Cyclin E Expression and Outcome in Pancreatic Ductal Adenocarcinoma

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Abstract

The association of high cyclin E expression with poor outcome in some cancers, in particular breast cancer, suggests that it may play an important role in tumor biology. Because the influence of cyclin E expression on outcome is yet to be examined in pancreatic cancer, we assessed the relationship between the expression of cyclin E, $p27^{Kip1}$, and survival in a large cohort of pancreatic cancer patients with long-term follow-up. Expression of cyclin E and $p27^{Kip1}$ was assessed by immunohistochemistry using tissue microarrays of tumor samples from 118 patients with pancreatic ductal adenocarcinoma (75 resections and 43 biopsies). High cyclin E expression (>10% positive nuclei) was identified in 39 of 118 (33%) patients. This was associated with poor prognosis on univariate analysis in the whole cohort ($P = 0.005$), as well as in the subgroup of 75 patients who underwent operative resection ($P = 0.04$). On multivariate analysis, high cyclin E expression was an independent predictor of poor survival in both the entire cohort ($P = 0.005$) and the resected subgroup ($P = 0.03$), and was superior to all tested clinicopathologic factors (tumor size, lymph node metastases, differentiation, margin involvement, and perineural invasion) as a marker of survival. Low $p27^{Kip1}$ expression (<5% positive nuclei) was present in 41 of 111 (37%) patients, but was not associated with survival, and coexpression of $p27^{Kip1}$ did not influence the association of high cyclin E expression with poor survival. High cyclin E expression is a strong independent predictor of poor outcome in patients with pancreatic cancer. Thus, if these data are confirmed in independent cohorts, measurement of cyclin E may add significant prognostic information to the currently used clinicopathologic variables and hence have potential clinical utility in the management of this disease. (Cancer Epidemiol Biomarkers Prev 2006;15(10):1941–7)

Introduction

Pancreatic cancer is the fourth leading cause of cancer death in men and women in Western societies, with a 5-year survival rate of <10% (1). Pancreatic cancer presents at an advanced stage, and, as a result, only 10% to 20% of patients are suitable for surgical treatment at the time of presentation (1). Clinical management of these patients is complicated by inconsistencies in the influence of conventional clinicopathologic variables on outcome, suggesting that some of these variables lack accuracy. In addition, preoperative assessment of some variables, such as lymph node metastases, is difficult. Whereas in other cancers, assessment of aberrations in gene expression that cosegregate with therapeutic response and outcome is being adopted to increase predictive power (e.g., estrogen receptor and *HER2/neu*, and potentially cyclin E in breast cancer), there remain no molecular markers of proven clinical utility in pancreatic cancer. This highlights the need to identify novel molecular markers of prognosis relevant to pancreatic cancer that may also have diagnostic and therapeutic utility.

Dysregulation of the normal cell cycle machinery is integral to the neoplastic process and there is now compelling evidence that the development and progression of most human cancers is associated with a high frequency of abnormalities in the retinoblastoma pathway that controls G_1-S phase progression (2). The elements in the retinoblastoma pathway that have been implicated in cancer include retinoblastoma itself, a tumor suppressor that in its underphosphorylated state represses the transcription of genes necessary for cell cycle progression, the cyclin-dependent kinases (CDK) that phosphorylate retinoblastoma during G_1 phase, and the cyclins and CDK inhibitors that regulate CDK activity. Cdk4 and Cdk6 are activated by the D-type cyclins, cyclins D1, D2, and D3, and initially phosphorylate retinoblastoma during G_1. Subsequent retinoblastoma phosphorylation by cyclin E-Cdk2 then relieves retinoblastoma inhibition of cell cycle progression and allows initiation of DNA synthesis. Recent studies have identified functions of cyclin E in addition to Cdk2 activation and retinoblastoma phosphorylation that may contribute to tumorigenesis; these include initiation of DNA replication, genomic instability, and centrosome amplification (3). The CDKs are regulated at multiple levels, including regulation of the abundance of the activating cyclins and two families of endogenous low molecular weight inhibitors. The INK4 family of CDK inhibitors includes $p16^{INK4A}$ and specifically targets cyclin D–associated CDKs. The CIP/KIP family includes $p27^{Kip1}$ and $p21^{WAF1/Cip1}$, which preferentially inhibit the activity of cyclin E-Cdk2 complexes. Many common cancers display deletion or mutation of retinoblastoma, overexpression of cyclins D1 or E, or reduced expression of $p16^{INK4A}$ or $p27^{Kip1}$ (2). In pancreatic ductal adenocarcinomas, alterations in cell cycle regulators (cyclins D1 and D3, $p16^{INK4A}$, $p21^{WAF1/CIP1}$, and $p27^{Kip1}$) have been reported at high frequencies (4) and are apparent in the precursor lesions of pancreatic cancer—pancreatic intraepithelial neoplasia (5-8) and intraductal papillary mucinous neoplasms (9, 10)—but do not reliably cosegregate with survival. Despite the association

