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Validation of histomolecular classification utilizing histological subtype, MUC1, and CDX2 for prognostication of resected ampullary adenocarcinoma

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Background: Outcomes for ampullary adenocarcinomas are heterogeneous, and numerous methods of categorisation exist. A histomolecular phenotype based on histology, caudal-type homeodomain transcription factor 2 (CDX2) staining and Mucin 1 (MUC1) staining has recently been tested and validated in two cohorts. We attempt to validate this classification in a large patient population.

Methods: Tissue samples from 163 patients with resected ampullary adenocarcinoma were classified based on histology and immunohistochemical expression of CDX2 and MUC1. A pancreaticobiliary histomolecular classification (PB) was defined as a sample with pancreaticobiliary histology, positive MUC1 and negative CDX2 expression.

Results: There were 82 deaths; median follow-up of 32.4 months; and median overall survival of 87.7 (95% CI 42.9–109.5) months. PB comprised 28.2% of the cases. Factors associated with overall survival were histological subtype ($P=0.0340$); T1/2 *vs* T3/4 ($P=0.001$); perineural ($P<0.0001$) and lymphovascular ($P=0.0203$) invasion; and histomolecular intestinal histomolecular phenotype (INT) *vs* PB phenotype (106.4 *vs* 21.2 months, $P<0.0001$). Neither MUC1 nor CDX2 was statistically significant, although MUC1 positivity defined as $\geqslant 10\%$ staining was significant ($P=0.0023$). In multivariate analysis, age (HR 1.03), PB phenotype (HR 2.26) and perineural invasion (PNI; HR 2.26) were associated with poor survival.

Conclusions: The prognostic ability of histomolecular phenotype has been validated in an independent cohort of ampullary adenocarcinoma patients.

The ampulla of Vater represents a small anatomic region into which three different epithelia (pancreatic, duodenal and biliary) converge. Owing to this fact, the exact tissue of origin responsible for ampullary adenocarcinomas has been uncertain. The known heterogeneity of clinical behaviour has led to a number of investigators to explore various histological and molecular characteristics in an attempt to prognostically stratify ampullary adenocarcinomas. (Yeo *et al*, 1998; Bouvet *et al*, 2000; O'Connell

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et al, 2008; Smith *et al*, 2008; Albores-Saavedra *et al*, 2009; Berberat *et al*, 2009; Hatzaras *et al*, 2010; Ang *et al*, 2014).

Kimura *et al.* (1994) were the first investigators to subclassify ampullary adenocarcinoma based on histologic features (intestinal *vs* pancreaticobiliary) and noted a survival advantage for those with intestinal phenotype. Over the past two decades, multiple investigators have sought to improve upon the histologic classification (Kitamura *et al*, 1996; Seno *et al*, 2002; Zhou *et al*, 2004; Hansel *et al*, 2005; Chang *et al*, 2007; Roh *et al*, 2007; de Paiva Haddad *et al*, 2010; Moriya *et al*, 2011; Ang *et al*, 2014). Intestinal phenotype tends to stain for MUC2, CK20, CDX2 (caudal-type homeodomain transcription factor 2) and occasionally CEA and CD10. In contrast, pancreaticobiliary phenotype stains positively for Mucin 1 (MUC1), CK7 and MUC5A. In general, patients with cancers whose features are more aligned with intestinal phenotype fare better than those with elements of either biliary or pancreatic phenotype (Kimura *et al*, 1994; Kitamura *et al*, 1996; Zhou *et al*, 2004; Hansel *et al*, 2005; Chang *et al*, 2007; Roh *et al*, 2007; Sessa *et al*, 2007; Westgaard *et al*, 2008; de Paiva Haddad *et al*, 2010; Moriya *et al*, 2011). In particular, positive staining for MUC2 and CDX2 tended to correlate with better survival, whereas MUC1 positivity correlated with worse survival. However, most studies included relatively few patients with variable definitions for intestinal and pancreaticobiliary subsets and were not validated in independent cohorts.

