

Tissue Biomarkers for Prognosis in Pancreatic Ductal Adenocarcinoma: A Systematic Review and Meta-analysis

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Abstract

Purpose: The management of pancreatic ductal adenocarcinoma (PDAC) continues to present a great challenge particularly with regard to prediction of outcome following pancreaticoduodenectomy. Molecular markers have been extensively investigated by numerous groups with the aim of enhancing prognostication; however, despite hundreds of studies that have sought to assess the potential prognostic value of molecular markers in predicting the clinical course following resection of PDAC, at this time, no molecular marker assay forms part of recommended clinical practice.

Experimental Design: We conducted a systematic review and meta-analysis of the published literature for immunohistochemistry-based biomarkers of PDAC outcome. A dual search strategy was applied to the PubMed database on January 6, 2010, to identify cohort studies that reported associations between immunohistochemical biomarker expression and survival outcomes in PDAC, and conformed to the REMARK (REporting recommendations for tumor MARKer prognostic studies) criteria.

Results: A total of 103 distinct proteins met all inclusion criteria. Promising markers that emerged for the prediction of overall survival included BAX (HR = 0.31, 95% CI: 0.71–0.56), Bcl-2 (HR = 0.41, 95% CI: 0.27–0.63), survivin (HR = 0.46, 95% CI: 0.29–0.73), Ki-67: (HR = 2.42, 95% CI: 1.87–3.14), COX-2 (HR = 1.39, 95% CI: 1.13–1.71), E-cadherin (HR = 1.80, 95% CI: 1.33–2.42), and S100 calcium-binding proteins, in particular S100A2 (HR = 3.23, 95% CI: 1.58–6.62).

Conclusions: We noted that there was incomplete adherence to the REMARK guidelines with inadequate methodology reporting as well as failure to perform multivariate analysis. Addressing the persistent incomplete adoption of these criteria may eventually result in the incorporation of molecular marker assessment within PDAC management algorithms. *Clin Cancer Res*; 17(10); 3316–31. ©2011 AACR.

Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the sixth most common cause of cancer-related death with 7,684 new cases registered for 2007 in the United Kingdom (1). It remains a therapeutic challenge: conventional cancer therapies have little impact on disease course; and patients with potentially resectable disease are unfortunately in the minority (10%–15%) because of extensive local spread or metastasis at presentation (2, 3). Clinicopathologic factors determined at pancreaticoduodenectomy (PD) including lymph node involvement, tumor grade, venous invasion,

and resection margin status establish risk stratifications among patients with PDAC (4–7). Unfortunately, these factors alone do not account for all of the observed variability in PDAC-related survival.

Although perioperative outcomes in terms of morbidity and mortality after major pancreatic resections have improved considerably, there have only been modest survival improvements. Recognition of the overwhelming biologic relevance of the systemic disease component of PDAC is mandatory if significant strides are to be made (8). Early recurrence following resection associated with tremendous morbidity is unacceptable in terms of quality of life and should be avoided. There is thus a need for superior markers of prognosis to enable the improved management of operable PDAC. Ideally, the status of such markers could be determined preoperatively, potentially augmenting current staging criteria and enhancing patient selection for PD in borderline resectable circumstances. Molecular analysis is clearly one source of such clinically useful biomarkers. Indeed, tumors with similar clinicopathologic characteristics can be shown to contain molecular differences, which may underpin variation in clinical behavior: this has been clearly shown in pancreatic and other cancers (9–12), with tumor subgroups classified by gene expression relating to disparate outcomes. We believe that review

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Translational Relevance

It was our aim to rigorously assess the pancreatic ductal adenocarcinoma (PDAC) immunohistochemical biomarker literature to determine the most clinically useful prognostic markers that may form components of future staging algorithms. We have combined the data of the most robust studies in a meta-analysis to further guide the potential clinical utility of biomarkers in PDAC. We have used established statistical methodology to interrogate the best of the available data; however, our meta-analysis is a unique study among the PDAC literature. We feel that this article is both important and immediately clinically relevant because currently, despite tremendous efforts in the field of biomarker discovery and development, for PDAC, molecular aids to the clinicopathologic toolbox are lacking. In addition to highlighting the immunohistochemical biomarkers that the literature best supports, we have drawn attention to areas where improvement is necessary in the pancreatic research field as determined by the REMARK (REporting recommendations for tumor MARKer prognostic studies) guidelines.

of the biomarkers associated with outcome following resected PDAC is the first step in the selection of markers that could have utility not only following resection but potentially preoperatively.

Immunohistochemistry (IHC) is a widely accepted and well-documented method for the characterization of gene expression at the protein level, while simultaneously allowing the interpretation of tissue and cellular architecture (13). In solid tumor pathology, almost all established diagnostic and prognostic markers are currently assessed by using this method (14), and biomarkers thus identified can be readily translated to routine clinical practice. Furthermore, the advent of tissue microarray (TMA) technology, which allows assessment of several hundred individual samples on a single slide, has extended the utility of IHC-based biomarker assessment by facilitating high-throughput analysis of candidate proteins across large tissue cohorts and by the standardizing staining conditions and interpretation thus substantially reducing expression misclassification (15). Although genomic studies including mRNA microarray experiments allow the parallel investigation of thousands of genes, TMA/IHC experiments enable serial investigation of targets in a candidate gene approach. The data from individual experiments are then combined into multi-marker prognostic discriminatory models.

Although reviews have been published on the prognostic utility of IHC markers in PDAC (16, 17), none have assessed the available data according to the REMARK (REporting recommendations for tumor MARKer prognostic studies) guidelines or methodologic assessment quality metrics (18). Tremendous variation exists in the experimental

procedures used, including antigen retrieval technique, antibody dilution, use of controls for antibody validation, observer variability in staining pattern description, cutoff point selection, and assignment of specimens to categories that could potentially influence the prognostic value of the proposed association. To ensure that a biomarker provides information additional to the routine prognostic markers, the study must adjust for clinicopathologic criteria. Studies that do not extend their analysis beyond univariate survival measures are therefore less valuable.

In this systematic review and meta-analysis we sought to determine candidate biomarkers with sufficient evidence to support prospective validation in a controlled clinical environment and furthermore identify functional pathways for which the data suggested a lack of involvement in PDAC prognosis or the need for additional investigation due to insufficient rigor among the previously conducted studies. We identified from the published literature a subset of candidate IHC-based protein predictors of outcome in PDAC that has been evaluated according to robust sampling and laboratory methods.

