Combining clinicopathological predictors and molecular biomarkers in the oncogenic K-RAS/Ki67/HIF-1α pathway to predict survival in resectable pancreatic cancer

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Background: The dismal prognosis of patients diagnosed with pancreatic cancer points to our limited arsenal of effective anticancer therapies. Oncogenic K-RAS hyperactivation is virtually universal in pancreatic cancer, that confers drug resistance, drives aggressive tumorigenesis and rapid metastasis. Pancreatic tumours are often marked by hypovascularity, increased hypoxia and ineffective drug delivery. Thus, biomarker discovery and developing innovative means of countervailing oncogenic K-RAS activation are urgently needed.

Methods: Tumour specimens from 147 pancreatic cancer patients were analysed by immunohistochemical (IHC) staining and tissue microarray (TMA). Statistical correlations between selected biomarkers and clinicopathological predictors were examined to predict survival.

Results: We find that heightened hypoxia response predicts poor clinical outcome in resectable pancreatic cancer. SIAH is a tumour-specific biomarker. The combination of five biomarkers (EGFR, phospho-ERK, SIAH, Ki67 and HIF-1α) and four clinicopathological predictors (tumour size, pathological grade, margin and lymph node status) predict patient survival post surgery in pancreatic cancer.

Conclusions: Combining five biomarkers in the K-RAS/Ki67/HIF-1α pathways with four clinicopathological predictors may assist to better predict survival in resectable pancreatic cancer.

Pancreatic cancer is the fourth leading cause of cancer-related death in the United States with a dismal 5-year survival rate at 5–6% (Siegel et al, 2013). Clinical symptoms associated with pancreatic ductal adenocarcinomas (PDAC) manifest by late diagnosis, local invasion and systemic metastasis—culminating in dismal prognosis and poor survival (Wolfgang et al, 2013). Surgical
Hypoxia reduces prognosis in pancreatic cancer

resection offers the best hope for increased survival if the disease is discovered before local and distant metastasis (Sohn et al., 2009). Adjuvant therapies confer limited or modest benefit (Conroy et al., 2011; Valle et al., 2014). As a result, there is an urgent need to discover new biomarkers aiding in prognosis and to develop curative strategies against pancreatic cancer (Maitra and Hruban, 2008).

The cancer genome-sequencing project reaffirmed the paramount importance of oncogenic K-RAS activation in human cancer (Vogelstein et al., 2013). The mitogen-activated protein kinase (MAPK/ERK) cascade is often activated in human cancer (Sebolt-Leopold and Herrera, 2004). Previous studies suggested that upregulation in phospho-ERK (that is, active ERK) is associated with reduced survival in pancreatic cancer (Javle et al., 2007). Ectopic expression of EGFR in pancreatic cancer has been linked to accelerated tumour growth, increased metastasis and poor survival (Ardito et al., 2012). Pancreatic cancer is well known to be desmoplastic, hypovascularised and treatment-resistant (von Hoff et al., 2009), and increased expression of hypoxia-inducible factor 1α (HIF-1α) may alter cellular metabolism, aid tumour growth and increase cancer survival in the hypoxic and nutrient-deprived tumour environment (Akakura et al., 2001; Sivak-Kroizman et al., 2013).

Many genes have been evaluated as prognostic biomarkers for pancreatic cancer, but most were unreliable and inconsistent in clinical validations (Sawyers, 2008; Costello et al., 2012; Winter et al., 2013). As tumorigenesis requires multiple molecular alterations to progress, a logical combination of several biomarkers in the main tumour-promoting signalling pathways may improve the prognostic accuracy. Here, we examined the prognostic value of six biomarkers: upstream receptors (EGFR and HER2), midstream kinase (phospho-ERK) and K-RAS downstream signalling ‘gatekeeper’ (SIAH E3 ligase; Schmidt et al., 2007; Ahmed et al., 2008), tumour proliferation index (Ki67) and hypoxic response indicator (HIF-1α) in human pancreatic cancer. Along with four clinicopathological predictors, we focused on the oncogenic K-RAS/Ki67/HIF-1α pathways by evaluating the prognostic value of these six aforementioned biomarkers. SIAH is a new tumour-specific biomarker in pancreatic cancer. We report that combining the five biomarkers (EGFR, phospho-ERK, SIAH, HIF-1α and Ki67) together with the four existing clinicopathological predictors (tumour size, pathological grade, margin and lymph node status) is clearly predictive for patient survival. Importantly, an increased expression of HIF-1α predict poor prognosis in pancreatic cancer.

