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 ≥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...
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 ≤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 cholangiocarcinomas (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.

**RESULTS**

**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 peroxide 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 MUC1 (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] × percentage of positive cells. Both definitions of MUC1 and CDX2 staining matched 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.

**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 (JHII; 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 JHII 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 round nuclei with scant fibrous cores) or mixed (>10% of each). Mixed histology was further simplified and categorised based on the predominant tissue component.
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 statistically significant factor in the multivariate model, although PNI and T stage were strongly correlated with 7.7% of T1 or T2 cases having PNI (HR 2.26, 95% CI 1.39–3.68) were the factors associated with OS (HR 2.26, 95% CI 2.40–3.65) and histomolecular criteria (B).

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 \( \geq 10\% \) was statistically significant with the median

| 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**       |     |     |                    |         |
| INT                           | 70  | 42.9| 109.5              | 0.03    |
| PB                            | 93  | 57.1| 43.4               |         |
| **T stage**                   |     |     |                    |         |
| 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**                   |     |     |                    |         |
| 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**          |     |     |                    |         |
| 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 \( \geq 35 \)         | 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, Muco 1; NE, not estimable; OS, overall survival; PB, pancreaticobiliary. 

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

Figure 1.
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 \( \geq 10\% \) 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 \( \geq 10\% \) 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 \( \geq 10\% \) 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 the MUC1 positivity defined as \( \geq 10\% \) tumour-staining criteria. In 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 cutoff 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 enrolled 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. A historical 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 \( \geq 10\% \) 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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**Table 2. Multivariate analysis for overall survival**

| Variable                  | HR   | 95% CI          | \( P \)-value |
|---------------------------|------|-----------------|---------------|
| Perineural invasion       | 2.26 | 1.39–3.68       | 0.0001        |
| 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.
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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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