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河田, 純

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Research Article

Clinicopathologic Features and Genetic Alterations in Mixed-Type Ampullary Carcinoma

Jun Kawata[a,b], Yutaka Koga[c], Shoko Noguchi[a,b], Yuki Shimada[a,b], Yutaka Yamada[a,b], Takeo Yamamoto[a,b], Koji Shindo[b], Masafumi Nakamura[b], Yoshinao Oda[a,*]

[a] *Department of Anatomic Pathology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan;* [b] *Department of Surgery and Oncology, Graduate School of Medical Sciences, Kyusyu University, Fukuoka, Japan;* [c] *Department of Pathology, National Hospital Organization Kyushu Cancer Center, Fukuoka, Japan*

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ABSTRACT

Mixed-type ampullary carcinoma is a subtype that combines intestinal-type (I-type) and pancreatobiliary-type (PB-type) lesions, but few studies have examined its clinicopathologic features and genetic alterations. The differences in genetic alterations between mixed type and other subtypes, as well as the genetic differences between I-type and PB-type lesions in the mixed type, remain unclear. In this study, we compared the clinicopathologic features and prognosis of 110 ampullary carcinomas classified by hematoxylin and eosin and immunohistochemical staining as follows: 63 PB-type, 35 I-type, and 12 mixed-type carcinomas. A comparative analysis of genetic mutations by targeted sequencing of 24 genes was also performed in 3 I-type cases, 9 PB-type cases, and I and PB-type lesions of 6 mixed-type cases. The mixed subtype had a poorer prognosis than the other subtypes, and there was also a similar tendency in the adjuvant group ($n = 22$). A total of 49 genetic mutations were detected in all 18 lesions for which genetic alteration was analyzed. No genetic mutations specific to the mixed type were found, and it was not possible to determine genetically whether the mixed type had originally been I or PB type. However, 5 of 6 cases had mutations common to both I and PB-type lesions, and additional mutations were found only in either I or PB-type lesions. In support of this, the mixed type more frequently exhibited genetic heterogeneity intratumorally than the other subtypes. Mixed-type tumors are histologically, immunohistochemically, and genetically heterogeneous, and this heterogeneity is associated with poor prognosis and may affect treatment resistance.

Introduction

Ampullary carcinoma is a relatively rare disease. It may arise from a complex site where the pancreatic duct, bile duct, and duodenum meet, but its detailed carcinogenesis and pathogenesis remain unclear. Histologically, Kimura et al[1] first classified it into the intestinal type (I type) and pancreatobiliary type (PB type), and this classification has since become widespread. Recently, a mixed type with features of I and PB types has been recognized, and in 2010, the World Health Organization (WHO) revised the criteria for the pathologic diagnosis of ampullary carcinoma into 3 distinct histopathologic subtypes based on morphologic and immunohistochemical (IHC) characteristics.[2] Furthermore, the fifth edition of the *WHO Classification of Tumours of the Digestive System* also continues to define the mixed type as having ambiguous features that are difficult to classify definitively as the I or PB type, whereas the gastric type (G type) with gastric-like mucin is included in the PB type.[3] Several studies[1,4-11] have shown that the prognosis of the PB type is worse than that of the I type, and a recent study[12]

* Corresponding author.
E-mail address: oda.yoshinao.389@m.kyushu-u.ac.jp (Y. Oda).

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suggested that postoperative adjuvant chemotherapy is more effective in the PB type, but there is no consensus on whether the mixed type has a better or worse prognosis than I and PB types.[5,10,13-15] Against this background, there is an urgent need for an accurate classification of histologic subtypes to determine prognosis, select the most appropriate treatment option, and develop a chemotherapy regimen.

The relationship between histologic subtype and genetic mutations of ampullary carcinoma has also been assessed in recent years. In a study by Yachida et al,[16] the I type showed an increased incidence of mutations in *APC*, *TP53*, and *KRAS* similar to colorectal cancer, whereas the PB type showed an increased incidence of mutations in *KRAS*, *TP53*, and *SMAD4* similar to pancreatic cancer. This suggests that the I and PB types are genetically distinct. Other studies[17,18] have also reported similar results. However, a study[14] has examined the clinicopathologic and genetic alterations in mixed-type ampullary carcinoma, and to the best of our knowledge, no studies analyzing and comparing the genetic alterations of the 2 lesions (I and PB-type lesions) in the mixed type have been reported.

In this study, we determined the subtypes of ampullary carcinoma based on the morphologic features and IHC staining and compared the clinicopathologic and molecular features of each subtype. We characterized protein expression in the primary lesion and metastatic lymph nodes and further investigated the genetic alterations and genetic heterogeneity comparing the findings among the 2 lesions of mixed-type cases.

Materials and Methods

Clinicopathologic Evaluation

A total of 110 patients with ampullary carcinoma who underwent surgery at the Kyushu University Hospital and related hospitals between 2001 and 2020 were included in this study. Clinical information was obtained from clinical medical charts. The stage was determined in accordance with the Union for International Cancer Control guidelines. Clinicopathologic findings (age, sex, tumor size, differentiation, vascular invasion, lymphatic invasion, perineural invasion, and the Union for International Cancer Control TNM stage) were evaluated. The Ethics Committee of the Kyushu University (Institutional Review Board: 2020-633) approved this study.

Classification of Histologic Subtype

Hematoxylin and eosin (HE)–stained formalin-fixed and paraffin-embedded tissue sections were used to evaluate histopathologic features to classify the cases into different subtypes (I, PB, G, and mixed) based on their resemblance to pancreatic/biliary or colonic carcinoma in accordance with the criteria proposed by Kimura et al[1] and endorsed by Albores-Saveedra et al[19] for ampullary carcinomas (Fig. 1). The I type has features similar to those of colon carcinoma and consists of tall columnar tumor cells with hyperchromatic and elongated nuclei. It has eosinophilic cytoplasm and may contain goblet cells. It also has necrotic debris in the lumen and may be accompanied by inflammatory cells. However, the PB type has features similar to those of pancreatic/cholangiocarcinoma and consists of cuboidal tumor cells with a high nuclear-to-cytoplasmic ratio, vesicular chromatin, and irregularly round nuclei. It exhibits small ductal structures that are sparsely distributed and surrounded by abundant desmoplastic stroma. The G type, introduced in the fifth edition of the *WHO Classification of Tumours of the Digestive System*, consists of tumor cells resembling the gastric foveolar epithelium, with basally oriented nuclei and pale mucinous cytoplasm (Fig. 1). Here cases in which the I and PB components comprised >25% of the sample