Received 4/18/06; revised 8/4/06; accepted 8/15/06.

Grant support: National Health and Medical Research Council of Australia, The Cancer Council NSW, the St Vincent's Clinic Foundation, the Royal Australasian College of Surgeons, the Prostate Cancer Foundation of Australia the R.T. Hall Trust, and fellowships from the Cancer Institute NSW (A.V. Biankin, S.M. Henshall, and E.A. Musgrove).

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doi:10.1158/1055-9965.EPI-06-0319

between aberrant cyclin E expression and outcome in other cancers (11-13), in particular the strong association of high cyclin E expression and poor survival in breast cancer (12, 13), the influence of cyclin E on outcome in pancreatic cancer has not been addressed.

Here, we report a strong association between high cyclin E expression and poor outcome in pancreatic cancer. Furthermore, high cyclin E expression was an independent prognostic factor in resected pancreatic cancer that was a better indicator of patient outcome than traditional clinicopathologic variables.

Materials and Methods

Tumor Samples and Patient Population. We identified a cohort of patients with a diagnosis of pancreatic ductal adenocarcinoma that underwent pancreatic resection or biopsy at Westmead Hospital, Concord Hospital, Royal Prince Alfred Hospital, or the St Vincent's Hospital Campus in Sydney, Australia, between 1972 and 2001. Multicenter ethical approval for data collection and tissue use was granted by the St Vincent's Hospital/University of New South Wales, and Royal Prince Alfred Hospital Ethics Committees, as well as specific approval from the Westmead Hospital and Concord Hospital Ethics Committees. Cyclin E and $p27^{Kip1}$ expression were evaluated in 118 patients. The clinicopathologic and survival data presented in Table 1 are confined to the whole cohort of 118 patients who had either pancreatic resection or biopsy, and a subgroup of 75 patients treated with operative resection. Eighty-two of this cohort were included in a previously described group of 139 patients examining prognostic factors in pancreatic cancer (14), whereas the remaining 36 have been accrued since July 1999.

Clinical variables, including sex, age at diagnosis, preoperative assessment of disease state, and type of operative procedure, were gathered retrospectively from patient records. Pathologic findings, including tumor size, Unio Internationale Contra Cancrum T stage, and lymph node status were obtained from the pathologists' original report. In addition, microscopic findings (tumor type, degree of differentiation,

Table 1. Clinicopathologic and outcome data for all patients in the cohort

Variable	*n* (%)	Whole cohort (*n* = 118)		*n* (%)	Resected cohort (*n* = 75)	
		Median survival (mo)	*P* (log rank)		Median survival (mo)	*P* (log rank)
Sex						
Female	54 (46)			31 (41)		
Male	64 (54)			44 (59)		
Age at diagnosis (y)						
Mean	64.8			62.3		
Median	66.7			65.0		
Range	34.4-89.7			34.4-82.6		
Treatment						
Resection	75 (64)	11.2				
Operative biopsy	43 (36)	4.7	<0.0001			
Outcome						
Follow-up (mo)		0-117			0.2-117	
Median		8.5			11.9	
30-d mortality	2			2		
Death from pancreatic cancer	105 (89)			62 (83)		
Death from other cause*	2 (2)			2 (3)		
Alive	7 (6)			7 (9)		
Lost to follow-up	4 (3)			4 (5)		
Stage						
I	27 (23)					
II	11 (9)	14.0†				
III	69 (59)					
IV	11 (9)	7.2	<0.0001			
Differentiation						
Well	9 (7)			6 (8)		
Moderate	66 (56)	9.5‡		44 (59)	12.9	
Poor	43 (37)	5.8	0.008	25 (33)	9.6	0.053
Tumor size (mm)						
>20				15 (20)	9.9	
≤20				60 (80)	17.1	0.04
Margins						
Clear				40 (53)	14.5	
Involved				35 (47)	8.5	0.002
Lymph node status§						
Negative				38 (51)	14.0	
Positive				36 (49)	9.5	0.02
Perineural invasion						
Negative				31 (41)	11.0	
Positive				44 (59)	11.2	0.11
Cyclin E expression						
Low (≤10% nuclear)	79 (67)	9.8		51 (68)	14.2	
High (>10% nuclear)	39 (33)	6.4	0.005	24 (32)	8.5	0.03
$p27^{Kip1}$ expression‖						
Low (<5% nuclear)	41 (37)	9.7		25 (35)	14.8	
High (≥5% nuclear)	70 (63)	7.4	0.42	47 (65)	10.1	0.38

