

Clinical, morphologic, and immunophenotypic characteristics of ampullary carcinomas with an emphasis on SMAD4 expression

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Background: The purpose of our study was to examine the relationship between clinicopathologic variables and morphologic subtypes in ampullary carcinoma, with an emphasis on the expression of *SMAD4* tumor suppressor gene.

Methods: Sixty-three cases of ampullary carcinomas resected between 2000–2011 were included in this study. Clinical characteristics and outcome data were recorded. Tumors were classified as pancreatobiliary or intestinal type based on morphology, and immunohistochemical (IHC) studies for cytokeratin 7 (CK7), cytokeratin 20 (CK20), cytokeratin 17 (CK17), and *SMAD4* were performed.

Results: Forty-nine percent of the ampullary tumors were pancreatobiliary, 29% were intestinal, and 22% were other variants. Tumors with pancreatobiliary morphology showed worse overall survival than those with intestinal morphology or other variants ($P=0.03$). A trend for higher stage, recurrence and less survival was seen in cases with negative *SMAD4* expression. In multivariate analysis, age group (≤ 60 vs. >60 years) and expression of CK17 were the most prognostic of survival.

Conclusions: Ampullary tumors with pancreatobiliary morphology have a worse overall survival, while negative *SMAD4* expression is associated with a trend of less survival.

Keywords: *SMAD4*; ampullary carcinoma; ampulla of Vater (AOV)

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Introduction

Carcinomas of the ampulla of Vater (AOV) are malignant epithelial neoplasms that are either centered in or completely replace the ampulla according to the World Health Organization (WHO) definition. Two main histologic subtypes of ampullary adenocarcinoma are recognized: pancreatobiliary and intestinal types, with the former carrying a worse prognosis including worse survival,

higher stage, and higher incidence of lymph node (LN) involvement (1,2).

Immunohistochemical (IHC) studies have been used to support the categorization of these tumors into a pancreatobiliary or intestinal phenotype, with cytokeratin 7 (CK7), cytokeratin 17 (CK17) and MUC1 expression supporting a pancreatobiliary phenotype (3-5), and cytokeratin 20 (CK20), MUC2, and CDX2 expression supporting an intestinal phenotype (4,6-9). However,

not all cases fit into these two categories. Tumors with mucinous, signet-ring cell, solid, and papillary morphology are often placed in a separate “other” category. In addition, some tumors show an IHC profile that does not entirely fit the classic phenotypes described for pancreatobiliary and intestinal type tumors. Because of this, Zhou *et al.* introduced an IHC category of “other type” for tumors with double positive or double negative CK7/CK20 profile (5). Clinical behavior of this subset of ampullary tumors is difficult to predict given the limited data available. Immunophenotyping using CK7, CK20, CDX2, MUC1 and MUC2 showed more enhanced subtyping of ampullary carcinoma once combined with histologic examination [hematoxylin and eosin (H&E)] (6), but still there are some cases that would not fit into this subtyping.

Carcinomas of the AOV are also a genetically heterogeneous group of tumors that may harbor different types of molecular alterations including *TP53* mutations (10), *Kras* mutations (11,12), *APC* mutations (10), *SMAD4* inactivation (13), and microsatellite instability (14,15). Although the 5-year overall survival lies between that of duodenal adenocarcinoma and pancreatic adenocarcinoma, there is great heterogeneity within this tumor group from both a clinical and histopathological spectrum (12). Identifying specific prognostic biomarkers is thus important to tailor treatment recommendations and future potential targeted therapies.

Inactivation of *SMAD4*, a tumor suppressor gene, has been shown to be an adverse prognostic factor in pancreatic adenocarcinomas (16). This type of genetic alteration has been identified in 55% of pancreatic adenocarcinomas, and though less frequent, it is also found in roughly a third of adenocarcinomas of the AOV (16-18). Determination of *SMAD4* expression by IHC has been shown to have good concordance with genetic status of *SMAD4* in pancreatic adenocarcinoma as demonstrated by Tascilar *et al.* (16), with lack of expression in cases with inactivation of the *SMAD4* gene.