Recently, Chang *et al.* (2013) used histology, CDX2 and MUC1 expression to classify patients with resected ampullary cancer. In their study, the pancreaticobiliary histomolecular phenotype (PB) was defined as having pancreaticobiliary histology, any MUC1 staining and negative CDX2 staining defined as a CDX2 H-index $\leqslant 35$. Cases not meeting this definition were considered intestinal histomolecular phenotype (INT). PB phenotype and lymph node positivity were both risk factors for poor overall survival (OS) in multivariate analysis, and these factors were verified across two separate validation cohorts. MUC1 is a transmembrane glycoprotein that is expressed in 66–98% of pancreatic adenocarcinomas and cholangiocarcinomas (Yonezawa *et al*, 2011), whereas CDX2 is a transcription factor that has a role in early intestinal differentiation (Silberg *et al*, 2000) and is commonly expressed in intestinal adenocarcinomas but infrequently seen in pancreatic (5%) or extrahepatic cholangiocarcinoma (5–22%; Hansel *et al*, 2005; Chang *et al*, 2007; Jun *et al*, 2014).

We sought to confirm the results obtained by Chang *et al.* (2013) in a large, independent cohort of patients with ampullary cancer. The ultimate aim of such an approach would be to establish a reliable, inexpensive method to provide better prognostication for resected ampullary adenocarcinomas.

MATERIALS AND METHODS

Patients. Tissue samples from 163 patients with resected ampullary adenocarcinoma from MD Anderson Cancer Center (MDACC; $N=111$) and from the Johns Hopkins Hospital (JHH; $N=52$) from 1992 until 2007 were obtained and analysed as outlined below. The patient variables, including demographic information, comorbidities, treatment course and outcome were obtained from the electronic medical records. This study was approved by the respective institutional review boards.

Sample selection. Tissue microarrays (TMAs) of tumour and nearby normal tissue were created from formalin-fixed, paraffin-embedded tumour blocks of 163 patients who previously underwent pylorus preserving pancreaticoduodenectomy for an ampullary adenocarcinoma at JHH and MDACC as previously described (Van Heek *et al*, 2004; Overman *et al*, 2013). In all cases, the original H&E slides from each surgical resection were reviewed to confirm the diagnosis of ampullary adenocarcinoma. H&E slides were also analysed by two Gastrointestinal Pathologists (HW and MG), and tumours were categorised based on whether the tissue appeared intestinal-like (tall columnar cells with elongated nuclei), pancreaticobiliary-like (rounded cells with rounded nuclei with scant fibrous cores) or mixed ($>10\%$ of each). Mixed histology was further simplified and categorised based on the predominant tissue component.

Immunohistochemistry. Immunohistochemical (IHC) stains were performed on 5-μm unstained sections from the TMA blocks. To retrieve the antigenicity, the tissue sections were treated at 100 °C in a steamer containing 10 mmol citrate buffer (pH 6.0) for 60 min. The sections were then immersed in methanol containing 0.3% hydrogen peroxidase for 20 min to block the endogenous peroxidase activity and were incubated in 2.5% blocking serum to reduce nonspecific binding. Sections were incubated for 90 min at 37 °C with primary antibodies: CDX2 (Biogenex, San Ramon, CA, USA; clone CDX-88) and MUC 1 (clone Ma695, Novocastra, Newcastle, UK). Standard avidin–biotin IHC analysis of the sections was performed according to the manufacturer's recommendations (Vector Laboratories, Burlingame, CA, USA). MUC1 staining was graded as 0 (negative), 1 (very focally positive, 1–9%), 2 (focally positive, 10–50%) and 3 (positive $>50\%$). A positive stain for MUC1 was defined as any positive staining (that is, 1, 2 or 3). Positive CDX2 samples were defined by an *H*-score $>35\%$, where H-score = intensity of staining [0 to 3] $\times$ percentage of positive cells. Both definitions of MUC1 and CDX2 staining match those of Chang *et al.* (2013). IHC staining was interpreted independently by two gastrointestinal pathologists (HW and NN for CDX2 and VA and BS for MUC1) with any differences resolved by consensus review. A pancreaticobiliary histomolecular phenotype (PB) is defined as a tumour with pancreaticobiliary histology, CDX2 negativity and any MUC1 positivity.