*Includes 30-d mortality.
†Well-differentiated and moderately differentiated tumors grouped together for analysis.
‡Stage I and II tumors versus stage III and IV.
§Lymph node status was only available in 74 patients in the resected cohort.
‖p27Kip1 expression was only available in 111 patients for the whole cohort and 72 patients in the resected cohort.

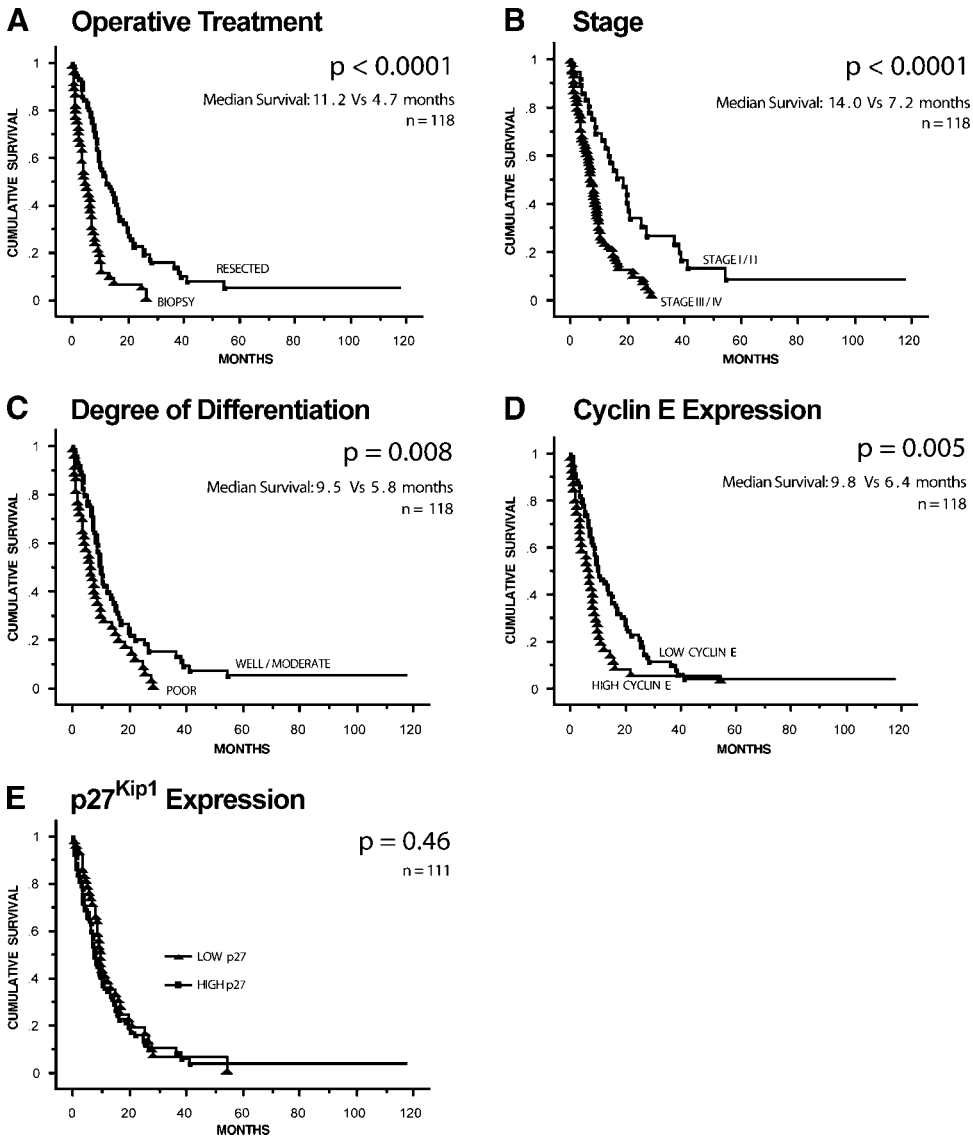

Figure 1. Kaplan-Meier survival curves for the whole cohort.

perineural invasion, and margin status) were independently reassessed by a pathologist (J.G.K.). Date and cause of death was obtained on a return-to-notifier basis from The Cancer Council NSW.