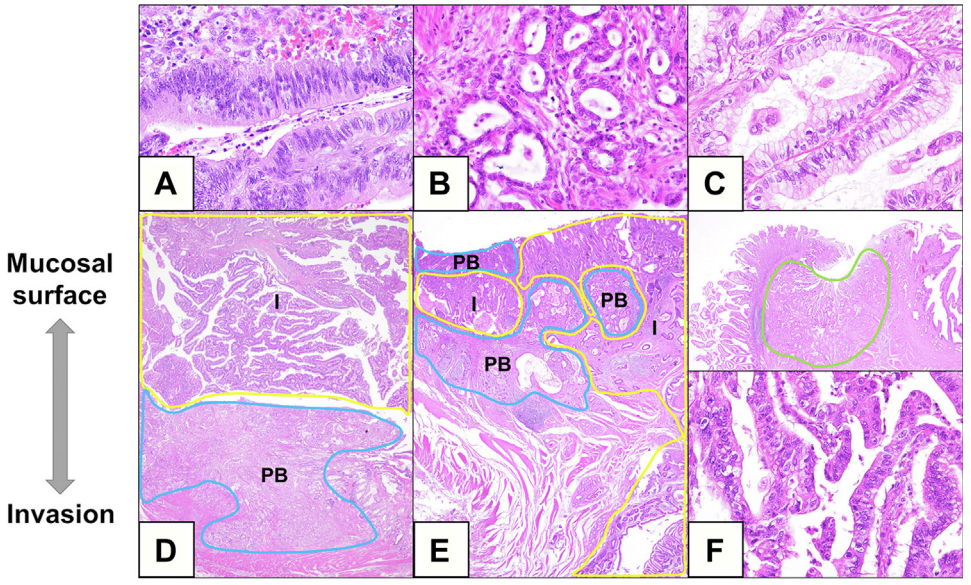

Figure 1.
(A) Ampullary carcinoma with intestinal features. Large tubules lined by tall columnar cells with elongated, pseudostratified, hyperchromatic nuclei. Luminal necrotic debris and acute inflammatory cells are also present. (B) Ampullary carcinoma with pancreatobiliary features. Small widely separated glands are lined by the low cuboidal eosinophilic epithelium with a high nuclear-to-cytoplasmic ratio, and round nuclei with vesicular chromatin and irregular nuclear contours, accompanied by abundant fibrotic stroma. (C) Ampullary carcinoma with gastric features. Tall columnar cells with basally oriented nuclei and pale mucinous cytoplasm reminiscent of gastric foveolar epithelium. (D-F) Definition of patterns by morphology in mixed-type ampullary carcinoma. (D) Separate pattern. Divided into I-type lesions in the duodenal mucosa and PB-type lesions in the invasion zones separately. (E) Random pattern. Divided into I and PB-type lesions, but the direction of lesion migration is unclear. (F) Unclassifiable pattern. Difficult to determine whether I-type lesion or PB-type lesion (intestinal architecture with pancreatobiliary cytology). I, intestinal; PB, pancreatobiliary.

area, or cases that were difficult to classify as the I or PB type, were also classified as the mixed type. This classification was performed independently by 2 pathologists (J.K. and Y.K.) who were blinded to the clinical findings. The sections to be examined were prepared to cover the entire lesion in each case. The average number of all slides reviewed in each case for this study was 30 (range, 10-54), of which the average number of slides with tumors was 7 (range, 1-28). Any disagreements in the classification were resolved by discussion until consensus was reached.

Immunohistochemical Subtyping

The subtype of ampullary carcinoma was determined based on morphologic evaluation upon HE staining, with reference to the results of IHC staining of the protein expression markers mentioned below. When morphologic evaluation and IHC findings did not agree, morphologic evaluation was given priority. Four protein expression markers were used: MUC1 (Ma695, 1:1; Leica), expressed in PB type carcinoma; MUC2 (Ccp58, 1:1; Leica), a marker of intestinal (goblet cell) differentiation; CDX2 (CDX2-88, 1:100; BioGenex), a transcription factor involved in intestinal epithelial cell growth and differentiation; and MUC5AC (CLH2, 1:200; Dako), a gastric foveolar mucin marker. Immunostaining was performed in accordance with the manufacturers' instructions. We arbitrarily defined IHC positivity when >10% of the tumors was positively stained.

Several IHC panels have been proposed for tissue subtyping in ampullary carcinoma, but no consensus has been reached regarding the appropriate criteria for their interpretation.[7,17,20,21] In this study, we followed the criteria chosen by most authors and defined MUC1-positive cases as the PB type, MUC2/CDX2-positive cases as the I type, and MUC5AC-positive cases as the G type. Ambiguous cases that were positive for MUC1 and MUC2/CDX2 and morphologically unclear in terms of whether they were I or PB type were considered the mixed type. In addition, in cases with lymph node metastasis, the same IHC analysis was also performed on metastatic lymph nodes to determine the subtype of cancer cells there.

Molecular Analysis

For targeted sequencing, a total of 18 ampullary carcinomas were selected, including 3 I type, 3 PB type without G type (pure PB type), 6 mixed type, and 6 PB type with G type (PBG type). In all cases, noncancerous tissues such as lymph nodes and normal duodenum were also prepared. For the mixed type, 6 cases in which I and PB-type lesions were clearly distinguishable were selected. A total of 6 cases each of mixed type and PBG type were extracted separately for each epithelial subtype. I, PB, or G-type lesion was separately prepared by manual microdissection or laser capture microdissection (LMD6500; Leica Microsystems) from 10-μm-thick sections of formalin-fixed and paraffin-embedded tissues. Genomic DNA was extracted using the FormaPure XL DNA Kit (Beckman Coulter) in accordance with the manufacturer's instructions.

A targeted sequencing approach using a custom-made panel was applied to analyze the entire coding regions of 24 genes, which were previously reported to be recurrently mutated in ampullary carcinoma: *ACVR1B*, *ACVR2A*, *APC*, *ARID2*, *BRAF*, *CDH10*, *CDKN2A*, *CNTN4*, *CTNNB1*, *ELF3*, *EPHA3*, *EPHA6*, *ERBB2*, *ERBB3*, *FBXW7*, *GNAS*, *KRAS*, *LOXHD1*, *RNF43*, *SMAD4*, *SOX9*, *TGFBR1*, *TGFBR2*, and *TP53*.[16] Library preparation was performed using the Agilent SureSelectXT Low Input Target Enrichment Protocol (Agilent). The barcoded libraries were sequenced using the HiSeqX system (Illumina). We identified candidate mutations that were present in >10% of distinct reads with coverage >30×. Variants were filtered against the matched normal tissues for each patient. Filtered variants were annotated using SnpEff software (http://snpeff.sourceforge.net/SnpEff.html). The COSMIC (http://cancer.sanger.ac.uk/cosmic) and ClinVar databases (https://www.ncbi.nlm.nih.gov/clinvar/) were consulted to classify variants as either pathogenic or a variant of unknown significance.