Statistics. Association between categorical clinical variables was determined by the Fisher's exact test. Survival curves were generated using the Kaplan–Meier method, and survival differences were determined with the log-rank test. The univariate Cox proportional hazards regression model for OS tested age, histological subtype, MUC1 staining, CDX2 staining, T stage, LN status, perineural invasion (PNI), lymphovascular invasion (LVI), neoadjuvant or adjuvant treatment and histomolecular phenotype. Cox proportional hazards models were fitted for multivariate analysis. After interactions between variables were examined, a backward stepwise procedure was used to derive the best-fitting model. The correlation coefficient (κ) was calculated according to the method of Cohen to estimate the interobserver agreement in MUC1 staining.

RESULTS

Cohort characteristics. Characteristics of the combined MDACC and JHH ampullary cohort are summarised in Table 1. Males represented 58.8% of cases. Minorities were under-represented as 4.9% were African American. The mean age was 64.4 years (range 28–88 years old). There were 82 events (death) out of 163 (50.3%) patients analysed during the study period. The median follow-up for the entire cohort was 32.4 (0.3–189.5) months. The median OS was 87.7 (95% CI 42.9–109.5) months. The 3- and 5-year OS estimates were 62.8% (95% CI 54.4–70.1%) and 54.4% (95% CI 45.6–62.2%), respectively, for all patients.

Clinicopathological and molecular prognostic factors. A PB histomolecular phenotype comprised 28.2% of the cases analysed. In univariate analysis, factors that were significantly associated with the median OS were intestinal *vs* pancreaticobiliary histology (109.5 *vs* 43.4 months, $P=0.0340$); T stage of 1 or 2 compared

Table 1. Clinicopathological parameters and outcome

Variable	No.	%	Median OS (months)	*P*-value
Median Age, years (range)			65 (28–88)	
Sex				
Male	96	58.8	108.3	0.24
Female	67	41.1	43.4	
Race				
African American	8	4.9	NE	0.85
Non-African American	155	95.1	87.7	
Histology				
INT	50	30.7	108.3	0.02
PB	75	46	36.4	
Mixed	38	23.3	171.6	
Histology, two tier[a]				
INT	70	42.9	109.5	0.03
PB	93	57.1	43.4	
T stage[b]				
1	21	13.5	N.E.	0.0006
2	60	38.7	106.4	
3	64	41.3	28.5	
4	10	6.5	63.8	
T stage[b]				
T1 or T2	81	52.3	114.1	0.0001
T3 or T4	74	47.7	32.7	
Lymph node				
Negative	71	44.1	108.3	0.07
Positive	90	55.9	61.8	
Perineural invasion				
Absent	110	71.4	100.1	<0.0001
Present	44	28.6	24.4	
Lymphovascular invasion				
Absent	98	64.5	106.4	0.02
Present	54	35.5	25.3	
Neoadjuvant therapy				
Yes	18	11.0	87.7	0.54
No	145	89.0	75.1	
Adjuvant therapy[c]				
Yes	43	39.5	146.2	0.82
No	48	44.0	100.0	
CDX2 expression				
H-score < 35	89	54.6	61.8	0.47
H-score ⩾ 35	74	45.4	98.1	
MUC1 expression				
Negative	56	34.4	106.4	0.05
Positive	107	65.6	43.4	
Histomolecular phenotype				
Intestinal	117	71.8	106.4	<0.0001
Pancreaticobiliary	46	28.2	21.2	

Abbreviations: INT, intestinal; JHH, Johns Hopkins Hospital; MDACC, MD Anderson Cancer Center; MUC1, Mucin 1; NE, not estimable; OS, overall survival; PB, pancreaticobiliary.
[a]Mixed histology classified by predominant histological type.
[b]Surgical stage (one case upon review was categorised as carcinoma *in situ*).
[c]Neoadjuvant and adjuvant therapy data were only available for the MDACC cohort and was fluropyrimidine-based in 46 cases. Note, JHH did not use neoadjuvant therapy; therefore, their cases were included in the neoadjuvant 'no' category.