MATERIALS AND METHODS

Ethical statement. This study was conducted with the ethical committee approval by the Mayo Clinic Institutional Review Board. Informed consent was obtained from pancreatic cancer patients before their surgeries at the Mayo Clinic.

Patients. Patients who underwent surgical resection for PDAC at the Mayo Clinic in Rochester, MN between 1985 and 2001 were included in this study (n = 147). Patients underwent either pancreaticoduodenectomy (n = 129), distal pancreatectomy (n = 8) or total pancreatectomy (n = 10). Clinical and pathologic variables were extracted from the patients’ medical records (Table 1). Nineteen patients (12.9%) were lost to follow-up; survival times were censored at the time of last known contact (2007).

Pathologic samples/tissue microarray (TMA). Biospecimens from 134 pancreatic cancer patients were used to construct this TMA. Owing to tumour heterogeneity, each patient’s tumour specimen was represented by three distinct, pathologically confirmed tumour cores (diameter = 0.6 mm) on the TMA. The numbers of TMA tissue cores and patients used in the statistical analyses are summarised (Table 2). Tumour biospecimens from 13 pancreatic cancer patients at distinct pathological stages (Hruban et al., 2000) were used to validate the TMA results. Normal pancreas and pancreatitis tissues were served as negative controls. All tissue cores and histology sections were carefully reviewed by an expert pathologist in a double blind manner and classified into normal pancreas (n = 7), pancreatitis (n = 20), PanIN-1 (n = 35), PanIN-2 (n = 20), PanIN-3 (n = 15), and adenocarcinoma ADCA 2 (n = 84), ADCA 3 (n = 161) and ADCA 4 (n = 33).

Five-micrometre sections were cut from each TMA slide and deparaffinised using standard techniques, and then placed in 1.0 mM EDTA (pH 8.0) for 30 min at 100 °C for antigen retrieval. Staining was conducted using monoclonal anti-EGFR antibody (pre-diluted, Dako, Glostrup, Denmark), monoclonal anti-phospho-ERK (1 : 750 dilution, Cell Signaling, Danvers, MA, USA), monoclonal anti-HER2 (pre-diluted, Dako), monoclonal anti-HIF-1α (1 : 250 dilution, Novus Biologicals, Littleton, CO, USA), monoclonal anti-Ki67 (1 : 100 dilution, Dako) and monoclonal anti-SIAH antibodies (1 : 40 dilution, Novus Biologicals) (Schmidt et al., 2007). A board-certified pathologist, with 32 years of experience in pancreatic cancer, scored and reviewed TMA slides and immunohistochemical (IHC) staining. Percent staining scores were measured for each core. Scores were averaged across three tissue cores from the same tumour to yield a single percent staining score representing each cancer patient. All IHC images were captured (×400) using Leica compound microscope and DC500 camera.

Statistical analysis. Descriptive statistics including the mean (s.d.), median (range) and frequency summarise the data (Table 1). Spearman’s correlation coefficients were computed to explore the correlations among clinicopathological variables and biomarker variables (Table 3). Survival time was calculated in days from the date of surgery to the date of death, or censored at the date of final contact as of 1 October 2007 if patients were lost to follow-up. Survival curves were analysed by the Kaplan–Meier method. In univariate analysis, the log-rank test was used to calculate the difference in survival for categorical variables, whereas the Cox proportional hazards (PH) model was used to examine the effect on survival for continuous variables. Multivariate survival analysis was carried out using the Cox PH models after controlling for the four clinicopathological predictors. The PH assumptions were verified by using the log-transformation plot and the goodness-of-fit of the models were checked by martingale and deviance residual plots. The time-dependent receiver operating characteristic (survival ROC) was applied to display and compare the sensitivity and specificity of the predictive models based on the multivariable survival analysis (Heagerty and Zheng, 2005). The survival ROC curves were used to accommodate the time-dependent nature and censoring in the survival data. All P-values were two-sided. A P-value of less than 0.05 was considered statistically significant. The statistical plots were generated using the SAS software Version 9.1.3 (SAS Institute Inc, Cary, NC, USA) and R software Version 2.6.1 (R Foundation for Statistical Computing, Vienna, Austria).