Materials and Methods

Search strategy

The aim of our search was to identify all primary research articles that assessed the utility of candidate protein expression levels, measured by IHC, as a prognostic factor among individuals with resected PDAC. Our search of the PubMed medical literature database on January 6, 2009, used the following separate search criteria:

1. [pancrea*] AND ([cancer OR adenocarcinoma OR carcinoma]) AND ([prognos*] OR [surviv*]) AND ([gene] OR [protein])
2. [pancrea*] AND ([cancer OR adenocarcinoma OR carcinoma]) AND [immunohisto*] AND ([prognos*] OR [surviv*])

N.B.J. inspected the title and abstract of citations to identify papers appearing to report the study of PDAC samples by IHC and obtained the full texts. Further PubMed searches by author name contributing to multiple manuscripts were carried out to ensure identification of papers that may have been omitted by the primary search strategy. Where several publications have derived from the same IHC dataset, only the manuscript describing the largest dataset was further analyzed. The search was limited to English language publications. Histologic microvessel density, although a prognostic factor enhanced by IHC, is a tumor morphologic characteristic rather than a molecular target (19).

Methodologic and validity assessment

Our inclusion criteria for this review were derived from the published guidelines (REMARK) for reporting IHC-based tumor marker studies (18) and made use of quality assessment criteria for evaluating IHC-based studies for inclusion in cancer-related meta-analyses that have been used in previous cancer biomarker evaluations

Table 1. Criteria for the inclusion of prognostic IHC studies

- 1 Prospective or retrospective cohort design with a well-defined study population with justification for excluded cases
- 2 Assay of primary resected PDAC tumor specimens
- 3 Clear description of methods for tissue handling and IHC, including antigen retrieval, selection, and preparation of both primary and secondary antibodies, as well as visualization techniques
- 4 A clear statement on the choice of positive and negative controls and on the outcome of the assay to ensure that the primary antibody used was a well-validated reagent
- 5 Statistical analysis using multivariate proportional hazards modeling that adjusted for clinical prognostic factors
- 6 Reporting of the resulting HRs including 95% CIs

(20). Studies were eligible according to the criteria listed in Table 1. Because other periampullary tumors (ampullary, distal cholangiocarcinomas, duodenal adenocarcinomas, intrapapillary mucinous neoplasms) have different clinical courses, studies focusing on these or not separating them from PDAC were excluded.

Data extraction

One author (N.B.J.) reviewed all eligible manuscripts resulting from the PubMed search. The extracted study characteristics included the number and type of PDACs studied, IHC methodology, and results. The surgical procedure carried out was either PD, distal pancreatectomy (DP), or total pancreatectomy. Factors recorded included authors' names, institution, year of publication, patient number, pathologic material examined, the use of whole sections or TMAs, clinical covariates incorporated in multivariate statistical analysis, outcomes, and evaluated proteins. Special attention was given to IHC methodologic data including primary antibody (including clone), dilution used, secondary signal amplification, method for evaluation of IHC staining, survival analysis thresholds and whether thresholds were established *a priori*, associated multivariate HR, 95% CI, and corresponding *P* value. When results were presented without CIs or standard error (SE), the *P* value was used to estimate the SE via the *z* statistic.

Statistical analysis

The evaluated proteins were sorted according to their major biological function. Function was determined by evaluation of the current scientific literature and then classified according to the 6 major acquired capabilities of cancer as outlined in the seminal work of Hanahan and Weinberg (21), including limitless replicative potential, insensitivity to growth inhibition signaling, apoptosis evasion, tissue invasion and metastasis, sustained angiogenesis, and self-sufficiency in growth signaling. To accommodate pancreatic differentiation markers, markers associated with DNA damage repair, and chemotherapy metabolism and altered immune response, the Hanahan–Weinberg classification system was supplemented by 3 additional categories: pancreatic differentiation, DNA damage repair, and altered immunocompetence.

For proteins evaluated appropriately in a single study, the summary HR (including 95% CI) represents the value reported in that study. For proteins that were evaluated

appropriately in multiple studies, fixed effects summary HR and 95% CI were calculated by using the generic inverse variance method and the random effects model according to the DerSimonian–Laird method (22). Inter-study heterogeneity was assessed by the I^2 statistic (23). An observed HR > 1 indicated worse outcome for the study group relative to the reference group and would be considered statistically significant if the 95% CI did not overlap 1, with $P < 0.05$. Meta-analyses were carried out by the REVMAN systematic review and meta-analysis software package version 5.0 (24).

Results

Excluded studies

Our search of the PDAC IHC prognostic literature yielded 1,992 manuscripts for consideration within this systematic review (Fig. 1). Title and abstract evaluation along with searches to identify further work from these authors and research groups identified 398 manuscripts apparently appropriate in terms of IHC evaluation of prognostic biomarkers in PDAC (Supplementary Table S1); for these manuscripts, full text articles were obtained. On careful review of study methodologies, 20 were excluded, as protein expression was not being assessed by IHC methods but rather gene expression assessment performed. The remaining 378 studies, which used IHC to measure protein expression in PDAC, were assessed in terms of study design. Seventy-one studies were either case series or cross-sectional studies with no formal investigation of outcome; instead these studies included correlations with clinicopathologic data only and were excluded.

Of the 307 cohort studies, 167 limited their analysis to only univariate log rank analysis or proportional hazards computations. Five studies contained data for a marker that were redundant within another study by the same authors (25–29). Eight studies provided incomplete information on the IHC methodology used (30–36). Two studies assessed only metastatic disease undergoing palliative therapy (37, 38). One study assessed only the predictive ability of the marker human equilibrative nucleoside transporter 1 (hENT1; ref. 39). Forty-five studies published multivariate analysis on robust datasets, but failed to publish HRs and 95% CIs; all were excluded. A large study that analyzed expression of multiple markers (10) unfortunately was not

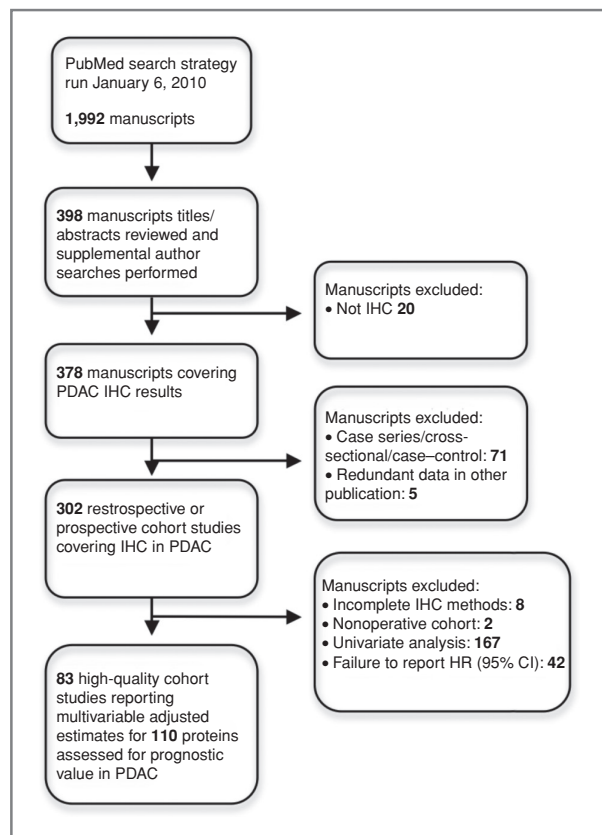


Figure 1. Flow diagram of the literature search and selection of included studies.

of a cohort design with unclear patient inclusion criteria and so was excluded (40).