Following a report by Yachida et al,[16] we identified mutations in *APC*, *ACVR2A*, *SOX9*, *ACVR1B*, *ARID2*, *EPHA6*, *BRAF*, *ERBB2*, *TGFBR1*, *LOXHD1*, *FBXW7*, *TGFBR2*, and *EPHA3* as those mainly found in the I type; mutations in *CDKN2A* and *CDH10* as those mainly found in the PB type; and mutations in *TP53*, *KRAS*, *SMAD4*, *CTNNB1*, *ERBB3*, *ELF3*, and *GNAS* as those found in both subtypes. We examined the relationship between each genetic mutation and the subtype (Supplementary Fig. S1).

Sanger Sequencing

To compare the mutations found in mixed types detected by targeted sequencing with those in metastatic lymph nodes, genomic DNA from metastatic lymph nodes was extracted and analyzed for mutations in metastatic lymph nodes by Sanger sequencing.

Generation of Mutant-Allele Tumor Heterogeneity Score

We used a mutant-allele tumor heterogeneity (MATH) algorithm to measure intratumoral heterogeneity (ITH) and explored its correlation with subtype. The tumor MATH score for each case was calculated following the method described by Mroz and Rocco.[22] The steps to determine the MATH score are summarized as follows: (1) calculating the mutant-allele fraction (MAF) for each locus as the proportion of mutant reads relative to total reads; (2) obtaining the absolute difference of each MAF from the median MAF value and then multiplying the median of these absolute differences by a factor of 1.4826, thus generating the median absolute deviation (MAD); and (3) calculating the MATH score as the proportion of the MAD relative to the median of the MAFs among the tumor's mutated genomic loci as follows: $\text{MATH} = \text{MAD/median} \times 100\%$.

Statistical Analysis

Statistical analyses were performed using JMP 14.0 software (SAS Institute Inc.). Categorical variables were compared using the Pearson χ^2 test or Fisher exact test, as indicated. Multivariate logistic regression analysis was used for multivariate analysis. The Kaplan-Meier analysis with log-rank test and Cox proportional hazards model analysis were used for survival analysis.

Results

Clinicopathologic Results, Subtyping, and Prognosis

The clinicopathologic characteristics of the 110 patients who underwent curative resection for ampullary carcinoma are presented in Table 1. These 110 patients comprised 53 men and 57

women, with a median age of 69.0 years (range, 32-93 years). The median tumor diameter was 20.0 mm (range, 5-65 mm). Nodal metastasis was present in 46 cases (41.8%). Furthermore, 22 (20%) patients received postoperative adjuvant chemotherapy. S-1 was administered as a first-line chemotherapy for 13 patients (1 I type, 10 PB type, and 2 mixed type); the remaining 9 patients (7 PB type and 2 mixed type) received gemcitabine chemotherapy.

In the fifth edition of the *WHO Classification of Tumours of the Digestive System*, the PB type was changed to "pancreatobiliary type or gastric-type adenocarcinoma," and we initially classified the gastric type separately in this study. The results of HE and IHC staining showed that 35 cases were I type, 51 were PB type with gastric type (PBG type), 12 were PB type without gastric type (pure PB type), and 12 were mixed type. Comparing PBG type and pure PB type, no significant differences in clinicopathologic characteristics and prognosis were observed (Supplementary Table S1, Supplementary Fig. S2). In addition, mutations in the *APC* gene, which had been reported to be characteristic of the I type,[13,16] were not found in either G or PB-type lesions of the PBG type (Supplementary Fig. S3). Therefore, the PB type was defined as the combination of PBG type and pure PB type, as described in the fifth edition of the *WHO Classification of Tumours of the Digestive System*. Of the 35 cases of the I type, 10 cases were partially stained with MUC5AC and contained gastric foveolar epithelium; the stained area of the sample was small, and therefore, all were included in the I type. No pure G -type was detected. Finally, 35 cases were determined to be I type, 63 cases PB type, and 12 cases mixed type (Table 2).

Of the 12 mixed-type cases, 5 had I-type lesions in the duodenal mucosa and PB-type lesions in the invasion zones separately accompanied by adenomas in 2 cases. We defined these as exhibiting a "separate" pattern. Four cases had a random mixture of I and PB-type lesions, which was defined as a "random" pattern. In the remaining 3 cases, it was difficult to determine whether there was an I-type lesion or a PB-type lesion; eg, cellular morphology indicated an I-type lesion but histologic morphology indicated a PB-type lesion or vice versa. These cases were defined as having an "unclassifiable" pattern (Fig. 1).

Table 1
Clinicopathologic parameters and outcome (N = 110)

Variable	No. of patients (%)	Overall survival *P* (log-rank)		Disease-free survival *P* (log-rank)	
		Univariate	Multivariate	Univariate	Multivariate
Overall	110 (100.0)				
Age (y), median (range)	69.0 (32-93)				
Sex, male/female	53/57				
Tumor size (mm), median (range)	20.0 (5-65)				
Differentiation					
Well	76 (69.1)	.0109*	.5475	.0878	
Moderate-to-poor	34 (30.9)				
Histologic subtype					
I, PB	98 (89.1)	.0134*	.097	.0487*	.0874
Mixed	12 (10.9)				
T stage (UICC)					
1,2	64 (58.2)	.0001*	.0747	.0003*	.5387
3	46 (41.8)				
Lymph node metastasis					
Negative	64 (58.2)	<.001*	.0483*	<.001*	.0023*
Positive	46 (41.8)				
Lymphatic invasion					
Negative	66 (60.0)	.0003*	.3468	.0006*	.7553
Positive	44 (40.0)				
Vascular invasion					
Negative	90 (81.8)	.0291*	.604	.0002*	.1326
Positive	20 (18.2)				
Perineural invasion					
Negative	83 (75.5)	.0264*	.92	.0009*	.6404
Positive	27 (24.5)				
Immunohistochemistry					
MUC1					
Negative	51 (46.4)	.0005*	.0564	.0135*	.39
Positive	59 (53.6)				
MUC2					
Negative	67 (60.9)	.7575		.7242	
Positive	43 (39.1)				
MUC5AC					
Negative	47 (42.7)	.6061		.4229	
Positive	63 (57.3)				
CDX2					
Negative	61 (55.5)	.9333		.6285	
Positive	49 (44.5)				

I, intestinal; PB, pancreatobiliary; UICC, Union for International Cancer Control.
* $P < .05$.