RESULTS

SIAH specifically decorates proliferating tumour cells in human pancreatic cancer. As the most downstream signalling ‘gatekeeper’ identified in the K-RAS pathway (Figure 1A), SIAH is a logical and potent anti-K-RAS drug target in cancer (Schmidt et al., 2007; Ahmed et al., 2008). SIAH-positive cells in pancreatic tumours are of epithelial origin in adult pancreas, and SIAH has a
predominantly nuclear expression pattern, highly specific to tumour cells (Figure 1B and D). SIAH staining was not detected in the normal pancreas, pancreatitis tissues, tumour stroma and infiltrating immune cells (Figure 1D and data not shown). Numbers of SIAH-positive tumour cells increase as human pancreatic tumours progress through well-defined neoplastic stages—PanIN-1, PanIN-2, PanIN-3 and ADCAs (Figure 1B, D and 2B), correlating SIAH expression with active tumour proliferation. Therefore, SIAH-dependent proteolysis in proliferating tumour cells may facilitate oncogenic K-RAS signalling.

| Table 1. Demographic, clinical and biomarker variables for pancreatic cancer-patient survival |
|---------------------------------|-----------------|-----------------|--------|------------------|
| Variables                       | Level           | Mean/frequency  | s.d./% | P-value*         |
| Age at surgery (year)           | Mean            | 63.1            | 12.0   | 0.077           |
| Gender                          | Male            | 80              | 54.4%  | 0.530           |
|                                 | Female          | 67              | 45.6%  |                  |
| Surgical procedure              | Standard pancreaticoduodenectomy | 77 | 52.4% | 0.542          |
|                                 | Pylorus-preserving pancreaticoduodenectomy | 52 | 34.4% |                  |
|                                 | Distal pancreatectomy | 8  | 5.4%  |                  |
|                                 | Total pancreatectomy | 10 | 6.8%  |                  |
| Complete resection              | Missing         | 1               |        | 0.584           |
|                                 | No              | 19              | 13%    |                  |
|                                 | Yes             | 127             | 87%    |                  |
| Tumour size                     | Mean            | 2.9             | 1.15   | 0.164           |
|                                 | £2 cm           | 44              | 20.9%  |                  |
|                                 | >2 cm           | 103             | 70.1%  |                  |
| Lymph node status               | N0              | 74              | 50.3%  | 0.343           |
|                                 | N1              | 73              | 49.7%  |                  |
| Positive margin                 | R0              | 127             | 86.4%  | 0.461           |
|                                 | R1/R2           | 20              | 13.6%  |                  |
| UICC stage                      | Stage IA        | 12              | 8.2%   | 0.703           |
|                                 | Stage IB        | 18              | 12.2%  |                  |
|                                 | Stage IIA       | 43              | 29.2%  |                  |
|                                 | Stage IIB/IV    | 74              | 50.4%  |                  |
| Tumour grade                    | 2               | 38              | 25.9%  | <0.001          |
|                                 | 3               | 73              | 49.7%  |                  |
|                                 | 4               | 36              | 24.5%  |                  |
| Extension                       | No              | 58              | 39.5%  | 0.284           |
|                                 | Yes             | 89              | 60.5%  |                  |
| Perineural invasion             | Missing         | 18              |        | 0.696           |
|                                 | No              | 74              | 57.4%  |                  |
|                                 | Yes             | 55              | 42.6%  |                  |
| Angiolymphatic invasion         | Missing         | 18              |        | 0.253           |
|                                 | No              | 98              | 76.0%  |                  |
|                                 | Yes             | 31              | 24.0%  |                  |
| Adjuvant therapy (chemotherapy and radiation therapy) | Missing | 7 | | 0.883 |
|                                 | No              | 26              | 18.4%  |                  |
|                                 | Yes             | 115             | 81.6%  |                  |
| Biomarkers                      | Number of patients | 123 | 2.79 | 0.556 |
|                                 | EGFR            | 127             | 42.30  | 0.235           |
|                                 | SIAH            | 119             | 39.33  | 0.171           |
|                                 | Ki67            | 131             | 36.22  | 0.185           |
|                                 | Phospho-ERK     | 100             | 38.27  | 0.005           |
|                                 | HIF-1α          |                 |        |                  |
| Survival                        | Median time (days) | 541 | (460 - | 615) |
| Vital status                    | Alive           | 19              | 12.9%  |                |
|                                 | Dead            | 128             | 87.1%  |                |