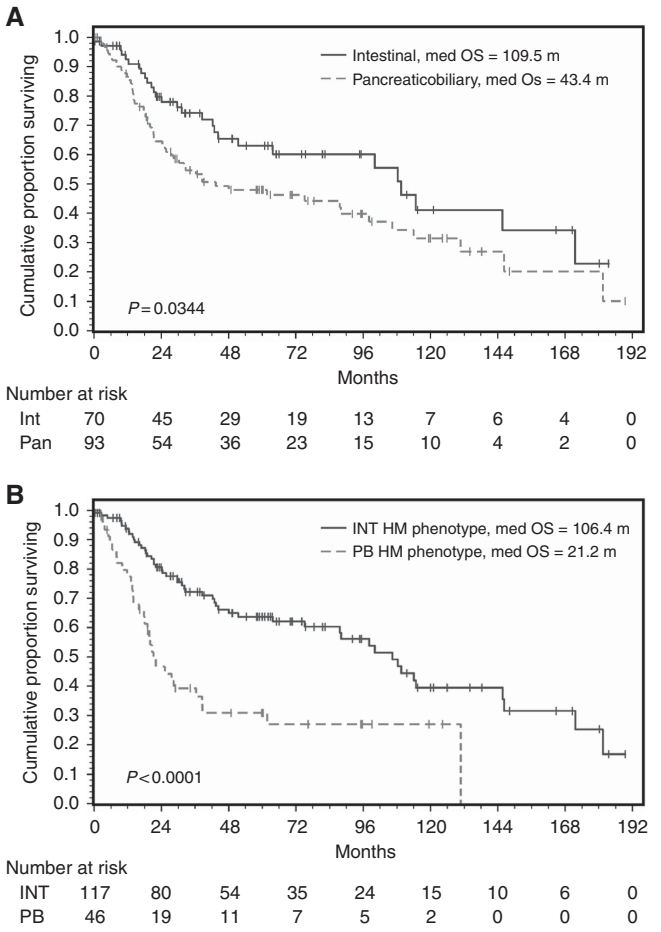

Figure 1. Kaplan–Meier curves estimating survival for patients based on overall histology (**A**) and histomolecular criteria (**B**).

with 3 or 4 (114.1 *vs* 32.7 months, $P = 0.0001$); absence of PNI compared with its presence (100.1 months *vs* 24.4, $P < 0.0001$); absence of LVI compared with its presence (106.4 *vs* 25.3 months, $P = 0.0203$); and INT *vs* PB histomolecular phenotype (106.4 *vs* 21.2 months, $P < 0.0001$). Figure 1 shows the Kaplan–Meier curves comparing the ability of histological subtype *vs* histomolecular phenotype to classify ampullary adenocarcinomas. Lymph node status, CDX2 staining and MUC1 staining did not statistically correlate with OS. Lymph node positivity did correlate with histomolecular subtype as 49% of INT histomolecular phenotype were lymph node-positive, whereas 75% of PB histomolecular phenotype were lymph node-positive, $P = 0.004$. A total of 28 cases with PB histology were reclassified as INT histomolecular phenotype due to either expression of CDX2 or lack of MUC1 expression. Survival for both groups was improved in comparison with the PB histomolecular phenotype cohort (Supplementary Figure 1).