Table 2
Clinical and pathologic comparison of subtype

	Total (N = 110)	I type (n = 35)	PB type (n = 63)	Mixed type (n = 12)	*P*		
					I vs Mixed	PB vs Mixed	I vs PB
Age, median (range)	69.0 (32-93)	67.0 (32-93)	70.0 (41-86)	69.0 (55-82)	.3859	.7765	.258
Sex, male/female	53/57	22/13	24/39	7/5	.7809	.192	.0186*
Tumor size (mm), median (range)	20.0 (5-65)	20.0 (6-47)	18.0 (5-55)	28.5 (10-65)	.0400*	.0322*	.8372
T stage 3a and 3b (UICC), n (%)	46 (42)	4 (11)	35 (56)	7 (58)	.0009*	.859	<.001*
Lymph node metastasis, n (%)	46 (42)	29 (83)	33 (52)	7 (58)	.0059*	.7048	.0006*
Lymphatic invasion, n (%)	44 (40)	8 (23)	30 (48)	6 (50)	.076	.8797	.0159*
Vascular invasion, n (%)	20 (18)	3 (9)	16 (25)	1 (8)	.9797	.1957	.0435*
Perineural invasion, n (%)	27 (25)	2 (6)	21 (33)	4 (33)	.0134*	1.0000	.0020*
Recurrence, n (%)	29 (26)	7 (20)	17 (27)	5 (42)	.1374	.3059	.4411

I, intestinal; PB, pancreatobiliary; UICC, Union for International Cancer Control.
* $P < .05$.

The relationship between clinicopathologic results and subtypes is shown in Table 2. Comparing the I and PB types, T stage ($P < .001$) and the rates of lymph node metastasis ($P = .0006$), lymphatic invasion ($P = .0159$), vascular invasion ($P = .0435$), and perineural invasion ($P = .0020$) were significantly higher in the PB type than in the I type. In addition, comparing the I and mixed types, tumor size ($P = .0400$), T stage ($P = .0009$), and the rates of lymph node metastasis ($P = .0059$) and perineural invasion ($P = .0134$) were significantly higher in the mixed type than in the I type. In a comparison of the PB and mixed types, T stage, lymph node metastasis, lymphatic invasion, vascular invasion, perineural invasion, and recurrence were similar in both, with the only significant difference being that tumor size was significantly greater in the mixed type than in the PB type ($P = .0322$).

Cox proportional hazards analysis (Table 1) showed that moderate-to-poor differentiation ($P = .0109$), mixed histologic subtype ($P = .0134$), stage T3 or higher ($P = .0001$), lymph node metastasis ($P < .001$), lymphatic invasion ($P = .0003$), vascular invasion ($P = .0291$), perineural invasion ($P = .0264$), and MUC1 positivity ($P = .0005$) were significantly associated with shorter overall survival (OS). In addition, mixed histologic subtype ($P = .0487$), stage T3 or higher ($P = .0003$), lymph node metastasis ($P < .001$), lymphatic invasion ($P = .0006$), vascular invasion ($P = .0002$), perineural invasion ($P = .0009$), and MUC1 positivity ($P = .0135$) were significantly associated with shorter disease-free survival (DFS). The multivariate model using Cox proportional hazards analysis included parameters with $P < .05$ in the univariate analysis using the log-rank test. In the multivariate analysis, only lymph node metastasis was significantly associated with shorter OS ($P = .0483$) and DFS ($P = .0023$).

The Kaplan-Meier survival analysis showed that the mixed type had a significantly poorer prognosis for both OS ($P = .0016$) and DFS ($P = .0025$) than the I type and tended to have a poorer prognosis than the PB type for both OS ($P = .0802$) and DFS ($P = .2775$) (Fig. 2A). Among those who received adjuvant chemotherapy postoperatively (n = 22), the mixed type tended to have a worse prognosis than the I (OS: $P = .2994$; DFS: $P = .2994$) and PB (OS: $P = .0821$; DFS: $P = .1769$) types (Fig. 2B).

Subtyping of Metastatic Lymph Nodes

In most cases of ampullary carcinoma in the current study, cancer cells in metastatic lymph nodes showed the same subtype as recognized in the primary lesion. However, 1 case of the I type was negative for MUC1 in the primary lesion but positive in the metastatic lymph nodes. Six cases of PB type were negative for CDX2 in the primary tumor but positive in the metastatic lymph nodes (Supplementary Table S2). Coexpression of MUC1 and CDX2 was also observed in 2 of 33 cases of the PB type and 3 of 7 cases of the mixed type (Supplementary Fig. S4).

Genetic Mutations of Intestinal and Pancreatobiliary Types of Ampullary Carcinoma

We found 49 mutations in 15 genes across all lesions of the I, PB, and mixed types (Fig. 3). Among these, the most frequently mutated gene was *TP53* (11), followed by *KRAS* (10), *APC* (4), *SMAD4* (3), *CTNNB1* (3), *ERBB3* (2), *ACVR1B* (2), *ERBB2* (2), *CDKN2A* (2), *FBXW7* (2), *ARID2* (1), *TGFBR2* (1), *ELF3* (1), *TGFBR1* (1), and *SOX9* (1). Comparing the subtypes, all 3 cases of the I type had mutations mainly found in the I type or mutations found in both I and PB types. However, among the 9 patients with the PB type, only 1 case had an *APC* mutation mainly found in the I type, but the others had mutations found in the PB type or both types.

Genetic Mutations of Mixed-type Ampullary Carcinoma

Regarding the genetic mutations of the mixed type, *TP53*, *KRAS*, *APC*, and *SMAD4* mutations were commonly found in both I and PB-type lesions of most mixed types (Fig. 3). *CTNNB1*, *ACVR1B*, *FBXW7*, *ARID2*, *TGFBR2*, and *ELF3* were present only in I-type lesions in 1 case each.

The genetic mutations of I and PB-type lesions and their changes in each case are shown in Figure 4. Cases 4, 5, 8, and 9 had a separate pattern, and only case 4 was associated with adenoma. Cases 4 and 6 had the same genetic mutation in 2 lesions and an additional genetic mutation in the PB-type lesion only. However, cases 5, 7, and 9 had the same genetic mutation in 2 lesions and an additional genetic mutation in the I-type lesion only. The remaining case 8 showed genetic mutations only in the I-type lesion and not in the PB-type lesion. Genetic mutation analysis of the unclassifiable pattern was not performed.

Five of the 6 mixed types had lymph node metastases, but 2 cases were excluded from the lymph node genetic mutation analysis because of insufficient amounts of DNA. The genetic mutations detected in the metastatic lymph nodes of the remaining 3 cases (cases 4, 6, and 9) were all consistent with those of the PB-type lesions (Fig. 5).