*Log-rank test for categorical variables and Cox proportional hazards model for continuous variables.
Hypoxia reduces prognosis in pancreatic cancer

The IHC staining of four signalling components in the K-RAS pathway. Tumour biospecimens were stained with anti-EGFR, anti-HER2, anti-phospho-ERK and anti-SIAH antibodies (Figure 1C and D, Table 2 and data not shown). The membrane receptors, EGFR and HER2 were evaluated by both extent and intensity of staining on a scale of 0–4 for their heterogeneous expression (Table 2). EGFR expression is summarised in Figure 1C and D and Table 2. Most tumour samples did not show appreciable HER2 expression (Table 2). Thus, HER2 was excluded in our statistical analyses. Phospho-ERK staining was scored by staining percentage on a scale of 0–100% positivity. Phospho-ERK expression was observed in the nuclei of tumour cells as well as some adjacent tumour stroma. SIAH staining was scored by percentage on a scale of 0–100% positivity (Figure 2F and Table 2). Representative HIF-1α staining in patients with distinct survival rates was shown (Figure 2D). The median expression level of HIF-1α was 40%. Patients expressing HIF-1α above 40% had a median survival time of 14.8 months, whereas patients expressing HIF-1α below 40% had a median survival time of 19.2 months with \( P = 0.004 \) (Figure 2C). Only 13.3% of patients with high HIF-1α expression survived 3 years or longer. Patients with HIF-1α below median 40% expression showed a statistically significant increase in 5-year survival compared with the patients with high HIF-1α expression (Figure 2C and D).

The IHC staining of Ki67 in resectable pancreatic cancer. The tumour proliferation index was measured and Ki67 staining was scored by percentage on a scale of 5–90% positivity (Figure 2F and Table 2). Only one patient did not display detectable Ki67 staining. Ki67 is expressed in tumour/cancerous cells; tumour stroma and immune cells showed no Ki67-positive staining. Ki67 expression increased with pathological grades (Figure 2F), marking proliferating tumour cells in pancreatic cancer. The median expression level of Ki67 was 40%. The median survival was 14.6 months for patients expressing Ki67 below 40% but 18.7 months for patients expressing Ki67 above 40% (Figure 2E). Ki67≥40% vs <40%; \( P = 0.046 \) (14.7 months vs 17.8 months; \( P = 0.046 \)). The results were not statistically significant (Table 2).

A combination of four clinicopathologic predictors and five biomarkers is associated with improved accuracy to predict patient survival post surgery. For clinical variables prognostic in pancreatic cancer, univariate analysis showed that tumour grade \(( P < 0.001)\) was a significant prognostic predictor of patient survival; the Kaplan–Meier survival curves indicated increased tumour grade associating with poor survival (Figure 2A). Other clinical variables including lymph node status, tumour extension beyond the pancreas, UICC clinical stage, margin status, perineural invasion, angiolymphatic invasion or adjuvant therapy were not significant prognostic factors of patient survival (Table 1).

| Biomarker | Total samples stained | Positive samples | Total patients stained | Positive patients | Median expression, range of positive samples |
|-----------|-----------------------|------------------|-----------------------|------------------|---------------------------------------------|
| EGFR      | 249                   | 192 (77%)        | 110                   | 94 (85%)         | 2 (0–4)                                     |
| HER2      | 287                   | 56 (19%)         | 117                   | 32 (27%)         | 0 (0–2)                                     |
| pERK      | 301                   | 219 (72%)        | 131                   | 106 (81%)        | 40% (5–90%)                                 |
| SIAH      | 315                   | 303 (96%)        | 127                   | 127 (100%)       | 30% (5–90%)                                 |
| Ki67      | 250                   | 237 (95%)        | 119                   | 118 (99%)        | 40% (5–90%)                                 |
| HIF-1α    | 210                   | 143 (69%)        | 100                   | 77 (77%)         | 40% (2–90%)                                 |