A multivariate Cox proportional hazards model was used to assess the effect of multiple factors on OS. There were 154 patients available for the final analysis. Age (HR 1.03, 95% CI 1.01–1.06), PB histomolecular phenotype (HR 2.26, 95% CI 2.40–3.65) and PNI (HR 2.26, 95% CI 1.39–3.68) were the factors associated with OS in this analysis (Table 2). The T stage did not remain a significant factor in the multivariate model, although PNI and T stage were strongly correlated with 7.7% of T1 or T2 cases having PNI, whereas 52.1% of T3 and T4 having PNI ($P < 0.0001$ by Fisher's exact test).

CDX2 and MUC1 cutpoint analysis. To explore the lack of statistically significant correlation of MUC1 and CDX2 in univariate analysis conducted above, optimal cutpoints for these two markers were explored. Graphical analysis of *H*-index scores for CDX2 demonstrated that an H-score of >35 was acceptable (Supplementary Figure 2). For MUC 1, a positive MUC1 stain of $\geqslant 10\%$ was statistically significant with the median

Table 2. Multivariate analysis for overall survival

Variable	HR	95% CI	*P*-value
Perineural invasion	2.26	1.39–3.68	0.001
PB histomolecular phenotype	2.26	2.40–3.65	0.0009
Age	1.03	1.01–1.06	0.016

Abbreviations: CI, confidence interval; HR, hazard ratio; PB, pancreaticobiliary.

OS of 32.7 *vs* 109.5 months, $P = 0.0023$ (Supplementary Table 1). In contrast to any positive staining, the MUC1-positive population reduced from 66 to 54% using the $\geqslant$10% criterion. Incorporating this criterion within the histomolecular classification resulted in a PB histomolecular phenotype representing 25.7% of the study population with a median OS of 21.1 months compared with 108.3 m for the INT histomolecular phenotype, $P < 0.0001$. In addition, utilising this MUC1 criterion resulted in an improved hazard ratio in multivariate modelling of 2.59 (95% CI 1.57–4.29) for the PB *vs* INT histomolecular phenotype (Supplementary Table 2). There was good intraobserver agreement (kappa = 0.69) for MUC1 interpretation utilising a $\geqslant$10% threshold.

DISCUSSION

In this study we validate the histomolecular classification by Chang *et al* (2013) in a large data set. Our results lend support to the clinical use of this new classification for ampullary adenocarcinomas. Utilising this histomolecular classification allows the identification of a particularly aggressive cohort of patients (PB), which comprised 28.2% of our patient population. Given the inherent challenges of an IHC criteria of 'any positive staining' along with our data demonstrating improved prognostication with a MUC1 positivity defined as $\geqslant$10% staining, we propose this definition for MUC1 positivity when applied to histomolecular subtyping of ampullary adenocarcinomas. Using this criterion, 25.2% of our population had a PB histomolecular phenotype with a median OS of 21.1 months in contrast to 108.3 months for the INT histomolecular phenotype, $P < 0.0001$.

In the prior report by Chang *et al.* (2013) the MUC1 positivity rate varied from 42.7 to 67.4% across the three studied cohorts. In two cohorts, reviewed by the same pathologists, the rates were 67.4 and 59.7%, whereas in the third cohort, reviewed by a separate group of pathologists, the rate was 42.7%. In our opinion, this variation reflects both the challenges with applying an 'any positive staining' criteria to IHC review and the difficulty with MUC1 staining in general. As we found increasing MUC1 staining to correlate with worse outcomes, we propose that MUC1 positivity be defined at a $\geqslant$10% tumour-staining criteria. This criterion is in alignment with prior work studying ampullary neoplasms in which a 10% threshold for MUC1 staining was utilised (Ohike *et al*, 2010).

Classifying ampullary tumours remains a challenge, and there is a reasonable level of disagreement by histology. In a recent study of 105 ampullary carcinoma cases concordance utilising histology alone was suboptimal with concordance among four gastrointestinal pathologists of $\kappa = 0.53$ for intestinal subtype, $\kappa = 0.48$ for pancreaticobiliary subtype, and $\kappa = 0.09$ for mixed subtype (Ang *et al*, 2014). In this study the authors propose the use of an IHC panel including CDX2, MUC1, MUC2 and CK20 to aid in cases that are histologically ambiguous. However, 18% of cases could not be classified by IHC, and no outcome data were reported. Thus, despite the presence of multiple studies evaluating various IHC markers for prognostication of ampullary carcinomas, the validation of this study of the prognostic impact of the histomolecular criteria has demonstrated these criteria to be a reliable methodology to prognosticate ampullary carcinomas.