Immunohistochemistry. Seven pancreatic cancer tissue arrays consisting of 2-mm-diameter tissue core biopsies were constructed from the archival tissue. Four-micrometer sections of the tissue arrays were dewaxed in xylene and rehydrated through graded alcohol concentrations. Antigen retrieval was achieved using EDTA buffer solution (pH 8.0) at high pressure in a microwave for 30 minutes. Slides were then transferred to a DAKO autostainer (DAKO Corporation, Carpinteria, CA) and endogenous peroxidase activity was quenched by 3% hydrogen peroxide treatment before incubation with mouse monoclonal antibody against cyclin E (clone 13A3, Novocastra, Newcastle-upon-Tyne, United Kingdom) or $p27^{Kip1}$ (clone 57, Transduction Laboratories, Lexington, KY). For evaluation of cyclin E protein expression, MDA-MB-157 human breast cancer cells (which display amplification and overexpression of cyclin E; ref. 15) and normal placenta were used as positive controls, and normal testis as a negative tissue control. Epithelial nuclei at the bases of normal duodenal crypts stained strongly, providing internal positive controls within the tissue arrays. IgG2a-negative mouse serum was used as a technical control to show antibody specificity. The positive control for $p27^{Kip1}$ staining was normal skeletal muscle, whereas normal spleen was used as a negative control. IgG1-negative mouse serum served as a technical control to show antibody specificity. The primary antibody was visualized using the DAKO Envision+ secondary detection system followed by color development using 3,3-diaminobenzidine (DAKO). Sections were counterstained using hematoxylin.

Up to four separate cores of pancreatic cancer were examined per patient. Staining was assessed by two independent blinded observers for each case (D.A.S. and J.G.K.). Standardization of scoring was achieved by comparison of scores between observers and by multiviewer microscope conferencing, where any discrepancies were resolved by consensus. The following criteria were used to dichotomize each antigen: Cyclin E expression was considered high if >10% of nuclei were positive; $p27^{Kip1}$ expression was considered high if ≥5% of nuclei were stained. Cyclin E expression scores clustered in two groups with the majority of scores ≤7%, and the remainder >15%. The bimodal distribution had a mean of 14% and a median of 5%. Based on this distribution of scores, a cut point of 10% was selected to discriminate the two groups. For $p27^{Kip1}$, published studies in pancreatic cancer have used either >1% or >5% (16, 17); we chose ≥5% as the threshold because this was closer to the median score for this cohort. When >1 core was present for a particular cancer, the highest scoring core was used in the analysis.