Abbreviations: HIF-1α = hypoxia-inducible factor 1α; IHC = immunohistochemical.

| Biomarker | Lymph node | Pathological grade | Extension | Tumour size | EGFR | SIAH | Ki67 | Phospho-ERK | HIF-1α |
|-----------|------------|--------------------|-----------|-------------|------|------|------|-------------|--------|
| Lymph node| 1          |                    |           |             |      |      |      |             |        |
| Pathological grade | 1 | | \( 0.22 \) | \( 0.17 \) | \( 1 \) | \( 0.16 \) | \( 0.11 \) | \( 0.76 \) | \( 1 \) |
| Extension | 1          |                    |           |             |      |      |      |             |        |
| Tumour size| \( 0.22 \) | \( 0.17 \) | \( 1 \) |             |      |      |      |             |        |
| EGFR      | \( 0.14 \) | \( 0.22 \)         | \( 0.13 \) | \( 0.12 \) | \( 1 \) |
| SIAH      | \( 0.11 \) |                    |           |             |      |      |      |             |        |
| Ki67      |            |                    |           |             |      |      |      |             |        |
| Phospho-ERK| \( 0.14 \) | \( 0.22 \)         | \( 0.13 \) | \( 0.12 \) | \( 1 \) |
| HIF-1α    | \( 0.14 \) | \( 0.22 \)         | \( 0.13 \) | \( 0.12 \) | \( 1 \) |

Abbreviation: HIF-1α = hypoxia-inducible factor 1α. Note: only coefficients \( > 0.10 \) or \( < -0.10 \) are shown and those significantly different from 0 are in bold.

Increased expression of HIF-1α is associated with shortened survival post surgery. HIF-1α expression was examined to assess the hypoxic response in pancreatic tumours. HIF-1α staining was scored by staining percentage on a scale of 2–90% (Figure 2D and Table 2). HIF-1α expression was quite heterogeneous. The greatest level of expression was examined to assess the hypoxic response in pancreatic cancer. However, low cytoplasmic expression levels were also detected. Representative HIF-1α staining in patients with distinct survival rates was shown (Figure 2D). The median expression level of HIF-1α was 40%. Patients expressing HIF-1α above 40% had a median survival time of 14.8 months, whereas patients expressing HIF-1α below 40% had a median survival time of 19.2 months with \( P = 0.004 \) (Figure 2C). Only 13.3% of patients with high HIF-1α expression survived 3 years or longer. Patients with HIF-1α below median 40% expression showed a statistically significant increase in 5-year survival compared with the patients with high HIF-1α expression (Figure 2C and D).

Making SIAH a useful and tumour-specific biomarker in pancreatic cancer.

The IHC staining of six biomarkers in the K-RAS/Ki67/HIF-1α pathways

### Table 2. IHC staining of six biomarkers in the K-RAS/Ki67/HIF-1α pathways

| Biomarker | Total samples stained | Positive samples | Total patients stained | Positive patients | Median expression, range of positive samples |
|-----------|-----------------------|------------------|-----------------------|------------------|---------------------------------------------|
| EGFR      | 249                   | 192 (77%)        | 110                   | 94 (85%)         | 2 (0–4)                                     |
| HER2      | 287                   | 56 (19%)         | 117                   | 32 (27%)         | 0 (0–2)                                     |
| pERK      | 301                   | 219 (72%)        | 131                   | 106 (81%)        | 40% (5–90%)                                 |
| SIAH      | 315                   | 303 (96%)        | 127                   | 127 (100%)       | 30% (5–90%)                                 |
| Ki67      | 250                   | 237 (95%)        | 119                   | 118 (99%)        | 40% (5–90%)                                 |
| HIF-1α    | 210                   | 143 (69%)        | 100                   | 77 (77%)         | 40% (2–90%)                                 |

Abbreviations: HIF-1α = hypoxia-inducible factor 1α; IHC = immunohistochemical.
Univariate analysis with the Cox proportional hazards model showed that HIF-1α expression has increased power to predict survival. HIF-1α expression was a significant predictor of survival (P < 0.005) with a hazard ratio of 1.19 (95% CI: 1.06–1.33) for a unit of 20% increment, indicating that a higher percentage of HIF-1α expression is associated with reduced survival (Table 1 and a P-value from a log-rank test for binary HIF-1α expression less than or equal to 0.05 were considered statistically significant as shown. (C) Venn diagram representation of single, double and triple staining of SIAH, phospho-ERK and EGFR expression on 234 tumour cores with a complete and overlapping set of the IHC staining, was shown. (D) Representative EGFR, phospho-ERK and SIAH staining in PanIN and ADCA stages are shown. SIAH marked tumour cells specifically. Note the lack of SIAH staining in normal pancreas and tumour stroma.