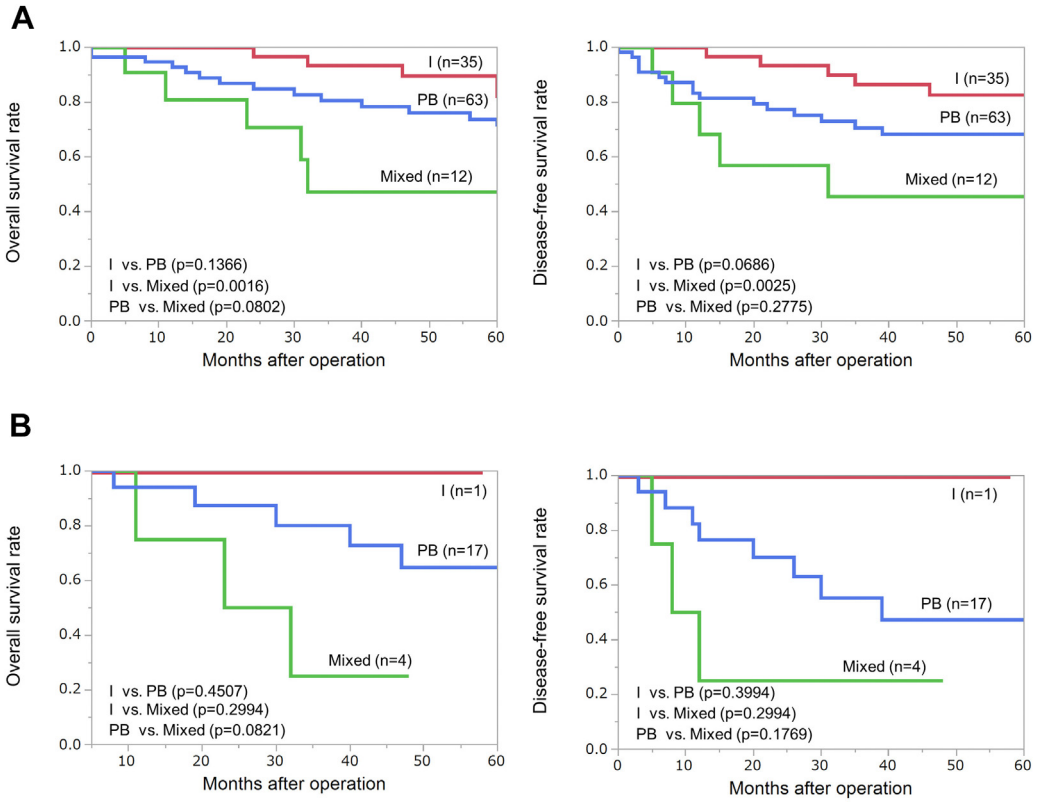

Figure 2.
Kaplan-Meier curves of patients with ampullary adenocarcinoma who underwent curative surgery according to the pathologic analysis. (A) All patients. (B) Adjuvant group. I, intestinal; PB, pancreatobiliary.

Mutant-Allele Tumor Heterogeneity Score in Ampullary Carcinoma

Overall, 17 MATH scores were acquired in this study. In the mixed-type group, MATH scores ranged from 11 to 85, with a mean ± SD of 44.5 ± 31.4. In the I-type group, MATH scores ranged from 5 to 45, with a mean ± SD of 19.6 ± 22.0; whereas in the PB type group, scores ranged from 0 to 57, with a mean ± SD of 11.9 ± 19.8. The distribution of MATH scores showed that ITH was significantly higher in the mixed type than in the PB type and tended to be higher than that in the I type (Supplementary Fig. S5).

Discussion

Few studies have reported on genetic mutations of mixed-type ampullary carcinoma. Meanwhile, previous studies[17,18] that did report on genetic mutations of this subtype only analyzed 1 lesion per case; therefore, it has remained unknown whether there are genetic variants specific to the mixed type. Furthermore, the mechanisms behind the development and progression of the mixed type have remained unclear because there are no reported studies comparing genetic mutations of the I and PB-type lesions in the same case. Here, we collected 110 ampullary carcinomas, for the first time surveyed gene mutations in each I-type lesion and each PB-type lesion, and compared the genetic alterations of these 2 lesions in 6 cases of mixed-type ampullary carcinoma by a targeted sequencing approach to analyze the entire coding region of 24 genes.

Overall, 5 of the 6 mixed types analyzed had common genetic mutations in the I and PB-type lesions, referred to as a "trunk mutation," with an additional genetic mutation in either lesion, referred to as a "branch mutation" (Fig. 4). The trunk mutations were consistent with those common to the I and PB types reported by Yachida et al,[16] except for the mutation in *APC* in case 9. Branch mutations in I-type lesions were mutations found in the I type or both types, but in the PB-type lesions, 2 were mutations found in both types, but 1 was in *ERBB2*, a mutation found in the I type. We hypothesized that, in the separate pattern, the I type existed first, and as a result of additional genetic mutation, the PB type was added. Meanwhile, in the random pattern, the I or PB type preceded the other type. Case 4, involving a separate pattern with adenoma, was consistent with this hypothesis, but in the other cases, additional genetic mutations may have added I-type lesions during the process of retrograde invasion of preexisting PB-type lesions into the duodenal mucosa. In the random pattern, it was not possible to predict the genetic mutation by morphologic findings in terms of whether the preceding lesion was I or PB type. Cai et al[23] reported that the molecular characteristics of tumor cells of the same adenocarcinoma component were not all the same in lung adenocarcinoma tissue and that histopathologic morphology might differ despite identical genetic mutation patterns, demonstrating inconsistency in genomic and morphologic variation. The results of this study suggest that, in ampullary carcinoma, it is difficult to determine the genomic variation based on morphologic findings alone in actual tumor tissue. However, in the analysis of 3 cases, the genetic mutations in the metastatic lymph nodes of the mixed-type cases were the same as those in the PB-type lesion of the primary tumor, suggesting the possibility that cancer cells in the PB-type lesion are more likely to spread to the lymph nodes.