There are limited data on *SMAD4* expression profiles in ampullary adenocarcinoma. A prior study of *SMAD4* expression in 140 adenocarcinomas of the AOV showed complete loss of *SMAD4* in 34% of cases with no difference between the different histologic subtypes (36% of intestinal, 37% of pancreatobiliary, and 29% of “other variants”) of ampullary carcinoma (13). Other clinicopathologic variables including pancreatobiliary morphology and histomolecular phenotype (1,3), stage (1,19), loss of CDX2 expression (7), vascular space invasion (7,19), and LN involvement (5,7,19) have been shown to be independent predictors of survival in ampullary carcinomas by various authors.

This study aims to describe the clinical characteristics and outcomes of ampullary carcinomas in regards to histologic subtypes, and evaluate the prognostic implications of *SMAD4* expression in these tumors.

Methods

Study cases and tumor specimens

This study was approved by the Institutional Review Board of the University of Florida. The study population comprised all ampullary carcinoma patients who had definitive surgical resection between Jan 2000–Aug 2011 at our institution. The following information was recorded from the Gastrointestinal Oncology Research Database: age, sex, clinical presentation, laboratory results of serum CEA and CA19.9, tumor stage (T), LN status (N), and recurrence. Clinical information about recurrence was available in 33 patients. Death status was obtained through a search of the national death index (NDI) performed on 4/1/2012.

Histologic evaluation

H&E slides available for all cases were reviewed by three independent pathologists (A Alkhasawneh, TZ Toro, and LV Duckworth). After review of all H&E slides, a representative block with invasive tumor was selected for IHC studies. Cases were classified as pancreatobiliary (glands with cuboidal lining), intestinal (glands with columnar lining and/or cribriform architecture) or other variants (cases that did not fit into either category) based on their morphologic characteristics on H&E (see *Figure 1*). The other variants category consisted of cases with mixed cuboidal/columnar epithelium, solid sheet-like growth pattern, mucinous tumors, and tumors with a prominent inflammatory component.

Immunohistochemistry

IHC studies for CK7, CK17, CK20, and *SMAD4* were performed using standard avidin-biotin-peroxidase complex procedure with a matching positive and negative control for each batch. A summary of the antibodies, clone, company, dilution, and antigen retrieval is listed in *Table 1*.

IHC studies for CK7, CK17, and CK20 were interpreted as positive in cases with cytoplasmic/membranous reactivity in $\geq 5\%$ of tumor cells and negative when $< 5\%$ of tumor cells were immunoreactive. *SMAD4* immunoreactivity was in general both nuclear and cytoplasmic, and four

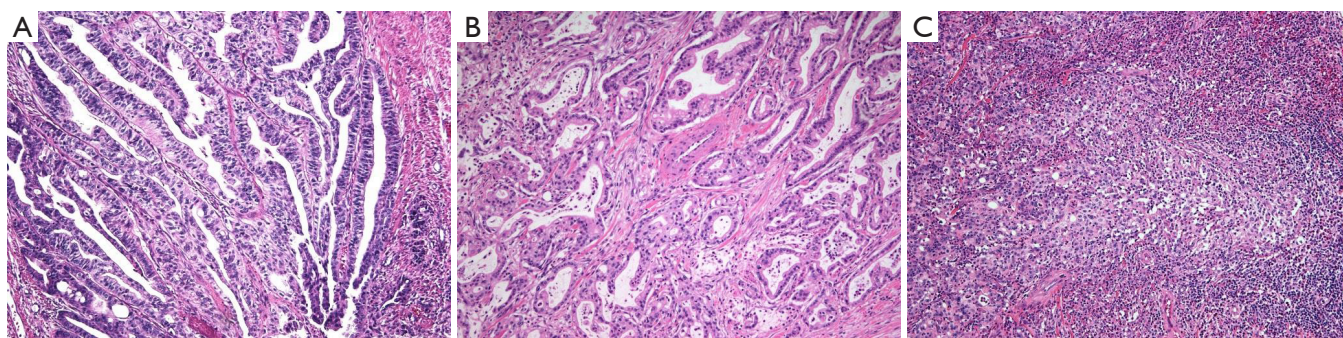


Figure 1 Histopathologic subtypes of ampullary adenocarcinoma. (A) Intestinal (glands with columnar lining and/or cribriform architecture); (B) pancreatobiliary (glands with cuboidal lining); (C) other variants. (A, B, C: 40×).