Survival ROC curves. Consistent with previous reports, clinical variables, such as tumour size and pathologic grade, are linked to survival in pancreatic cancer (Tables 1 and 4). Our biomarker analysis revealed that HIF-1α expression may be associated with reduced survival in pancreatic cancer. Here, we proposed five models for predicting patient survival: (A) five biomarkers of Ki67, HIF-1α, EGFR, phospho-ERK and SIAH, (B) clinicopathologic only, (C) clinicopathologic variables plus Ki67, (D) clinicopathologic variables plus HIF-1α, (E) clinicopathologic variables plus the five biomarkers. To evaluate predictive accuracy of these models, time-dependent survival ROC curves were constructed for 3-year survival predictions (Figure 3A). Area under the curve (AUC) for 3-year survival was used to numerically compare the performance of these five models. The AUC increased from 0.685 for five biomarkers only (Model A) to 0.751 for the clinicopathologic variables alone (Model B), and to 0.773 with the addition of Ki67 (Model C), and to 0.788 with the addition of HIF-1α (Model D), respectively (Figure 3B). The AUC combining the four clinicopathologic variables plus the five molecular biomarkers together (Model E) was 0.850 (Figure 3B).

A time-dependent AUC(t) curve for continuous time points was also plotted to investigate the predictive accuracy of the five models with survival. The data suggest that the absent or decreased HIF-1α
is associated with a good prognosis for long-term survival; and the combination of the five molecular biomarkers (EGFR, phospho-ERK, SIAH, Ki67 and HIF-1α) in the K-RAS/HIF-1α pathway and the four clinicopathologic variables (tumour size, pathological grade, margin and lymph node status) can improve prognostic accuracy of patient survival post surgery (Figure 3B).

**DISCUSSION**

Ninety-five percent mortality rates in pancreatic cancer have shown little improvement despite intense research efforts over 40 years. Patient prognosis appears to be influenced in large part by pancreatic tumour genome biology and cancer cell dissemination rather than by adequate resection (Wolfgang et al, 2013). Favourable clinicopathological prognostic indicators include small tumour size, negative nodal status, negative resection margin and well-differentiated carcinomas. Evaluation of numerous molecular biomarkers as prognostic factors has achieved inconsistent results (Sawyers, 2008; Costello et al, 2012; Winter et al, 2013). Discovery and validation of logical and useful biomarkers to improve prognosis and guide effective therapies is important to combat pancreatic cancer. Here, we report that SIAH is a tumour-specific biomarker in pancreatic cancer (Figure 1); HIF-1α shows some promise as a prognostic variable (Figure 2); and individually, the selected five biomarkers, EGFR, phospho-ERK, SIAH, Ki67 and HIF-1α are weakly prognostic or not at all, but when combined them with the four clinicopathological predictors (tumour size, pathological grade, margin status
and lymph node status) together, it yields increased prognostic accuracy in resectable pancreatic cancer (Figure 3). One important caveat is that only resected pancreatic tumours were examined in this study. As ~85% of pancreatic cancer patients have incurable diseases without surgical options, we do not know whether the K-RAS/Ki67/HIF-1α pathway biomarkers will synergise with clinicopathologic predictors in inoperable pancreatic cancers. With rapid autopsy programmes in pancreatic cancer, we hope to analyse and validate our findings on HIF-1α, EGFR, phospho-ERK, SIAH and Ki67 alone and in combination with clinical parameters in the context of systemic metastasis in the future.