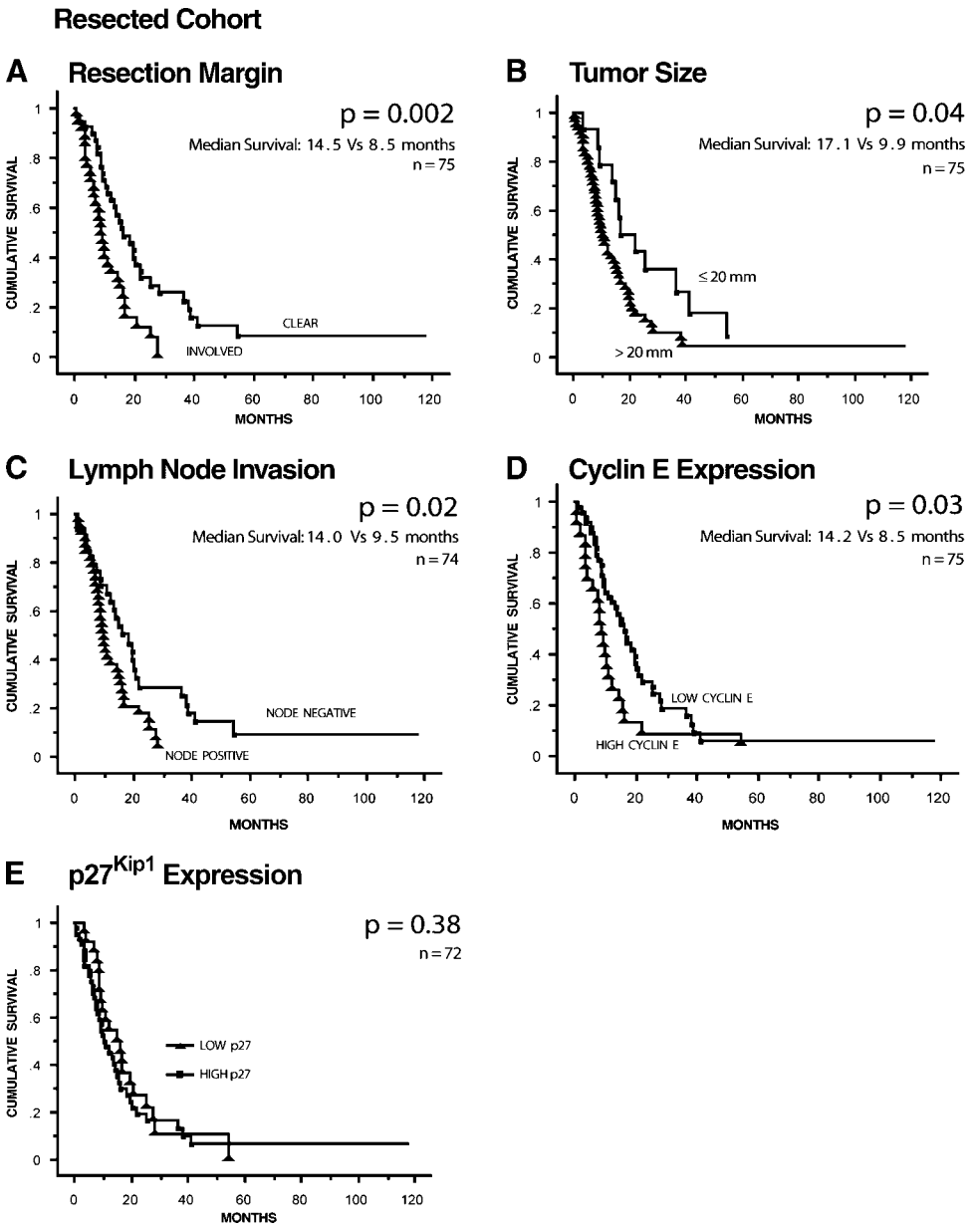

Figure 2. Kaplan-Meier survival curves for patients who underwent pancreatectomy.

Statistical Analysis. The association between cyclin E or $p27^{Kip1}$ protein expression and clinicopathologic variables, including clinical stage, degree of differentiation, operative resection, perineural invasion, tumor size, lymph node invasion, and surgical margin involvement were evaluated using nonparametric statistical tests (Kruskal-Wallis or Mann-Whitney).

Both univariate and multivariate analyses were done to assess the association of cyclin E and survival in relation to covariates using Statview 5.0 Software (Abacus Systems, Berkeley, CA). $P < 0.05$ was considered to be statistically significant. The outcome variables were assessed as time-to-event, which was defined as the difference between the time of diagnosis and the time of death.

For univariate analysis, event-survival curves were constructed using the Kaplan-Meier method for each cyclin E category. The differences in survival times between categories were compared using the two-tailed, log-rank statistic. In addition, survival curves for the coexpression of cyclin E with $p27^{Kip1}$ were plotted. Cox proportional hazards models were used to estimate hazard ratio (and its 95% confidence interval) associated with each risk factor and covariate. Those factors that were prognostic on univariate analysis were then assessed in multivariable models to identify factors that were independently prognostic.