Table 1 Immunohistochemical studies (IHC) antibody specification

Antibody	Clone/code	Company	Dilution	Antigen retrieval
CK7	OV-TL 12/30	DAKO Cytomation (Carpinteria, CA, USA)	1:100	Trilogy; water bath (95 °C, 25 min)
CK17	Clone E3	DAKO Cytomation (Carpinteria, CA, USA)	1:40	high pH 9.0; water bath (95 °C, 30 min)
CK20	Ks20.8	DAKO Cytomation (Carpinteria, CA, USA)	1:50	Trilogy; water bath (95 °C, 25 min)
SMAD4	EP618Y	Millipore (Billerica, MA, USA)	1:200	No antigen retrieval

categories were reported [adapted from Tascilar *et al.* (16)], as follows: absent (no staining), trace (weak reactivity relative to surrounding pancreas), focal (two cell population with a subset of clearly negative cells) and diffuse (strong staining comparable to surrounding pancreas) as illustrated in *Figure 2*. Cases with absent, trace or focal SMAD4 expression were considered negative and those with diffuse SMAD4 expression were considered positive. Internal positive controls included background fibroblasts, lymphocytes, and non-neoplastic pancreatic tissue.

Statistical analysis

The clinical characteristics and IHC data were summarized by sample size and the corresponding sample proportion. Chi-square exact test was used to test the association between clinical characteristics with IHC variables. Overall survival curves were compared by log rank test, and the corresponding cumulative survival rates were estimated using Kaplan-Meier method. All statistical analysis was performed using SAS 9.2 software.

Results

Clinicopathologic characteristics

Sixty-three cases were identified and all were included in this

analysis. Our patient cohort presented at a mean age of 68, with a slight male predominance (55%). Detailed clinicopathologic characteristics including age, sex, clinical presentation, laboratory data, and tumor parameters are summarized in *Table 2*. Thirty-two of sixty-three patients (51%) had died at the time of this study with a mean survival of 23 months (range, 0.6–111 months) and an overall survival of 49%.

Tumor histopathologic subtypes and IHC profile

Nearly half of our cases (49%) were pancreatobiliary subtype, 29% were intestinal subtype, and 22% were other variants. Nearly all cases of pancreatobiliary type demonstrated a CK7+/CK20– profile in tumors, while most intestinal type were CK20+ and half of the intestinal type were CK7+. Tumors of other variants category showed a variety of IHC profiles including six CK7+/CK20–, five dual positive (CK7+/CK20+), and three dual negative (CK7–/CK20–) tumors. CK17, a marker seen in tumors of the pancreatobiliary tract, was present in 51% (32/63) of our cases. Though most of these cases showed pancreatobiliary morphology (69%), three had intestinal morphology, and seven had other variants morphology.