Included studies

A total of 83 high-quality cohort studies from 34 independent research groups met the eligibility criteria for this systematic review by presenting multivariable survival estimates for differential levels of candidate protein expression as measured by IHC in operative PDAC cohorts (41–123). Supplementary Table S2 illustrates all of these retrospective cohort studies. All evaluated IHC staining in formalin-fixed paraffin-embedded tissue blocks. In 68 of the 81 studies, IHC was carried out on individual whole-slide tissue sections. In the remaining 15 studies, TMAs were created by using 2-mm (44, 85, 100, 103), 1.5-mm (46, 48, 84), 1.0-mm (74), or 0.6-mm (61, 64, 69, 75, 83) diameter cores from representative tissue regions; one study provided no core size details (116). For 2 studies, only automated scoring techniques were used (42, 48). Sixty studies documented whether staining assessment was blinded to outcome status. The size of the sample size ranged from 30 (48) to 300 (61). The study of Biankin and colleagues (44) included 600 tumor samples, a combination of a test set (162 tumor samples including 76 resections), and validation set (439 tumors including 296

resections). Sixteen studies included 50 or less patients, 40 studies examined between 51 and 100 individuals, 21 studies examined between 100 and 200 individuals, and 6 studies examined more than 200 individuals. The study size figure included in Supplementary Table S2 represented the component of each cohort undergoing resection with curative intent. The overall median study size was 73 (mean, 102). Including only resections carried out for pancreatic head lesions by PD the median study size was 62 (mean, 80). Eighteen studies only considered patients undergoing PD, with a further 5 including PD subgroup analysis (43, 44, 85, 100, 103).

Twenty-one clinicopathologic factors were incorporated in one or more of the included studies' multivariate analyses. The most commonly included prognostic factor was lymph node involvement with tumor grade being included in 54 (68%) and 53 (66%) studies (Fig. 2). Other common adjustments were made for tumor stage (41%), Union Internationale Contra Cancrum stage (41%), age (40%), tumor size (34%), and resection margin status (33%). Ten studies considered less than 3 clinical covariates, 50 considered between 3 and 5 covariates, and 21 studies included more than 5 covariates.

These 83 studies present data on 103 unique proteins; 43 of the studies analyzed the prognostic impact of a single protein marker, the remaining 40 evaluated between 2 and 17 proteins. Only one study reported multivariate HRs for a large number of proteins (40, 44), which included 17 proteins. This study examined the markers within the setting of a separate training and test TMA cohort combining data from a number of their previous studies. Stratified by outcome, data are available on 83 proteins for overall survival (OS) and 15 proteins for disease-free survival (DFS).

For 89 of the 103 biomarkers, a multivariate HR and associated 95% CI were available from a single study only. For the remaining 14 markers, outcome data were available from 2 or more studies and were combined by using both fixed effects general inverse variance and DerSimonian-Laird random effects modeling resulting in a single summary HR and 95% CI (Fig. 3). For 11 studies, the fixed effects summary point estimates and 95% CI yielded a more conservative result than the random effects estimate. For the remaining 3 studies, the fixed effects summary point estimate and the 95% CI were identical to the random effects summary statistic.

Overall survival

The 79 proteins evaluated for OS are sorted according to Hanahan–Weinberg functional capabilities modified to include pancreatic differentiation markers, immunocompetence, markers associated with DNA damage repair, and chemotherapeutic metabolism (Table 2). Of the 79 candidate markers, 67 (84%) had a significant association with outcome at $P < 0.05$. Five of the 6 original Hanahan–Weinberg functional capabilities along with the additional groups were represented by at least 3 markers statistically associated with OS. Of

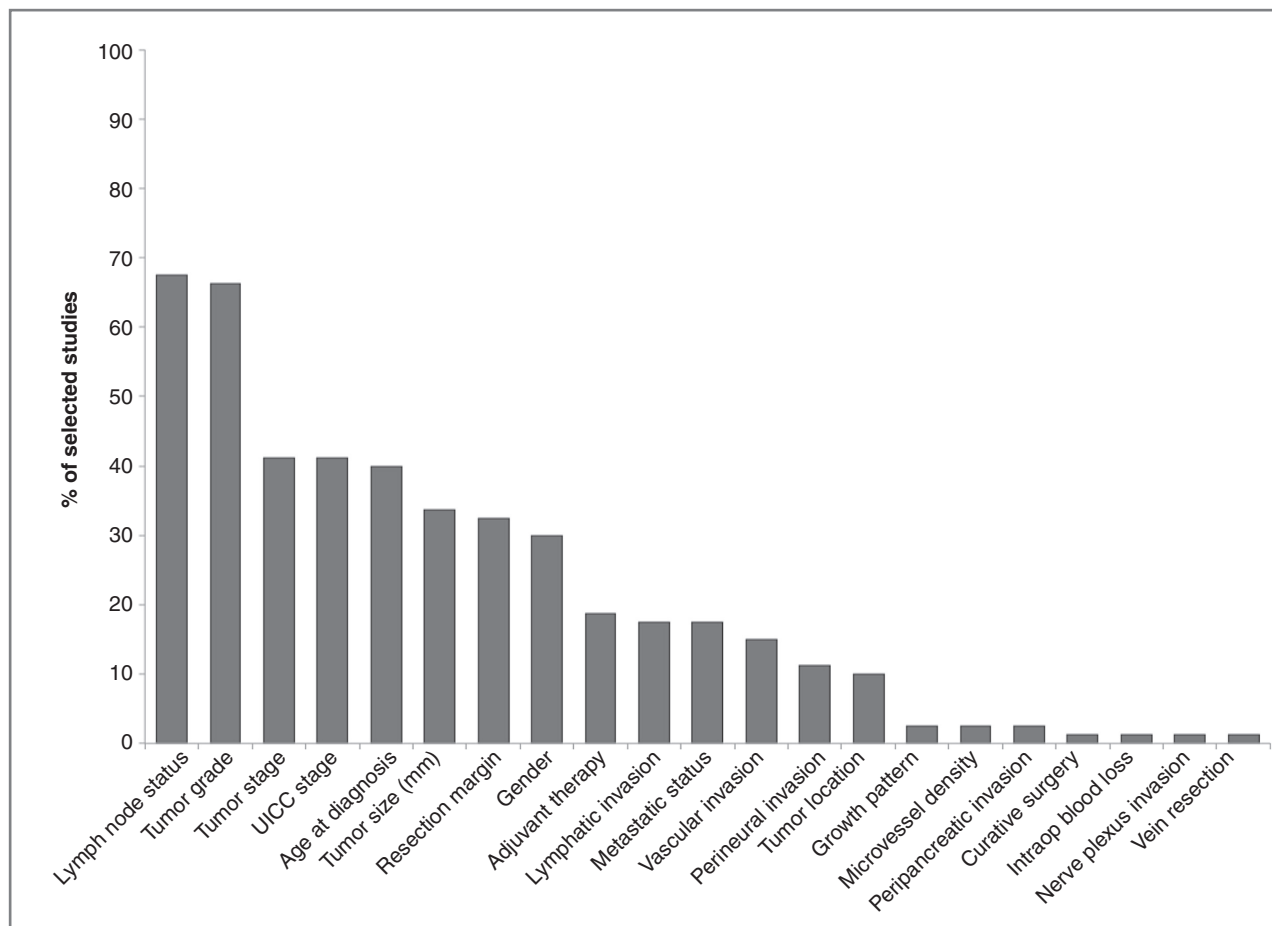


Figure 2. Characteristics of studies included in this meta-analysis. Frequencies with which adjustments were made for various clinicopathologic parameters.

