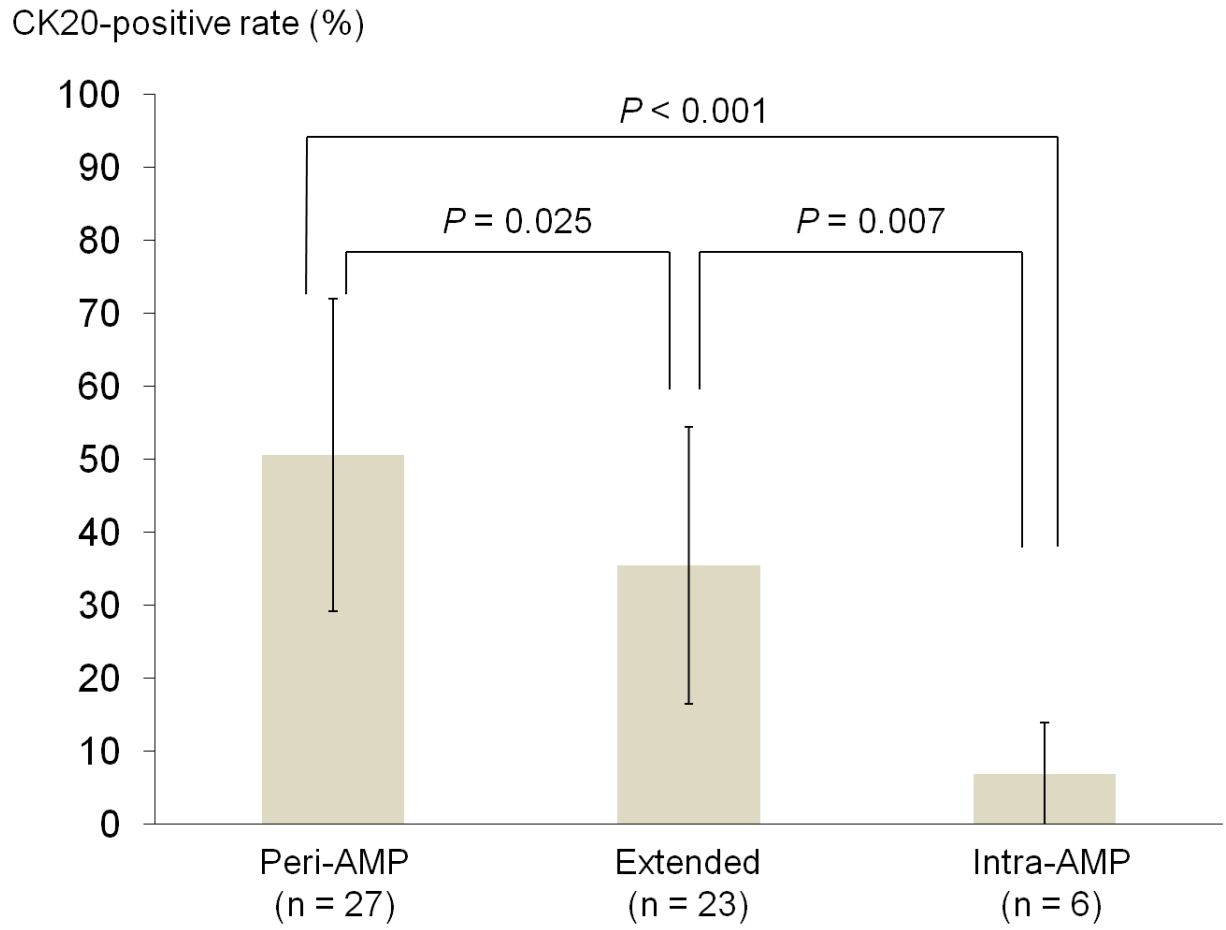
CK20-positive rate (%)
100
90
80
70
60
50
40
30
20
10
0
P < 0.001
P = 0.025
P = 0.007
Peri-AMP
(n = 27)
Extended
(n = 23)
Intra-AMP
(n = 6)

Fig 4

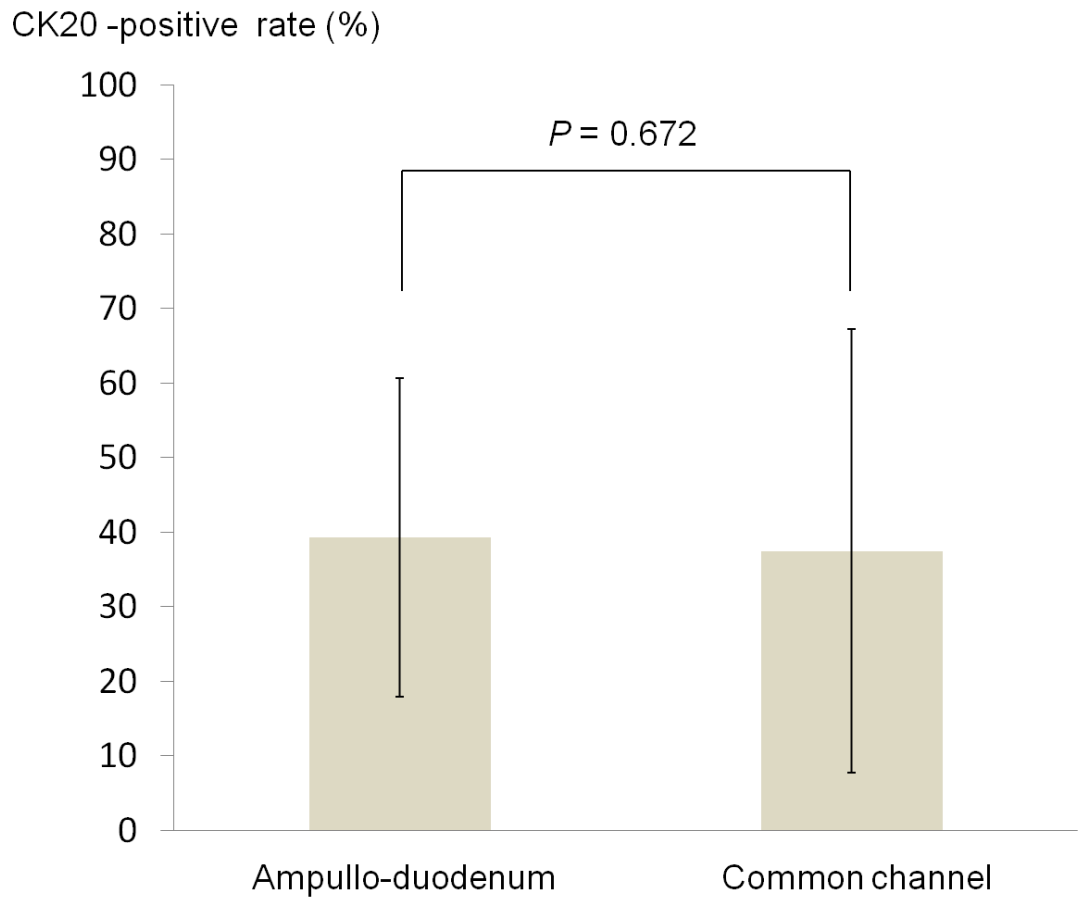
CK20 -positive rate (%)
100
90
80
70
60
50
40
30
20
10
0
P = 0.672
Ampullo-duodenum
Common channel

Fig 5