Results

Cohort Characteristics. The cohort consisted of 118 patients with a histologic diagnosis of pancreatic ductal adenocarcinoma (64 males and 54 females). Clinicopathologic and survival data for these patients are presented in Table 1. The mean age at diagnosis was 64.8 years (range 34.4-89.7 years). Tissue was available from 75 patients who had undergone pancreatic resections and 43 who had intraoperative biopsies. A large proportion of the cancers were either moderately (66 of 118, 56%) or poorly (43 of 118, 37%) differentiated with only 9 (7%) well-differentiated tumors. Well-differentiated and moderately differentiated tumors were grouped together for analysis. The majority of patients had advanced stage disease (23% Unio Internationale Contra Cancrum stage I, 9% stage II, 59% stage III, and 9% stage IV). Stage I and II tumors were grouped together for analysis and compared with stage III and IV tumors, which were also grouped together.

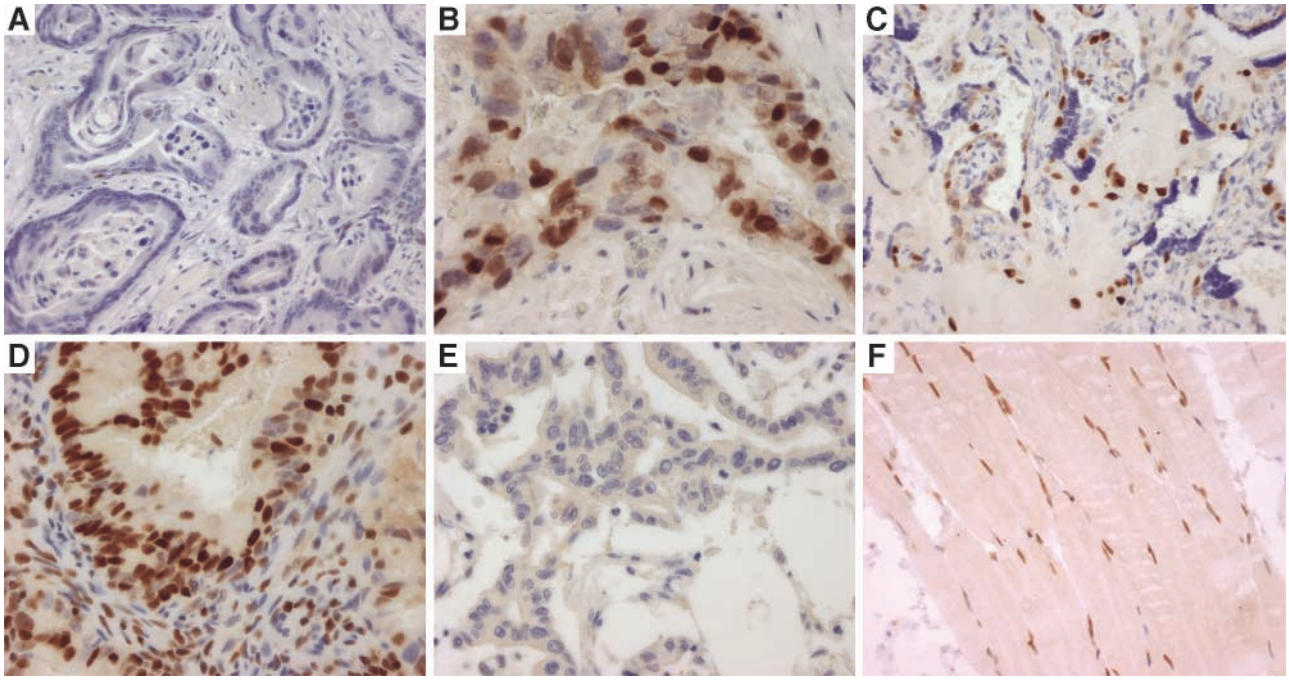

Figure 3. Pancreatic ductal adenocarcinoma. **A.** Low cyclin E expression. **B.** High cyclin E expression. **C.** Cyclin E expression in placenta. **D.** High $p27^{Kip1}$ expression. **E.** Low 27^{Kip1} expression in pancreatic cancer. **F.** 27^{Kip1} expression in smooth muscle.