SMAD4

Clinical characteristics according to SMAD4 expression are

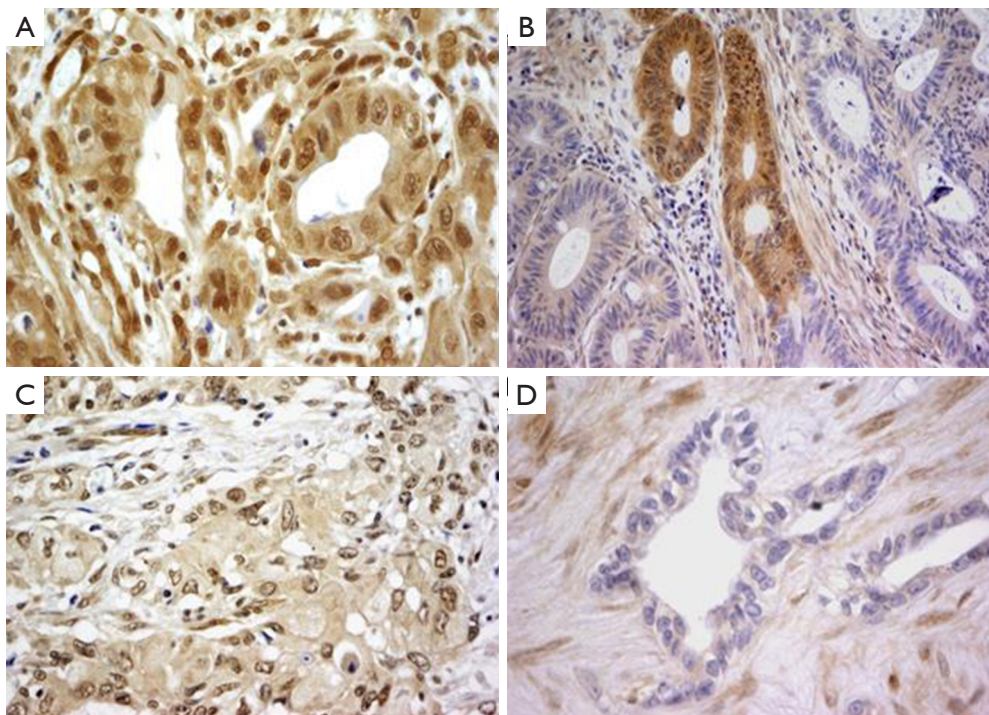


Figure 2 SMAD4 immunohistochemical studies (IHC) expression patterns in ampullary adenocarcinoma. (A) Diffuse (strong staining comparable to surrounding pancreas); (B) focal (two cell population with a subset of clearly negative cells) (100 \times); (C) trace (weak reactivity relative to surrounding pancreas); (D) absent (no staining). (A, C, D: 200 \times).

summarized in *Table 3*. Similar clinicopathologic features were seen among patients with absent or trace SMAD4 expression in comparison to patients with focal or diffuse SMAD4 expression (data not shown). Complete absence of SMAD4 by IHC was seen in 22 (35%) cases, showing a higher predominance of SMAD4 loss in pancreatobiliary type than in intestinal type tumors (48% *vs.* 22%). Fifty (79%) of ampullary adenocarcinomas had negative (absent, trace or focal) SMAD4 expression by IHC, and 52% were pancreatobiliary type, 22% intestinal type, and 26% other variants type.

Correlation of clinicopathologic variables with outcomes

Tumors with pancreatobiliary morphology showed a worse overall survival than tumors with intestinal and other variants type ($P=0.03$) (see *Table 3*, *Figure 3*).

Negative (absent, trace or focal) SMAD4 expression by IHC portrayed a trend for higher tumor stage, recurrence, and death, though none of these parameters was statistically significant (*Table 4*, *Figure 4*). After multivariate analysis, CK17 positivity and age >60 were found to be independent predictors of shorter survival ($P=0.0092$ and $P=0.0038$, respectively).

Discussion

SMAD4 is a nuclear transcription factor that is inactivated in a subset of malignant neoplasms of the pancreatobiliary tract and is an independent predictor of poor prognosis in pancreatic adenocarcinomas (16,18). Loss of SMAD4 is seen in 55% of pancreatic and distal common bile duct, 36% of ampullary, and 15% of proximal bile duct carcinomas (13,20). In colorectal cancer, loss of SMAD4 expression correlated with poor survival, but did not correlate with tumor stage, type, or growth pattern (21). In gliomas, higher WHO grade and lower survival rates were seen in patients without SMAD4 staining (22).