the cell-cycle proteins evaluated, only cyclin E achieved statistical significance ($P = 0.0021$). Of the cell-cycle regulators, p21 ($P = 0.004$) was significantly associated with outcome but p27 ($P = 0.47$) and p53 ($P = 0.14$) were not. Regarding the TGF- β signaling pathway, TGF- β 1 ($P = 0.015$) and SMAD7 ($P = 0.014$) had a significant prognostic relationship, but SMAD4 ($P = 0.21$) did not. All the 3 apoptosis markers evaluated, Bcl-2 ($P < 0.0001$), Bax ($P = 0.001$), and survivin ($P = 0.0001$), were associated with OS and all had at least 2 studies available for analysis. Twenty-two markers associated with limitless replicative potential were eligible for examination, of which Ki-67 and pAKT had been investigated in more than a single high-quality study. Of these, only Ki-67 maintained statistically significant associations with outcome ($P = 0.005$). Of the eligible markers associated with tissue invasion and metastasis, all 19 markers were significantly associated with overall mortality including E-cadherin, which was evaluated in 2 studies ($P = 0.009$). Regarding angiogenesis, 10 of 11 eligible markers were also significantly associated with, including COX-2 ($P = 0.002$), VEGF ($P = 0.002$), PD-ECGF ($P = 0.007$), for which data were pooled. The 5 eligible transcription

factors (HOXB2, HMGA, SP-1, LMO2, LMO4) were all significantly associated with outcome ($P < 0.05$) in a single study. All eligible markers associated with pancreatic differentiation along with the stem cell marker CD133 from a single study were significantly associated with survival.

Disease-free survival

Fifteen proteins, which covered only 2 of the original Hanahan-Weinberg capabilities, as well as DNA damage repair and chemotherapeutic metabolism-associated factors were assayed for DFS, and 6 significant associations were found (Table 3). All but one marker, Annexin II, had also been assessed for impact on OS. For the 14 markers assessed for both outcomes, the results were concordant in 9 (64%). ERCC, FGF, and p53 were not associated with either outcome. CXCR4 ($P = 0.054$), Ki67 ($P = 0.38$), VEGF ($P = 0.39$), hCNT3 ($P = 0.52$), RMM1 ($P = 0.265$) were associated with overall mortality but not with DFS. Differential levels of VCP/p97, MAPK4K, TROP 2, Synucleiny, PDECgf, and hENT1 were predictive of both overall mortality and DFS with similar direction and magnitude.

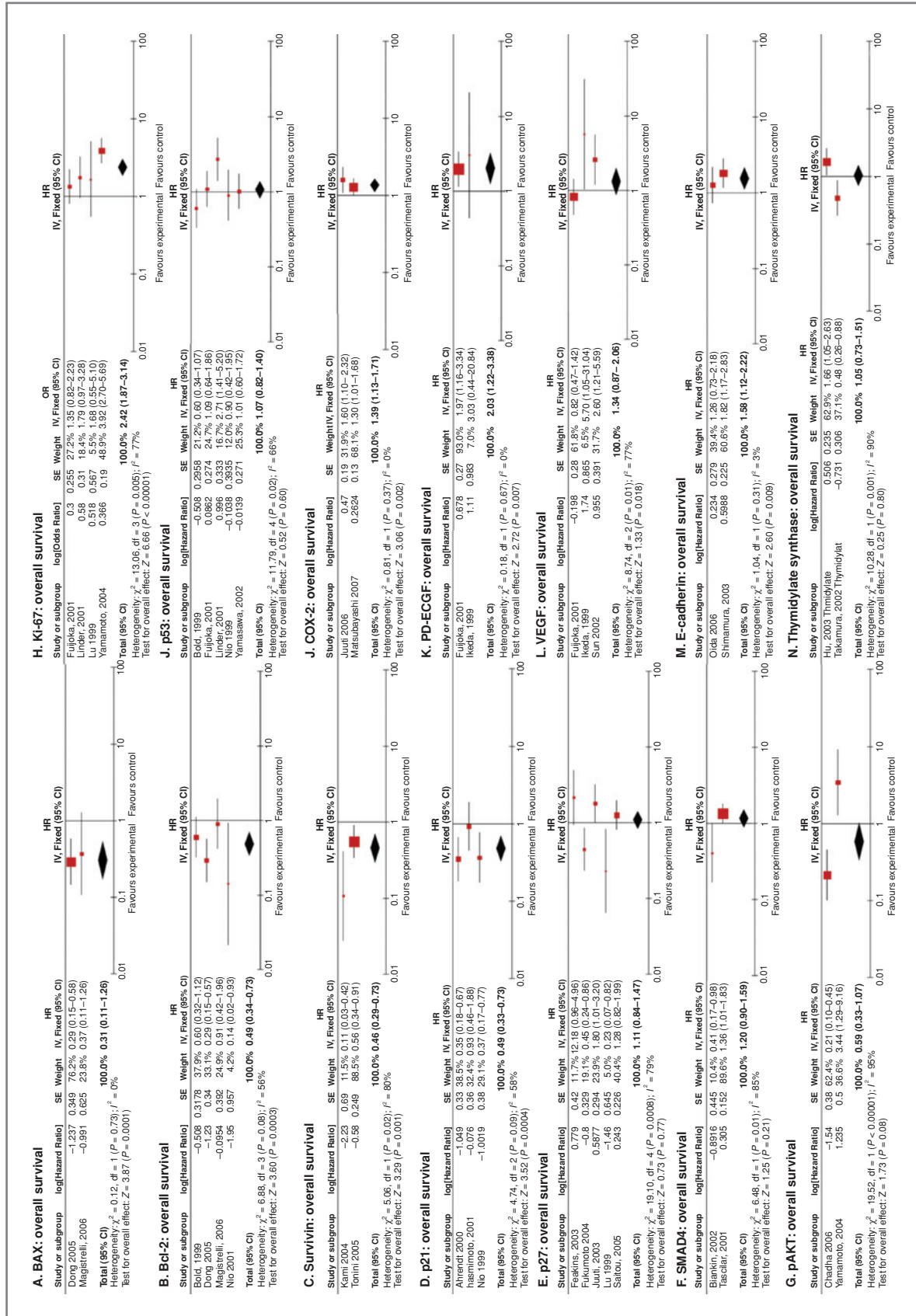


Figure 3. Forest plots of the data from the contributing studies for each of the biomarkers with 2 or more studies available for comparison. For each study, HR, 95% CI, and relative weight are shown. SEs are shown for log of the HR. Combined fixed effects HRs and tests for heterogeneity (I^2) were based on the generic inverse variance (IV) method.