The median follow-up for the cohort was 8.5 months (range 0-117.4 months). Of the 118 patients, seven patients were alive at the census date (September 21, 2002), 105 had died from pancreatic cancer, 2 due to other causes (postoperative deaths), and 4 were lost to follow-up. The overall median survival was 8.5 months and the disease-specific survival was 8.7 months. The overall disease-specific 1-year survival rate was 35%, whereas only one patient survived longer than 5 years. The actuarial 5-year survival rate for patients who underwent resection was 10%. On univariate analysis for the whole cohort, those patients that underwent pancreatectomy survived longer (log-rank $P < 0.0001$) as did those with a lower clinical stage (log-rank $P < 0.0001$) and those with non–poorly differentiated tumors (log-rank $P = 0.008$; Table 1; Fig. 1A-C). For those patients who underwent pancreatectomy, those with a tumor size of <20 mm survived longer (log-rank $P = 0.04$), as did those with surgical margin clear of tumor (log-rank $P = 0.002$) and those without lymph node involvement (log-rank $P = 0.02$; Table 1; Fig. 2A-C). However, neither degree of differentiation (log-rank $P = 0.053$) nor perineural invasion (log-rank $P = 0.11$) influenced survival.

Cyclin E Expression. Nuclear cyclin E immunostaining was identified in 101 of 118 patients; the remainder had no detectable staining (Fig. 3A-B). Beyond a threshold of 10%, high expression of cyclin E was detected in 39 of 118 (33%) of the whole cohort and 24 of 75 (32%) of the resected cohort. On univariate survival analysis, high cyclin E expression was associated with shortened survival in the whole cohort (log-rank $P = 0.005$; hazard ratio, 1.8; 95% confidence interval, 1.2-2.7) with a median survival of 6.4 months for patients with high cyclin E expression, compared with 9.8 months for those with low cyclin E expression. Similarly, high cyclin E expression was associated with reduced survival in the patients who underwent resection (median survival 8.5 versus 14.2 months, log-rank $P = 0.03$; hazard ratio, 1.8; 95% confidence interval, 1.1-3.1; Table 1; Figs. 1D and 2D).

Cyclin E did not cosegregate with any clinicopathologic variables assessed (data not shown). We next did multivariate analysis on the whole and resected cohorts, including all clinicopathologic variables that were significant predictors of survival on univariate analysis. Cyclin E overexpression was an independent marker of prognosis in both the whole

Table 2. Multivariate analysis for clinicopathologic variables and cyclin E expression in the whole and resected cohorts of pancreatic cancer

	Variable	Hazard ratio (95% confidence interval)	*P*
A. Whole cohort ($n = 118$)	High cyclin E expression	1.71 (1.12-2.63)	0.0128
	Operative resection	2.74 (1.79-4.19)	<0.0001
	Stage III/IV vs I/II	1.95 (1.91-3.19)	0.0079
	Poor differentiation	1.56 (1.04-2.35)	0.0335
B. Resected cohort ($n = 74$)	High cyclin E expression	2.39 (1.30-4.37)	0.0048
	Tumor size >20 mm	2.22 (1.07-4.58)	0.0315
	Margin involvement	1.92 (1.03-3.56)	0.0388
	Lymph node involvement	1.18 (0.62-2.22)	0.6069
C. Resected cohort ($n = 75$)	High cyclin E expression	2.48 (1.39-4.41)	0.0021
	Tumor size >20 mm	2.26 (1.09-4.68)	0.0276
	Margin involvement	2.09 (1.19-3.66)	0.0104

NOTE: Group C is the resolved model of group B, eliminating redundant variables.

cohort and the resected subgroup (Table 2). Other independent predictors of poor outcome in the whole cohort were poor differentiation, advanced clinical stage (stages III or IV versus stages I or II), and operative biopsy versus resection. In the resected subgroup, in addition to high cyclin E expression, tumor size >20 mm and positive surgical margins were independent markers of poor prognosis. In Table 2, group A shows the resolved model for the whole cohort, whereas group B shows the multivariate model for resected tumors before resolution to the final model shown in group C.

Coexpression of cyclin E and $p27^{Kip1}$. Because cyclin E functionally interacts with $p27^{Kip1}$ and this interaction seems to be prognostically significant in breast cancer (11-13), we evaluated the influence of cyclin E and $p27^{Kip1}$ coexpression on outcome. Low $p27^{Kip1}$ expression alone, i.e., <5% of nuclei positive for $p27^{Kip1}$, did not correlate with any clinical or pathologic variables, nor was it associated with survival in either the whole cohort ($P = 0.42$) or in those patients that underwent resection ($P = 0.38$). Differential expression of $p27^{Kip1}$ did not significantly alter the association of cyclin E with survival in patients with high cyclin E expression (log-rank $P = 0.73$) or low cyclin E expression (log-rank $P = 0.93$).