What this study does not address is whether there is a better method to classify these tumours based on an assay that can be considered closer to their underlying biology. Lessons from breast cancer may serve as a relevant example, in which subtypes differing based upon the cell of origin (luminal A, luminal B, ERBB2-overexpressing, basal, and so on) can be grouped based on gene expression-based classification (Perou *et al*, 2000), which corresponds fairly well with IHC detection of the key predictive markers such as the oestrogen receptor (ER) and HER2/neu. Although the biological understanding of ampullary adenocarcinomas is limited, a recent study that performed unsupervised gene expression clustering of ampullary adenocarcinomas identified two subgroups best classified as intestinal-like and biliary-like groups that were distinct from pancreatic adenocarcinoma (Overman *et al*, 2013). Such work supports the two-tiered classification of ampullary adenocarcinomas as defined in this study. Future efforts – perhaps using genomics or proteomics – to understand the molecular and biological basis of the histomolecular subgroups are needed.

Owing to the large sample size, TMAs were utilised for staining assessment, and despite the use of multiple cores per case, the possibility of variations in staining across the full tumour specimen cannot be determined. However, previous studies have shown that the results of TMA-based studies with two to three cores from each tumour are comparable to those using full sections. The suboptimal interobserver agreement for ampullary histological subtype determination that has been reported represents a limitation of the histological subtype-based prognostic classifier validated in this report. Our efforts to identify an optimal cutpoint for both CDX2 and MUC1 represent unvalidated findings and future efforts to further validate the optimal criteria for MUC1 positivity are needed.

How this study aids clinical decision-making regarding the choice of therapy – both in adjuvant and advanced settings – is an open question. The randomised phase III European Study Group for Pancreatic Cancer (ESPAC)-3 enroled resected periampullary adenocarcinomas, of which 297 were ampullary carcinomas, and randomized patients to observation *vs* chemotherapy (either bolus 5-FU or gemcitabine; Neoptolemos *et al*, 2012). After adjusting for prognostic factors, adjuvant chemotherapy improved OS, HR 0.75, $P = 0.03$. However, differences by chemotherapy type were not seen and stratification by histological subtype was not reported as histological subtype determination was only carried out on 162 (55%) of the ampullary cases (Neoptolemos *et al*, 2012; Overman *et al*, 2012). Within this subgroup of ampullary cases an intestinal subtype demonstrated an improved DFS ($P = 0.01$), but not OS ($P = 0.28$). As in the ESPAC-3 study, other studies investigating ampullary subtypes had not investigated the predictive impact of such subtypes with regard to the type of chemotherapy. Despite this lack of data, the concept of ampullary subtype determined adjuvant chemotherapy (for example, FOLFOX for INT histomolecular phenotype and gemcitabine/cisplatin for PB histomolecular phenotype) has merit and should be explored in a clinical trial. In the metastatic setting the utilisation of histomolecular subtype determination with regard to the choice or sequence of systemic chemotherapy agents would represent a reasonable approach, given the limited studies and lack of consensus guidelines regarding systemic chemotherapy for metastatic ampullary carcinoma (Valle *et al*, 2010).

In conclusion, we validate the prognostic utility of a histomolecular classification of ampullary adenocarcinomas. This combination of histological and IHC classification of ampullary adenocarcinomas should be incorporated into clinical practice. Defining MUC1 positivity as $\geqslant$10% staining should provide improved objectivity. Future efforts to understand the biological bases of ampullary subgroups are needed as is the incorporation of these subgroups into any future clinical trials.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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