Table 2. Proteins related to OS according to the study of resected PDAC

Protein	Total no. (PD)	Reference group	Compartment	HR (95% CI)	P	Reference
Evading apoptosis						
Bax ^a	126 (113)	<10% cells +	C, N	0.31 (0.17–0.56)	0.0001	49, 80
Bcl-2 ^a	227 (183)	<5% cells +	C, N	0.41 (0.27–0.63)	<0.0001	27, 49, 80, 90
Survivin ^a	119 (106)	<10% cells +	C, N	0.46 (0.29–0.73)	0.001	68, 113
Insensitivity to antigrowth signals						
14-3-3 σ	300	Compared with ND	M, C	1.4 (0.9–2.20)	0.14	61
IEX-1 ^a	78 (48)	<25% cells +	C	1.84 (1.22–2.82)	0.004	97
GADD45	72 (38)	<50% cells +	M, C	1.38 (0.78–2.45)	0.26	123
p21 ^a	148 (37)	No stain	N	0.49 (0.33–0.73)	0.09	41, 89, 57
p27	352 (336)	<20% cells +	N	1.11 (0.84–1.47)	0.47	77, 55, 96, 51
SMAD4	300	No stain	C	1.20 (0.91–1.59)	0.21	43, 111
SMAD7 ^a	71	Compared with ND	C	0.39 (0.18–0.83)	0.014	117
TGF- β 1 ^a	61(31)	<30 cells +	C	0.44 (0.23–0.86)	0.015	57
Limitless replicative potential						
Akt ^a	65 (50)	Immunoscore <100	C	0.40 (0.20–1.10)	0.043	47
pAkt	104 (89)	Relative to ND	C	0.59 (0.33–1.07)	0.08	437 120
Caveolin ^a	79	<50% cells +	M, C	1.88 (1.04–3.39)	0.036	106
Cyclin E ^a	75	<10% cells +	N	2.48 (1.39–4.41)	0.0021	103
EGFR	65 (50)	Immunoscore <100	M	1.80 (0.80–4.20)	>0.05	47
Ezrin ^a	73	No stain	M, C	2.93 (1.58–2.30)	0.02	122
Erk	65 (50)	Immunoscore < 100	C	0.80 (0.30–2.10)	>0.05	47
pErk ^a	65 (50)	Immunoscore < 100	C	3.52 (1.41–9.01)	0.003	47
GPR54	53 (38)	No stain	C	1.22 (0.74–2.0)	0.43	86
HDGF ^a	50	Compared with ND 90% cells +	N, C	0.26 (0.08–0.65)	0.0026	115
Her2 ^a	129 (88)	Hercept test score 0–1	M	1.81 (1.07–3.04)	0.025	72
Ki-67 ^a	84	<10% cells +	N	2.42 (1.87–3.14)	0.005	54, 77, 76, 120
MAP4K4 ^a	66	<10% cells +	C	2.16 (1.10–4.23)	0.025	74
Metastin ^a	53 (38)	No stain	C	2.08 (1.1–4.7)	0.03	86
P53	287 (213)	<1% cells +	N	1.26 (0.93–1.70)	0.14	76, 90, 54, 123
PPAR ^a	129	No stain	N	2.46 (1.04–5.78)	0.039	73
Rel65/P65 ^b	82	Semiquantitative score <6	N, C	RR 3.49	0.02	118
S100A2 ^a	162 (75) 439 (296)	<30% cells +	C	3.23 (1.58–6.62) 1.87 (1.25–2.81)	0.0014 0.0024	44
S100A4 ^a	72	No stain	C	1.81 (1.01–3.27)	0.048	94
S100A6 ^a	60	No stain	C	2.85 (1.43–5.71)	0.003	116
Skp2 ^a	46 (35)	< 20% cells	N	6.05 (1.43–25.1)	0.014	50
VCP/p97 ^a	83	Less than ND	C	2.42 (1.11–2.26)	<0.01	121
Transcription factors						
LMO2 ^a	164	<10% cells +	N	0.43 (0.28–0.66)	<0.001	88
LMO4 ^a	120 (75)	<50% cells +	N	0.46 (0.21–0.99)	0.049	85
HMGA ^a	89 (83)	No stain	N	12.5 (2.70–57.5)	0.001	75
SP-1 ^a	42	<20% cells +	N	2.99 (1.06–8.46)	0.039	65
Tissue invasion and metastasis						
α 5 β 1 Integrin ^a	31	<15% cells +	C	4.10 (1.15–14.7)	0.030	98
α 6 β 1 Integrin ^a	42 (35)	<50% cells +	C, M	0.55 (0.29–0.78)	0.026	99
Actinin-4 ^a	173	Relative to ND	M, C	2.23 (1.61–3.39)	0.000009	70
ADAM9 ^a	59	No stain	M, C	2.85 (1.21–6.71)	<0.05	56
Claudin 18 ^a	166	No stain	M	0.52 (0.32–0.84)	0.013	69
CXCR4 ^a	71	Immunoscore \leq 3	C	2.54 (1.27–5.1)	0.001	79

(Continued on the following page)

Table 2. Proteins related to OS according to the study of resected PDAC (Cont'd)