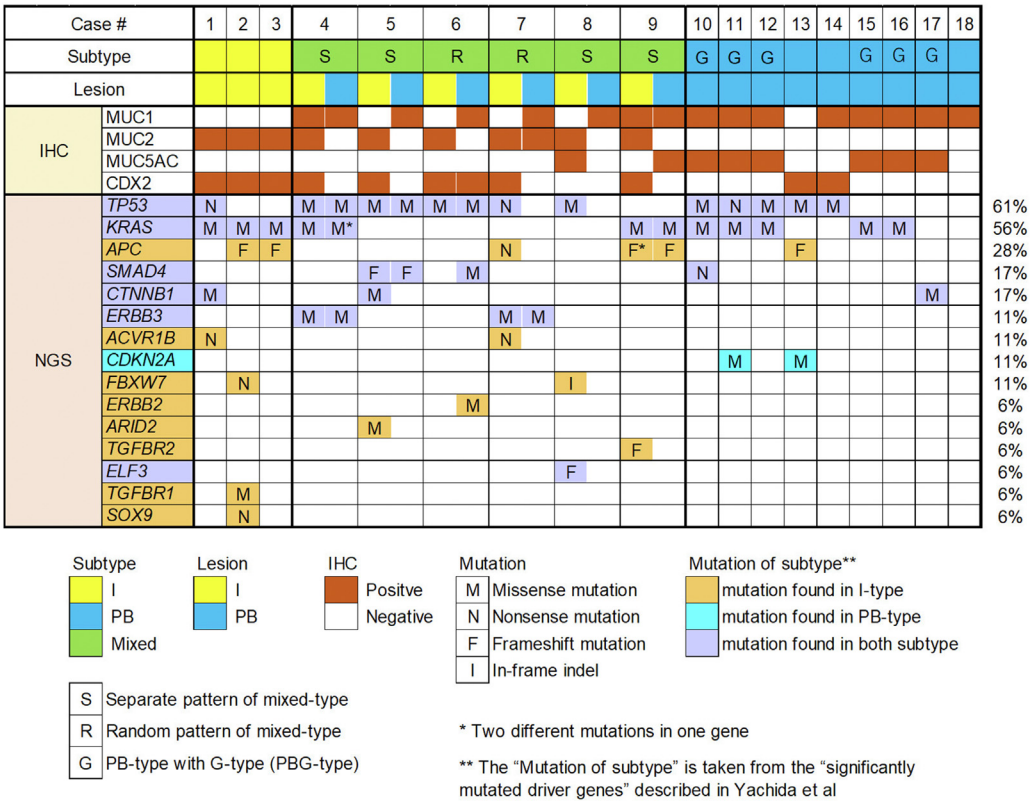

Figure 3.
Genetic mutations and protein expression of each case. I, intestinal; IHC, immunohistochemical; NGS, next-generation sequencing; PB, pancreatobiliary.

ITH is a hallmark of cancer, which is characterized by the presence of different subpopulations of cancer cells with distinct genetic, phenotypic, or behavioral characteristics within the same tumor.[24,25] ITH is closely linked to tumor resistance to treatment, and tumors with high ITH exhibit greater drug resistance.[26] In the present study, the MATH score developed by Mroz et al[22] was used to evaluate genetic ITH. Independent studies have shown that high MATH scores are associated with poor prognosis in patients with various cancers, and in our study, mixed types with high MATH scores were associated with poor prognosis.[22,27-31] Furthermore, the postoperative adjuvant chemotherapy group showed a similar trend toward poor prognosis, although the difference was not significant because of the small number of cases. Although the mixed morphologic findings did not allow

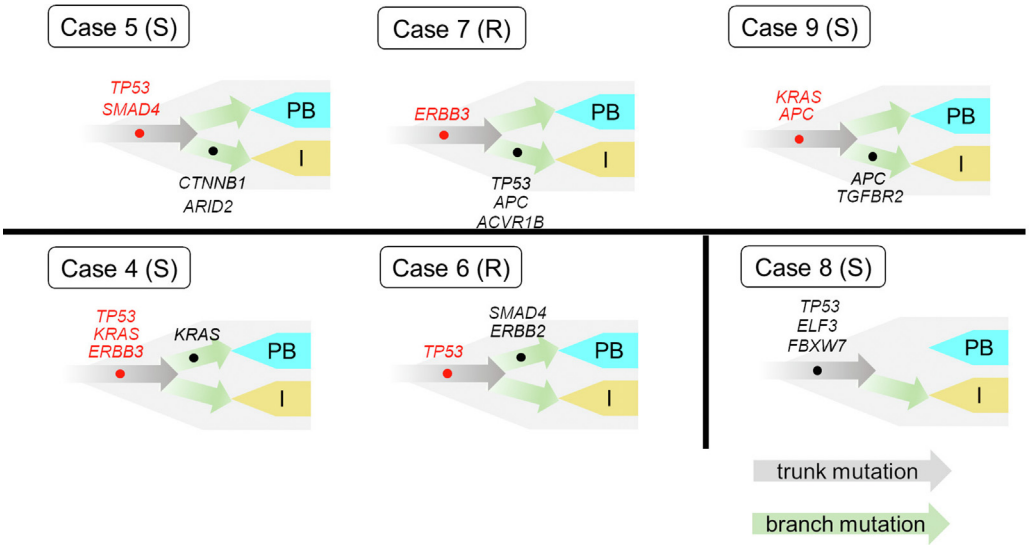

Figure 4.
Cases 5, 7, and 9: cases harboring the same mutation in 2 lesions, with additional mutations in the I-type lesion. Cases 4 and 6: cases harboring the same mutation in 2 lesions, with additional mutations in the PB-type lesion. I, intestinal; PB, pancreatobiliary; R, random pattern; S, separate pattern.

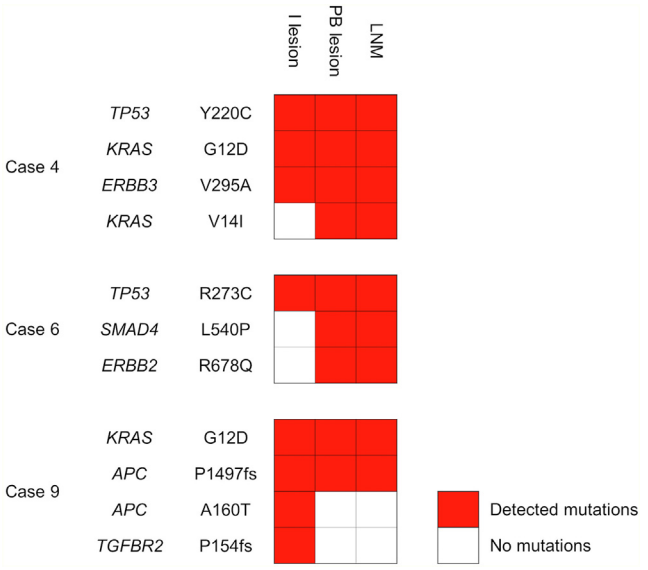

Figure 5.
Comparison of genomic mutations between primary tumors and lymph node metastases of mixed-type ampullary carcinoma. I, intestinal; LNM, lymph node metastasis; PB, pancreatobiliary.

inferences to be made about the specific genetic mutation, mixed types were expected to have higher ITH, suggesting that they may be related to treatment resistance.