The role of *SMAD4* in signaling and tumor progression is more complex. For instance, nuclear and cytoplasmic expression of SMAD4 in prostatic adenocarcinoma was shown to correlate with aggressive tumor behavior (higher grade, higher stage, and postsurgical biochemical recurrence) (23). In breast carcinoma, a trend of longer survival was noted in SMAD4-negative patients (24). Also, strong SMAD4 expression was found to be an independent factor for predicting malignant transformation in oral hairy leukoplakia (25).

Table 2 Clinicopathologic characteristics of ampullary adenocarcinoma cases

Parameter	Value and range [%]
Age, years	
Mean [range]	68.1 [22–86]
≤60	13 [21]
>60	50 [79]
Gender	
Male	35 [55]
Female	28 [45]
Jaundice	
Present	31 [49]
Absent	32 [51]
Abdominal pain	
Present	22 [35]
Absent	41 [65]
CEA levels (n=25)	
Normal (≤4.3 ng/mL)	19 [76]
Elevated (>4.3 ng/mL)	6 [24]
CA 19.9 (n=35)	
Normal (≤35 U/mL)	18 [51]
Elevated (>35 U/mL)	17 [49]
Pathologic stage	
T1/T2	17 [27]
T3/T4	46 [73]
Lymph node status	
Negative	34 [54]
Positive	29 [46]
Recurrence (n=33)	
No	19 [58]
Yes	14 [42]

Immunohistochemistry for SMAD4 is a highly specific and sensitive marker for *SMAD4* gene inactivation. Tascilar *et al.* showed that uniform SMAD4 expression in pancreatic adenocarcinoma was associated with wild-type *SMAD4* gene, while absent or non-uniform (trace or focal staining) SMAD4 expression was associated with *SMAD4* gene inactivation, and correlated with worse survival (16). However, two of the four (50%) patients with focal SMAD4 expression in this study were wild-type for the *SMAD4* gene. Also, Wilentz *et al.* (26) found diffuse SMAD4 expression in the majority of wild-type *SMAD4* cases and loss of SMAD4 expression in *SMAD4* inactivated patients. However, one of the two patients with focal SMAD4 expression had wild-type *SMAD4* gene. These findings raise concern about the relationship between focal SMAD4 expression and gene status, and suggest a more complex mechanism of *SMAD4* gene regulation. In our cohort, similarity in tumor behavior in patients with diffuse and focal SMAD4 expression was noted (data not shown), but no statistical significance was found in relation to survival.

In our study, 35% of ampullary adenocarcinomas showed complete absence of SMAD4, similar in incidence to McCarthy *et al.* (13). However, we found that a substantial number of cases (n=28; 44%) showed either trace or focal expression of SMAD4 by IHC. We found a trend for worse survival and biologic behavior between patients with diffuse SMAD4 expression and patients with absent, trace, or focal SMAD4 expression. For example, the death rate was higher for patients with negative (absent, trace or focal) SMAD4 expression compared to patients with positive (diffuse) SMAD4 expression (62% *vs.* 31%). Although not statistically significant, the survival trend is limited by our small sample size and must be validated in a larger patient cohort. Higher pathologic stage (T3/T4) and

Table 3 Immunohistochemical profile and clinical outcomes of ampullary adenocarcinoma according to histopathologic subtype

Variable	Pancreatobiliary (n=31) [%]	Intestinal (n=18) [%]	Indeterminate (n=14) [%]
CK7 (+)	29 [94]	9 [50]	11 [79]
CK17 (+)	22 [71]	3 [17]	7 [50]
CK20 (+)	3 [10]	15 [83]	5 [36]
Negative SAMD4 (absent/trace/focal)	26 [84]	13 [72]	11 [79]
Stage T3/T4	26 [84]	10 [56]	10 [71]
LN metastasis	17 [55]	4 [22]	8 [57]
Recurrence	8 [47]	1 [14]	5 [56]
Deceased	20 [65]	6 [33]	6 [43]

LN, lymph node.