Protein	Total no. (PD)	Reference group	Compartment	HR (95% CI)	P	Reference
Cytokeratin 20 ^a	76 (67)	No stain	M	2.15 (1.13–4.12)	0.02	83
Dysadherin ^a	125	<20% cells +	M	2.17 (1.14–4.14)	0.019	102
E-cadherin ^a	197	>90% cells +	M	1.80 (1.33–2.42)	0.0001	94, 102
Galectin-3 ^a	104	Staining less than pancreatic nerve	C	2.06 (1.23–3.46)	0.006	101
Laminin γ 2 ^a	48	No stain	C, M	2.41 (1.18–4.93)	0.0161	107
Li-cadherin ^a	102	<25% cells +	M	2.04 (1.16–3.61)	0.01	109
Maspin ^a	229	<5% cells +	N, C	2.43 (1.36–4.34)	0.01	46
MMP7 ^a	70	<30% +	C	3.09 (1.22–10.8)	0.022	119
PAI-2 ^a	46 (39)	No stain	C	2.72 (1.13–6.53)	0.001	104
PGP9.5 ^a	65	<50% cells +	C	0.37 (0.21–0.65)	0.0006	112
SPARC (stromal) ^a	299	<10% cell +	S	1.89 (1.31–2.74)	0.001	64
Syndecan (stromal) ^a	144	<5% area +	S	1.70 (1.21–2.38)	0.002	67
Synuclein- γ ^a	62	<10% cells +	C, N	3.4 (1.51–7.51)	0.003	58
TROP2 ^a	197 (105)	Immunoscore <4	M	1.8 (1.1–3.0)	0.009	52
UPAR ^a	42 (35)	<50% cells +	C	0.49 (0.28–0.82)	0.006	99
Angiogenesis						
APN/CD13 ^a	50	<10% cells +	M, C	0.4 (0.19–0.84)	0.016	63
COX-2 ^a	485 (384)	Immunoscore 0/+ <10% cells	C	1.39 (1.13–1.71)	0.002	84, 66
DKK-3 ^a	154	No stain	C	0.6 (0.40–0.94)	0.024	53
FGF	104 (80)	Immunoscore <1	C, S	1.56 (0.92–2.66)	0.098	54
FLT-1 ^a	76 (30)	AQUA	C, M	9.87 (2.04–47.7)	0.004	48
HIF-1 ^a	58	No stain	C	2.22 (0.99–4.99)	0.05	105
Midkine ^a	75	<10% cells +	C	2.14 (1.29–3.71)	0.003	79
PD-ECGF ^a	144	No stain	C	2.03 (1.22–3.38)	0.007	54, 62
PEDF ^a	80 (61)	No cytoplasmic stain	C	0.39 (0.22–0.70)	0.0016	114
Tissue factor ^a	113 (78)	<25% cells +	C, M	2.01 (1.21–3.34)	0.008	92
VEGF	202 (178)	<10% cells +	C	1.34 (0.87–2.06)	0.18	105, 63, 54
Pancreatic differentiation and stem cell-like function						
CD133 ^a	80	No stain	C	2.15 (1.21–3.87)	0.009	79
HOXB2 ^a	59	<20% cells +	N	5.01 (2.36–10.6)	<0.0001	100
MUC-4 ^a	135 (116)	<5% cells +	M, C	2.13 (1.24–3.65)	0.006	96
PDX-1 ^a	35	>50% cells +	C	0.53 (0.28–0.95)	0.03	71
Chemotherapy						
ERCC	64	AQUA	C	1.54 (0.80–2.94)	0.194	42
GLUT-1 ^a	60 (44)	No stain	M	2.81 (1.1–8.0)	0.034	95
hCNT3 ^a	45	Median scoring <150	C	2.65 (1.19–5.87)	0.017	82
hENT1 ^a	45	Median scoring <80	C	3.42 (1.44–8.81)	0.005	82
OPRT	99 (40)	No stain	C	0.90 (0.61–1.35)	0.62	91
RRM1 ^a	64	AQUA	N	1.89 (1.01–3.48)	0.046	42
Thymidylate synthase	132 (116)	\leq 2 score	C	1.05 (0.73–1.51)	0.8	60, 108
Altered immunocompetence						
CD74 ^a	68	<70% cells +	C	2.00 (1.3–3.2)	0.003	87
ILR1	31	<15% cells +	C	1.64 (0.38–7.02)	0.506	98
PD-L1 ^a	51	<10% cells +	C	2.29 (1.12–4.68)	0.022	93
RCAS ^a	80 (61)	<5% cells +	C, M	3.09 (1.33–7.21)	0.009	59

NOTE: Summary of the multivariate HRs with 95% CIs for the eligible proteins, ranked according to Hanahan–Weinberg functional capabilities.

Abbreviations: C, cytoplasm; M, membrane; N, nuclear; S, stromal; ND, normal distribution.

^aFor associations representing data from single study, *P* values calculated by multivariate Cox proportional hazards modeling. For associations representing data from multiple studies, combined summary HRs are those calculated for the fixed effects general inverse variance method. Proteins with a statistical significance of *P* < 0.05 are marked.

^bPrognostic significant only node negative cases (*n* = 26).

Table 3. Proteins related to DFS according to the study of resected PDAC

Protein	Total no. (PD)	Reference group	Compartment	HR (95% CI)	P	Reference
Limitless replicative potential						
Ki-67	104 (80)	<10% cells +	N	1.25 (0.76–2.04)	0.38	54
MAP4K4 ^a	104 (80)	<10% cells +	C	2.44 (1.33–4.50)	0.004	74
P53	104 (80)	<1% cells +	N	1.06 (0.61–1.81)	0.845	54
VCP/p97 ^a	83	Less than normal	C	2.28 (1.09–2.15)	<0.01	121
Tissue invasion and metastasis						
Annexin II ^a	62	<30% cells +	C	2.72 (1.36–5.47)	0.047	110
CXCR4 ^a	71	Immunoscore \leq 3	C	1.86 (0.99–3.49)	0.054	79
Synuclein- γ ^a	62	<10% cells +	C, N	2.80 (1.26–6.02)	0.011	58
TROP2 ^a	197 (105)	Immunoscore <4	M	1.8 (1.10–2.90)	0.014	52
Angiogenesis						
FGF	104 (80)	Immunoscore <1	C, S	1.62 (0.97–2.73)	0.068	54
PD-ECGF ^a	104 (80)	No stain	C	1.97 (1.16–3.35)	0.012	54
VEGF	104 (80)	<10% cells +	C	0.77 (0.43–1.39)	0.391	54
Chemotherapy						
ERCC	64	AQUA	C	1.42 (0.77–2.63)	0.194	42
hCNT3 ^a	45	Median scoring <150	C	2.09 (0.99–4.42)	0.52	82
hENT1 ^a	45	Median scoring <80	C	3.17 (1.43–6.73)	0.004	82
RRM1 ^a	64	AQUA	N	1.39 (0.78–2.48)	0.265	42

NOTE: Summary of the multivariate HRs with 95% CIs for the eligible proteins, ranked according to Hanahan–Weinberg functional capabilities.

Abbreviations: C, cytoplasm; M, membrane; N, nuclear; S, stromal.

^aProteins with a statistical significance of $P < 0.05$ are marked.

Discussion

The need for independent prognostic molecular markers that can be easily evaluated by IHC following potentially curative resection of PDAC prompted this systematic review and meta-analysis. The subsequent aim was identification of proteins that correlate with outcome that could be validated in cohorts and most importantly in preoperative samples potentially augmenting current clinical management algorithms. Using stringent inclusion and exclusion criteria examining patient selection, laboratory and statistical methodology (18, 20), we identified 83 high-quality cohort studies publishing multivariate survival analysis for 103 unique proteins. The individual biomarker assay data were organized according to OS and DFS, including functional grouping including the Hanahan–Weinberg acquired capabilities of cancer (21).