Mixed-type ampullary carcinoma consists of PB and I-tpye lesions, which are positive for MUC1 and MUC2/CDX2, respectively, on IHC staining. In addition, in this study, there were cases with the coexpression of MUC1 and CDX2 in metastatic lymph nodes and the primary tumor, indicating the IHC heterogeneity of the mixed type. Similar to the molecular biological results, the mixed type is a heterogeneous tumor both morphologically and phenotypically. Our proposed relationship among I, PB, and mixed types is shown in Figure 6.

An accurate diagnosis of histologic subtypes contributes not only to prognostic prediction but also to appropriate selection of therapeutic approaches to improve clinical outcomes. Moekotte et al[12] reported that PB and/or mixed types may benefit from gemcitabine-based adjuvant therapy, but the I type did not show any survival benefit from adjuvant chemotherapy. Moreover, Kapp et al[32] reported a case of lung and liver recurrence after resection of ampullary carcinoma treated with FOLFOX and FOLFIRI, but after reevaluation of the histologic subtype and confirmation of the PB type, chemotherapy with gemcitabine and nab-paclitaxel

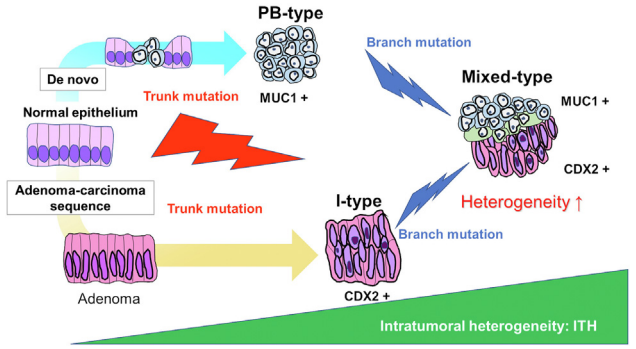

Figure 6.
Schema of progression of mixed-type ampullary carcinoma. I, intestinal; PB, pancreatobiliary.

was successfully performed. Furthermore, Schiergens et al[33] suggested that the PB type should be treated with particular chemotherapy regimens applied in pancreatic cancer and the I type with those applied in colorectal cancer. In the present study, a comparison between PB type and mixed-type ampullary carcinoma cases that received postoperative adjuvant chemotherapy showed that the mixed type tended to have a worse prognosis (Fig. 2B). ITH based on the MATH score was significantly higher in the mixed type than in the PB type (Supplementary Fig. S5). An accurate pathologic diagnosis of mixed-type cases with high ITH is important, and intense multidrug treatment regimens for mixed types may improve prognosis.

Another noteworthy result in our study is the classification of gastric type. In the fifth edition of the *WHO Classification of Tumors: Digestive System Tumours*, "pancreatobiliary type" was changed to "pancreatobiliary type or gastric type."[3] However, few reported studies have examined in detail the genetics and clinicopathologic features of gastric-type ampullary carcinoma. In this study, we compared the differences in genetic mutations and clinicopathologic features between cases with and without gastric type in the PB type. There were no significant differences between these 2 types in prognosis and molecular biological analysis (Supplementary Fig. S2, Supplementary Table S1), resulting in no inconsistency in the inclusion of gastric type in the PB type. In intraductal papillary mucinous neoplasm of the pancreas, areas with gastric-type epithelium can be associated with the other subtypes, suggesting that the gastric type might be a common precursor of the other types.[34] However, in this study, there was no accumulation of genetic mutations during progression from the gastric type to PB type (Supplementary Fig. S3).

Our study had several limitations. All data were derived from retrospective studies, and therefore, potential biases including in patient selection may have been unavoidable. In addition, the sample size was relatively small because of the rarity of ampullary carcinoma, in particular the mixed type. Several studies[5,10,13-15] have reported on the prognosis of the mixed type, but only Asano et al[14] reported that the mixed type had a poorer prognosis than the I and PB types. Individual studies included only a small number of mixed-type cases, and therefore, the prognosis of the mixed type should be evaluated in prospective clinical trials in a larger cohort study.

As in previous studies,[17,18] we could not find any genetic variants characteristic of the mixed type, but we might have found them if we had been able to analyze the genes of the unclassifiable pattern of the mixed type. In this study, a targeted panel was used to analyze genetic alterations, but no genetic alterations were found in the PB-type lesions in cases 8 or 18. Other genetic or epigenetic alterations may be involved, the identification of which may require a different approach. Furthermore, quantifying genomic ITH from bulk genomics comes with several caveats and limitations. Bulk genomics are a result of processing a complex admixture of cancer and noncancer cells simultaneously. The proportion of tumor cells in the admixture (tumor purity) depends on the tumor extraction details and strongly confounds the results. In the present study, the variant allele frequency differed between mixed-type I and PB-type lesions, with PB-type lesions having a lower variant allele frequency than I-type lesions in most cases (Supplementary Table S3). This may be because of the strong desmoplasia of the PB-type lesions, which reduced the tumor cell percentage during DNA extraction.

In conclusion, our results show that mixed-type ampullary carcinoma is a subtype distinct from the I and PB types in terms of its genetic variation. Mixed-type tumors were shown to be histologically, immunohistochemically, and genetically heterogeneous,

indicating that these features not only predict patient prognosis but may also influence resistance to treatment. These results suggest that ampullary carcinoma requires a personalized clinical approach based on the nature of the tumor. Future studies, including randomized controlled trials for each subtype, will be needed to select ideal candidates for treatment, including chemotherapy.

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Author Contributions

J.K. performed the research and wrote the paper. Y.K., S.N., Y.S., Y.Y., T.Y., and K.S. contributed to the research design and slide review. M.N. and Y.O. designed the research and gave final approval of the manuscript. All authors read and approved the final paper.

Data Availability

Data supporting this study's findings are available from the corresponding author upon reasonable request.

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Declaration of Competing Interest

The authors report no relevant conflicts of interest.

Ethics Approval and Consent to Participate

This study was approved by the Ethics Committee of the Kyushu University (institutional review board: 2020-633).