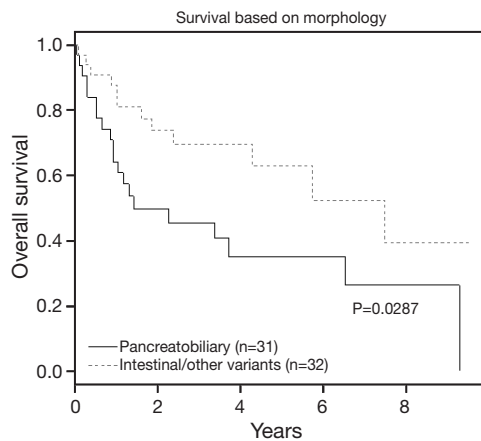


Figure 3 Overall survival based on histologic subtypes of ampullary adenocarcinoma.

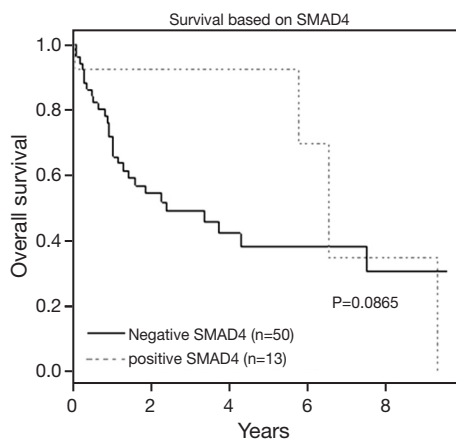


Figure 4 Overall survival based on SMAD4 immunohistochemical expression.

more recurrence were also noted in patients with negative SMAD4 expression in comparison to positive SMAD4 cases. Moreover, among patients who recurred, most had SMAD4 negative tumors (86%).

CK17, a low molecular weight keratin often used as a marker of pancreatobiliary origin, was expressed in 51% of the cases in our study. The majority of CK17-positive tumors demonstrated pancreatobiliary morphology by H&E (69%, $P=0.002$) and showed a higher incidence of LN metastasis (59%, $P=0.04$). A prior study showed greater CK17 expression in pancreatobiliary-type ampullary adenocarcinoma, but the relationship between CK17 expression with survival was not evaluated (4). Since

Table 4 SMAD4 expression in relation to clinical variables

Variable	SMAD4		P value
	Negative (n=50)	Positive (n=13)	
Age			1.00
≤60	10	3	
>60	40	10	
Gender			0.35
Female	24	4	
Male	26	9	
Jaundice			0.54
No	24	8	
Yes	26	5	
Serum CEA (ng/mL)			0.60
≤4.3	15	4	
>4.3	4	2	
Serum CA 19-9* (U/mL)			0.04*
≤35	11	7	
>35	16	1	
Morphology			0.54
Pancreatobiliary	26	5	
Intestinal	11	3	
Other variants	13	5	
Stage			1.00
T1/T2	11	6	
T3/T4	39	7	
Recurrence*			0.67
No	14	5	
Yes	12	2	
Lymph node status			0.54
Negative	26	8	
Positive	24	5	
Current status			0.35
Alive	19	9	
Deceased	31	4	

*, denotes statistical significance.

CK17 expression appears to be an independent predictor of shorter survival, this marker may be useful in risk stratification of ampullary adenocarcinomas.

In summary, our study demonstrated poorer outcomes for ampullary adenocarcinoma containing pancreaticobiliary morphology and negative SMAD4 expression. Negative SMAD4 expression was associated with higher tumor stage, recurrence, and death. Additional, larger scale studies are needed to validate the prognostic significance of SMAD4 in

ampullary adenocarcinoma.

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Footnote

Conflicts of Interest: Abstract was presented at the 102nd Annual United States and Canadian Academy of Pathology meeting, Baltimore, MD, March 2013.

Ethical Statement: This study was approved by the University of Florida Institutional Review Board and ethics committee (FWA00005790) with a waiver of informed consent given the de-identified nature of the data collected (Protocol ID# 452-2011).

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