Discussion

In this study, we present the first report concerning cyclin E expression and outcome in patients with pancreatic cancer. High cyclin E expression was associated with a poor prognosis on univariate analysis, and was the most influential prognostic factor on multivariate analysis compared with clinicopathologic variables in patients that underwent pancreatectomy. These findings suggest a potentially important role for cyclin E in the clinical behavior of pancreatic cancer supporting similar data seen in breast cancer where high cyclin E expression is a powerful predictor of outcome (12, 13). Hence, cyclin E expression is a potentially clinically useful prognostic marker for pancreatic cancer creating scope for the application in pancreatic cancer of novel therapeutic strategies being developed that target aberrant cyclin E.

Numerous studies have assessed the prognostic value of clinicopathologic variables in pancreatic cancer (18-22). Only involved surgical margins and large tumor size are consistently associated with poor patient outcome (14). These factors are either difficult to identify or indeterminable preoperatively. The prognostic significance of molecular markers in pancreatic cancer, including growth factors and their receptors, cell cycle regulators, oncogenes, tumor-suppressor genes, apoptotic factors, angiogenic factors, and stromal factors, have been assessed (14, 23, 24), but at present have limited clinical utility. Given the importance of cell cycle deregulation in carcinogenesis, and that aberrant expression of any other cell cycle regulatory molecules important in pancreatic cancer (cyclin D1, p53, $p16^{INK4A}$, $p21^{WAF1/CIP1}$, and $p27^{Kip1}$) has not been reliably associated with outcome to date (reviewed in ref. 23),[5] the association of high cyclin E expression and poor prognosis in pancreatic cancer presented here suggests a potentially important role for cyclin E deregulation in pancreatic cancer.

Oncogenic effects of cyclin E are usually attributed to its ability to promote cell cycle progression through phosphorylation of retinoblastoma but recent data provide increasing evidence for induction of genetic instability as a likely contributor (3). Cyclin E overexpression in cultured cells leads to chromosome loss, apparently via generalized chromosomal instability (25). Potential mechanisms for this effect include interference with the assembly of a prereplication complex at origins of replication (26), and centrosome amplification, which can contribute to aneuploidy by induction of mitotic spindle defects (27). Aneuploidy has been reported as a poor prognostic factor in some studies (28), and it would be of interest to examine the relationship between aneuploidy and cyclin E expression.

Three previous studies have examined cyclin E expression in pancreatic cancer. The different cut points for staining scores between all the studies make comparisons difficult. Schraml et al. (29) examined the expression of cyclin E using immunohistochemistry in 128 tumor types and identified high expression of cyclin E in one of eight pancreatic cancer samples. Although amplification of cyclin E using fluorescence *in situ* hybridization was seen in some tumor types, it was not detected in their samples of pancreatic cancer. Other studies, both using a cut point of 5%, reported higher rates of cyclin E overexpression in smaller cohorts: Al-Aynati et al. (7) identified cyclin E overexpression in 75% of 38 pancreatic cancer samples, and Yue et al. (30) reported cyclin E overexpression in 69% of 32 pancreatic cancers. At a cut point of 10%, we identified high cyclin E expression in 33% of 118 pancreatic cancers. It is likely that these differences in incidence are due to the different cut points used, rather than a true difference in cyclin E expression between cohorts, with our large cohort containing sufficient numbers to detect a bimodal distribution, which, in turn, cosegregated with outcome. Only one study has assessed cyclin E expression in pancreatic intraepithelial neoplasia (8), the precursor lesion of pancreatic cancer, where cyclin E overexpression was seen in 25% of advanced (PanIN-3) lesions (7).

In summary, we have shown that high cyclin E expression is an independent prognostic factor in pancreatic cancer. In patients who underwent pancreatectomy, high cyclin E expression was a superior predictor of survival than clinicopathologic variables, including tumor size, margin involvement, and lymph node status. Hence, cyclin E is a potentially clinically useful prognostic marker in pancreatic cancer, suggesting a simple, well-described test that can be implemented in the routine pathology laboratory in a disease where there are few useful markers of prognosis. If confirmed in other independent cohorts, these data create scope for the potential application of novel therapies targeting aberrant cyclin E, a marker of poor prognosis cancers, in pancreatic cancer.

Acknowledgments

We thank Dr. Catherine Langusch for her help with the maintenance of the Garvan Institute of Medical Research Pancreatic Cancer tissue bank and clinical database.

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