On the basis of functional capabilities, those facilitating invasion and metastases were most likely associated with prognosis with numerous subclasses displaying statistically significant results for both OS and DFS. In total, 21 markers were significantly associated with OS survival outcome, however, all within single studies except for E-cadherin for which data from 2 studies were included. Three markers were examined for DFS impact (TROP2, Synuclein γ , CXCR4) and showed concordant results. Certainly there is evidence supporting the prognostic roles of cadherins with loss of E-cadherin, overexpression of LI-cadherin, and

dysadherin-influencing outcome; however, despite numerous studies investigating catenins none were eligible (33, 124, 125). Although matrix metalloproteinases have been heavily investigated as therapeutic targets, unfortunately, only one study was eligible for inclusion. A further study showing MMP7 prognostic influence failed to report HRs adequately (126). There is considerable evidence for the prognostic value of the UPA/UPAR system (104, 127–129); however, only 2 studies were eligible for analysis (99, 104).

Regulators of angiogenesis that influenced overall mortality included 11 candidates—COX-2, HIF-1, FGF, PEDF, VEGF, PD-ECGF, FLT-1, APN/CD13, midkine, DKK-3, and tissue factor—highlighting the importance of this functional grouping in PDAC progression. COX-2 was investigated in 2 studies with elevated levels associated with significantly worse outcome ($P = 0.002$). Despite VEGF being a heavily studied marker, only 3 studies (54, 62, 105) were eligible, a combination of which did not suggest that overexpression influenced OS (HR = 1.34, 95% CI: 0.87–2.06; $P = 0.18$), with a similar result for DFS. PD-ECGF had been assessed in 2 eligible studies and increased expression was associated with worse outcome (HR = 2.03, 95% CI: 1.22–3.38; $P = 0.007$; refs. 54, 62).

Among the 22 proteins associated with limitless replicative potential, pAkt and Ki-67 seem to be most consistently associated with OS. Despite more than 40 studies assessing p53 prognostic impact, few identified a significant association. Pooling the eligible datasets assessing OS

impact failed to support a prognostic role for p53 ($P = 0.14$); likewise, DFS was unaffected. In terms of cyclins and cyclin-dependent kinases (CDK) only cyclin E had been suitably investigated relating to outcome in a single study (HR = 2.03, 95% CI: 1.22–3.38; $P = 0.0021$). Further validation of cyclin E by the authors in an additional cohort failed to support the finding (44). CDK inhibitors were statistically associated with mortality. Elevated levels of P21/WAF1 were associated with improved OS across multiple studies ($P < 0.004$) whereas P27/KIP1 expression was not ($P = 0.47$).

Loss of the tumor suppressor SMAD4 is an early event in PDAC; however, combining 2 studies, each reporting prognostic impact but with opposing direction (43, 111), did not reveal any prognostic impact on OS ($P = 0.21$). A further component of the TGF- β pathway, TGF- β 1 itself was an independent predictor of outcome ($P = 0.015$). The proteins associated with apoptosis survivin, BCL-2, and Bax were consistently significantly associated with overall outcome in multiple studies.

From the small number of studies investigating pancreatic differentiation markers, 4 were eligible for inclusion. Expression of the mucins (MUC-1, -4) were associated with worse OS following resection. Regarding transcription factors, although HOXB2 strongly prognostic in an initial test cohort this was not proven in a validation set (44). The presence of both LMO2 and LMO4 has recently been associated with improved outcome following resection, whereas a similar association has been shown for HMGA and SP-1. The frequency of statistical significant associations among transcription factors suggests that altered transcriptional regulation is a pivotal step in the regulation of PDAC-related survival. S100 calcium-binding proteins have been identified by microarray experiments to be differentially expressed in PDAC. Three members, S100A2, S100A4, and S100A6, were eligible for inclusion and all associated with poorer outcome. Of particular interest is S100A2, which remained prognostic following interrogation in a validation cohort (44). These molecules seem to have multiple effects and may modulate cell cycle, motility, and invasion (130).

Cyclin D1 and p16, both integral cell-cycle regulators, were presented in the literature by 12 and 9 studies, respectively; however, none were eligible for inclusion. Twenty-four studies reported on HER-2 data; however, only 1 was eligible for this review (72). Other underrepresented signal transduction components include 13 studies reporting for epidermal growth factor receptor (131–133), c-kit (134–136), PTEN (137), and c-myc (138–140). The Wnt signaling pathway, important in embryogenesis and gastrointestinal cancer development, was underrepresented with even β -catenin, investigated in 7 studies having no representation. Likewise, recent studies have identified deregulation of pathways important in vertebrate pancreas development, including Notch (141) and Hedgehog (142), in the development and progression of PDAC. Limited assessment of the prognostic utilities of these pathways has begun (143, 144).

Markers associated with chemotherapeutic agent metabolism may yield prognostic information as well as predictors of adjuvant therapy response. hENT1 is a ubiquitous protein and principal cellular transporter of gemcitabine, which is a predictor of response to gemcitabine (39), and also a predictor of both DFS and OS (82). RRM1 seems to be a key determinant of gemcitabine resistance and it was significantly related to decreased OS following R0 resections but did not relate to DFS (42). Low RRM1 also associated with improved response to gemcitabine following recurrence (42).

Our strengths lie within the broad, unbiased search of the PDAC literature and the application of standardized systematic review and meta-analysis techniques to objectively identify manuscripts containing data sufficiently robust to be summarized. By not considering redundant study data we have attempted to avoid repeated inclusion of cohorts from different publications. However, there were limitations. We did not extend the search to unpublished data that would likely include increased proportions of null results. While limiting our strategy clearly risks publication bias, these alternative sources are unlikely to contain sufficient methodology. We did not contact authors of excluded manuscripts due to inadequate methodology or inadequate result presentation. The restriction to English language articles possibly favors the positive studies (145).

We endeavored to focus on studies assessing prognostic markers following resection with curative intent, excluding studies with a considerable proportion of metastatic disease. Furthermore, our interest lay in evaluating prognostic markers following PD, which the vast majority of studies focused on. However, resected tail lesions were included within this meta-analysis, a group with a worse prognosis, and therefore represents a confounding factor.

These data are limited: 89 proteins the data presented were from only a single study, which in 16 cases included less than 50 samples. False-positive as well as false-negative results therefore cannot be excluded. Validation of the results of these single reports by independent study is certainly warranted, and it is hoped that this review highlights the most appropriate candidates. For those studies evaluating the 14 markers studied in more than a single study, factors related to immunostaining heterogeneity across studies along with categorization and statistical adjustment for the clinicopathologic factors included within multivariate analysis may have contributed to measurement error of biomarker association. Although the majority of authors corrected for established prognostic variables, variations in adjustment method contribute to inaccuracy associated with risk estimation. In particular, variation in resection margin involvement as a result of nonstandardized pathology reporting likely contributes to variation across the studies (146).

Variability in protein expression assessment must be considered a potential source of bias. Only 2 included studies used automated image capture and analysis techniques (42, 48). The remaining authors determined staining levels visually, with obvious potential for incorrect

classification especially among the 23 studies (28%), which did not report blinding of the scorers from clinicopathologic details. The high proportion of independently prognostic biomarkers identified by studies using TMAs (44, 46, 61, 85, 100, 103, 116) highlights the importance of this high-throughput methodology allowing appropriate tissue resource rationing but also improving IHC standardization and reproducibility in large cohorts.