We have demonstrated that increased HIF-1α expression correlates with increasing pathological grades, consistent with previous findings in pancreatic cancer (Kitada et al, 2003; Shibaji et al, 2003; Guillaumond et al, 2013). HIF-1α overexpression is a negative prognostic variable in several human cancers (Semenza, 2003; Wilson and Hay, 2011). Here, we demonstrate that increased HIF-1α expression correlates with increasing pathological grades and poor prognosis, and HIF-1α is overexpressed in 69% of pancreatic tumour specimens in this study within 147 patients, consistent with the results of two prior, small-scale studies (Zhong et al, 1999; Kitada et al, 2003). In the first study, HIF-1α was found to be overexpressed in four out of five primary pancreatic cancer specimens (Zhong et al, 1999). In the second study, overexpression of HIF-1α was detected in 59.2% (29 out of 49 cases) of pancreatic cancer specimens (Kitada et al, 2003). Previous studies have found correlations between HIF-1α expression and larger tumour size, advanced UICC stage, and metastatic disease in pancreatic cancer (Kitada et al, 2003; Shibaji et al, 2003; Chang et al, 2011). Our results have shown that high HIF-1α expression levels in pancreatic adenocarcinomas—with staining percentages above 40%—signify a notable decrease in patient survival. Furthermore, survival ROC analysis suggests that adding HIF-1α expression alone to the list of existing clinicopathologic parameters will strengthen clinical prediction of survival in pancreatic cancer (AUC 0.751 vs 0.788; Figure 3). The data suggest that decreased Ki67 may have a good prognosis for short-term survival, within a year or so, whereas the lack of or the decreased HIF-1α may have a good prognosis for long-term survival. The different onset and distinct dynamics of Ki67 and HIF-1α expression patterns may reflect the altered biology and successful adaptation of pancreatic cancer cells to the

### Table 4. Cox PH regression models with four clinical variables and five biomarkers

| Predictors                  | Level | Hazard ratio | 95% CI    | P-value |
|-----------------------------|-------|--------------|-----------|---------|
| Lymph node positive         | No    | 1.00         | —         | —       |
|                            | Yes   | 0.915        | 0.536     | 1.560   | 0.745   |
| Pathological grade          | 2     | 1.00         | —         | —       | —       |
|                            | 3     | 2.665        | 1.329     | 5.340   | 0.006   |
|                            | 4     | 3.944        | 1.736     | 8.960   | 0.001   |
| Extension                   | No    | 1.00         | —         | —       | —       |
|                            | Yes   | 1.280        | 0.748     | 2.190   | 0.368   |
| Tumour size                 |       | 1.180        | 0.907     | 1.530   | 0.219   |
| EGFR                        |       | 1.056        | 0.895     | 1.250   | 0.519   |
| SIAH                        |       | 0.998        | 0.980     | 1.020   | 0.822   |
| Ki67                        |       | 1.010        | 0.993     | 1.030   | 0.247   |
| Phospho-ERK                 |       | 0.997        | 0.989     | 1.000   | 0.443   |
| HIF-1α                      |       | 1.005        | 0.998     | 1.010   | 0.149   |

Abbreviations: CI = confidence interval; HIF-1α = hypoxia-inducible factor 1α.

Figure 3. A combination of four clinical markers and five biomarkers have more accurate prognostic value in predicting 3- or 5-year patient survival in resectable pancreatic cancer. (A) Three-year survival prediction: time-dependent survival ROCs at 3 years based on five predictive models are shown. Five biomarkers only (black line) and clinicopathological predictors only (red dotted line) served as the controls. Ki67 in combination with the clinical predictors (green dotted line) have improved predictive power, as with HIF-1α in combination with the clinical predictors (blue dotted line). When all five biomarkers are combined with the clinical predictors together (aqua/cyan dotted line), there is a significant improvement in predicting patient survival post-pancreatic surgeries. (B) Five-year survival prediction: AUC(t) plots for the first 5 years based on five predictive models are shown (black line). Five biomarkers only (black line) and clinicopathological predictors only (red dotted line) served as the controls. Ki67 in combination with the clinical predictors (green dotted line) have an improved predictive power, as with HIF-1α (blue dotted line). When all five biomarkers are combined with the four clinical predictors together (aqua/cyan dotted line), there is a significant improvement in predictive power based on AUC analysis.
Hypoxia reduces prognosis in pancreatic cancer

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