Supplementary Material

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References

1. Kimura W, Futakawa N, Yamagata S, et al. Different clinicopathologic findings in two histologic types of carcinoma of papilla of Vater. *Jpn J Cancer Res*. 1994;85:161–166.
2. Bosman FT, Carneiro F, Hruban RH, Theise ND. *WHO Classification of Tumours of the Digestive System*. 4th ed. IARC; 2010.
3. WHO Classification of Tumours Editorial Board. *WHO Classification of Tumors: Digestive System Tumours*. 5th ed. IARC; 2019.
4. Carter JT, Grenert JP, Rubenstein L, Stewart L, Way LW. Tumors of the ampulla of Vater: histopathologic classification and predictors of survival. *J Am Coll Surg*. 2008;207:210–218.
5. Kohler I, Jacob D, Budzies J, et al. Phenotypic and genotypic characterization of carcinomas of the papilla of Vater has prognostic and putative therapeutic implications. *Am J Clin Pathol*. 2011;135:202–211.
6. Kim WS, Choi DW, Choi SH, Heo JS, You DD, Lee HG. Clinical significance of pathologic subtype in curatively resected ampulla of Vater cancer. *J Surg Oncol*. 2012;105:266–272.
7. Chang DK, Jamieson NB, Johns AL, et al. Histomolecular phenotypes and outcome in adenocarcinoma of the ampulla of Vater. *J Clin Oncol*. 2013;31:1348–1356.
8. Westgaard A, Pomianowska E, Clausen OPF, Gladhaug IP. Intestinal-type and pancreatobiliary-type adenocarcinomas: how does ampullary carcinoma differ from other periampullary malignancies? *Ann Surg Oncol*. 2013;20:430–439.
9. Okano K, Oshima M, Yachida S, et al. Factors predicting survival and pathological subtype in patients with ampullary adenocarcinoma. *J Surg Oncol*. 2014;110:156–162.
10. Zimmermann C, Wolk S, Aust DE, et al. The pathohistological subtype strongly predicts survival in patients with ampullary carcinoma. *Sci Rep*. 2019;9:12676.
11. Xia T, Wu X, Mou Y, et al. Clinicopathological prognostic factors and chemotherapeutic outcome for two histopathological types of ampulla of Vater adenocarcinoma. *Front Oncol*. 2021;11:616108.
12. Moekotte AL, Malleo G, van Roessel S, et al. Gemcitabine-based adjuvant chemotherapy in subtypes of ampullary adenocarcinoma: international propensity score-matched cohort study. *Br J Surg*. 2020;107:1171–1182.
13. Chakraborty S, Ecker BL, Seier K, et al. Genome-derived classification signature for ampullary adenocarcinoma to improve clinical cancer care. *Clin Cancer Res*. 2021;27:5891–5899.
14. Asano E, Okano K, Oshima M, et al. Phenotypic characterization and clinical outcome in ampullary adenocarcinoma. *J Surg Oncol*. 2016;114:119–127.
15. Liu F, Shen D, Ma Y, Song Q, Wang H. Identification of ampullary carcinoma mixed subtype using a panel of six antibodies and its clinical significance. *J Surg Oncol*. 2019;119:295–302.
16. Yachida S, Wood LD, Suzuki M, et al. Genomic sequencing identifies ELF3 as a driver of ampullary carcinoma. *Cancer Cell*. 2016;29:229–240.
17. Gingras MC, Covington KR, Chang DK, et al. Ampullary cancers harbor ELF3 tumor suppressor gene mutations and exhibit frequent WNT dysregulation. *Cell Rep*. 2016;14:907–919.
18. Harthimmer MR, Stolborg U, Pfeiffer P, Mortensen MB, Fristrup C, Detlefsen S. Mutational profiling and immunohistochemical analysis of a surgical series of ampullary carcinomas. *J Clin Pathol*. 2019;72:762–770.
19. Albores-Saveedra J, Henson DE, Klimstra DS. *Tumors of the Gallbladder, Extrahepatic Bile Ducts and Ampulla of Vater. Atlas of Tumor Pathology*. Armed Forces Institute of Pathology; 2000.
20. Ang DC, Shia J, Tang LH, Katabi N, Klimstra DS. The utility of immunohistochemistry in subtyping adenocarcinoma of the ampulla of Vater. *Am J Surg Pathol*. 2014;38:1371–1379.
21. Mafficini A, Amato E, Cataldo I, et al. Ampulla of Vater carcinoma: sequencing analysis identifies TP53 status as a novel independent prognostic factor and potentially actionable ERBB, PI3K, and WNT pathways gene mutations. *Ann Surg*. 2018;267:149–156.
22. Mroz EA, Rocco JW. MATH, a novel measure of intratumor genetic heterogeneity, is high in poor-outcome classes of head and neck squamous cell carcinoma. *Oral Oncol*. 2013;49:211–215.
23. Cai W, Lin D, Wu C, et al. Intratumoral heterogeneity of ALK-rearranged and ALK/EGFR coaltered lung adenocarcinoma. *J Clin Oncol*. 2015;33:3701–3709.
24. McGranahan N, Swanton C. Biological and therapeutic impact of intratumor heterogeneity in cancer evolution. *Cancer Cell*. 2015;27:15–26.
25. Dagogo-Jack I, Shaw AT. Tumour heterogeneity and resistance to cancer therapies. *Nat Rev Clin Oncol*. 2018;15:81–94.
26. Sharma A, Cao EY, Kumar V, et al. Longitudinal single-cell RNA sequencing of patient-derived primary cells reveals drug-induced infidelity in stem cell hierarchy. *Nat Commun*. 2018;9:4931.
27. Mroz EA, Tward AD, Pickering CR, Myers JN, Ferris RL, Rocco JW. High intratumor genetic heterogeneity is related to worse outcome in patients with head and neck squamous cell carcinoma. *Cancer*. 2013;119:3034–3042.
28. Hou Y, Li T, Gan W, et al. Prognostic significance of mutant-allele tumor heterogeneity in uterine corpus endometrial carcinoma. *Ann Transl Med*. 2020;8:339.
29. Mao H. Clinical relevance of mutant-allele tumor heterogeneity and lung adenocarcinoma. *Ann Transl Med*. 2019;7:432.
30. Ma D, Jiang YZ, Liu XY, Liu YR, Shao ZM. Clinical and molecular relevance of mutant-allele tumor heterogeneity in breast cancer. *Breast Cancer Res Treat*. 2017;162:39–48.
31. Zhang J, Yan S, Liu X, et al. Gender-related prognostic value and genomic pattern of intra-tumor heterogeneity in colorectal cancer. *Carcinogenesis*. 2017;38:837–846.
32. Kapp M, Kosmala A, Kircher S, Luber V, Kunzmann V. Exceptional response to nanoparticle albumin-bound paclitaxel and gemcitabine in a patient with a refractory adenocarcinoma of the ampulla of Vater. *Case Rep Oncol*. 2016;9:15–24.
33. Schiergens TS, Reu S, Neumann J, et al. Histomorphologic and molecular phenotypes predict gemcitabine response and overall survival in adenocarcinoma of the ampulla of Vater. *Surgery*. 2015;158:151–161.
34. Adsay NV, Merati K, Basturk O, et al. Pathologically and biologically distinct types of epithelium in intraductal papillary mucinous neoplasms: delineation of an "intestinal" pathway of carcinogenesis in the pancreas. *Am J Surg Pathol*. 2004;28:839–848.