Traditional survival analysis techniques (Kaplan–Meier, log-rank test) rely on variable dichotomization into high or low values or splits into multiple bins. Cutoff point establishment is a source of considerable interstudy heterogeneity. For most markers, immunostaining cutoff points were arbitrarily selected and varied between studies even for the same marker. p21 expression was deemed high when expression was evident in more than 5% of cancer cells in the study by Ahrendt and colleagues (41); however, a threshold of 10% was used in another study (44). Consensus adoption among the pancreatic research community of cutoff points could facilitate result replication. A solution is the use of image analysis software to calculate expression as a continuous parameter (147). However, algorithmic interpretation of PDAC pathology remains difficult due to dense stromal response and diffuse growth patterns.

All the eligible studies were retrospective in design; however, a prospective assessment of a predictive biomarker was included. TMA-evaluated hENT1 expression was significantly associated with both OS and DFS by multivariate analyses in patients receiving adjuvant gemcitabine, but not 5-FU (39). The analysis benefited from a clearly defined cohort with rigorous follow-up that received 2 uniform treatment regimens. This provides a strong example of treatment decision guidance using a simple single biomarker, transportable to any pathology department.

The execution of this systematic review and meta-analysis has highlighted the gaps in IHC-based PDAC prognostic biomarker research. Despite an initially large number of studies there were limited numbers with high-quality data. A number of factors may contribute to this paucity. First, large sample size is difficult to achieve, as only 10% to 15% of patients undergo PD. Many studies therefore combine primary operable cases, biopsy-derived samples, and DPs. Second, unlike large-scale genome-wide studies with parallel candidate assessment, IHC analyses must begin with candidate nominations based on *a priori* biological rationales, followed by their prioritization for execution in serial assays. Clearly, research trends lend significant selection bias. Examination of Ki-67 and VEGF was supported by their established roles in cancer progression (148–150). Genes intimately involved in pancreatic carcinogenesis are another source of investigation, for example, SMAD4 (111). Likewise, proteins not linked directly to PDAC involvement are less rigorously studied, for example, CXCR4 (81).

To avoid the biased *a priori* selection of markers, an unbiased mRNA expression profiling microarray approach can select markers; however, this approach will be unful-

filled if no validated antibodies exist. Many transcripts resulting from microarray experimentation lack functional characterization and proteins are unlikely to have commercial antibodies. Although a gene expression microarray meta-analysis failed to identify significant profile overlap (10), the platform has identified numerous overexpressed transcripts, which IHC validation has established as prognostic markers, including S100 calcium-binding proteins (44, 116) and claudin 18 (69).

The number of proteins eligible for analysis in this systematic review was limited by the number of studies that lacked one or more of the inclusion criteria. The dual PubMed keyword search criteria along with manual curation of the literature identified approximately 2,000 articles, from which 398 manuscripts were selected for detailed consideration. This was reduced to 85 studies covering 103 unique proteins for which 83 had sufficient descriptive statistics available to allow inclusion. For 71 studies, analysis was restricted to cross-sectional correlations with clinicopathologic criteria including tumor stage, grade, and size for which many proteins achieved statistical significance. Unfortunately, protein expression was not pursued to formal survival analysis despite pathologic correlation not guaranteeing prognostic utility.

Unfortunately, the most distressing source of loss is the failure to conduct multivariate analysis following initial univariate survival association in 167 studies. In a further 42 cases, multivariate analysis was executed in an otherwise robust study; however, the HRs and 95% CIs were omitted. The REMARK guidelines state the investigation must include established clinicopathologic prognostic factors as part of a multivariate model and report the resulting HRs regardless of statistical significance (18). Since the 2005 REMARK criteria, 51 univariate and 16 multivariate studies have been published suggesting that recommendation uptake has been slow among the pancreatic cancer research community; however, the situation is improving with numerous high-quality publications in 2009. As already highlighted, the negative studies reported less detailed results, making them unlikely to be evaluated. Although we excluded studies that did not include multivariate analysis, we note that for many robust markers including UPAR and survivin multiple studies supported their prognostic role without adjustment for clinical variables (129, 151), providing further validation for these significant markers.

Most, if not all, proteins are subject to modulation by intricate molecular interactions within pathways inducing cellular effects that support tumor progression. Rather than targeting single markers, which potentially only evaluate the marginal effects of individual proteins on prognosis, multimarker phenotypes, defined as combinations of pathologic and/or tumor markers, may better identify poor prognostic subgroups. Of 84 studies, 11 assessed the prognostic utility of multiple markers (28, 49, 57, 80–82, 86, 89, 90, 94, 102, 123). Notably, apoptotic marker combinations were more powerful than single protein evaluation (49, 80, 90). A combination of DNA damage repair

enzymes also outperformed single marker estimations (82). Bcl-2 and lymph node status combined was more powerful than pathologic status alone (27). Further studies, in which no individual marker was prognostic, yielded independently prognostic groups of markers, for example, KLK6 and KLK10 (152).

Clearly, adequate validation has yet to occur for the majority of IHC biomarkers, highlighting the necessity for independent cohort validation studies. The limitations of retrospectively accrued cohorts may lead to conflicting results, and greater insights would be gained from prospective clinical trials. To date, no prognostic markers for PDAC have been investigated in this manner, although hENT1 independently predicted gemcitabine response in a large prospective clinical trial (39). Only following successful validation of these biomarkers in the setting of a clinical trial, could management decisions be potentially influenced. This requires marker assessment in preoperatively collected samples, with endoscopic ultrasound (EUS) fine needle aspiration (FNA) being the standard method (153). The utility of potential prognostic and predictive biomarkers must be rigorously tested in cytologic samples as adaptation and optimization of IHC techniques will be necessary (154, 155). This could potentially enhance PD selection preoperatively with resection for cancers judged to harbor low-risk while directing high-risk cases to alternative treatment, for example, neoadjuvant therapy.

Summary

This systematic review of the IHC-based PDAC molecular prognostic marker literature supports the involvement

of S100 calcium-binding proteins, multiple regulators of tissue invasion and metastasis, regulators of angiogenesis, and proteins associated with pancreatic development in modulating PDAC outcome following resection. The clinical utility of these markers is reliant on appropriate validation in adequately powered prospective cohorts. Clearly, the high attrition rate of potential studies for integration into this review highlights the limitations in terms of quality and consistency of published studies. If we are to achieve clinically useful prognostic biomarkers for PDAC the collective pancreatic cancer research community should address the persistence of incomplete adoption of the 2005 REMARK guidelines (18). The highlighted shortcomings may explain why molecular prognostic markers have yet to be incorporated within management algorithms and furthermore may help determine markers most suited to validation in EUS FNA specimens.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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