Differential cell cycle and proliferation marker expression in ductal pancreatic adenocarcinoma and pancreatic intraepithelial neoplasia (PanIN)

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Summary

Aims: Pancreatic cancer is an aggressive tumour following a multistep progression model through precursors called pancreatic intraepithelial neoplasia (PanIN). Identification of reliable prognostic markers would help in improving survival. The aim of this study was to investigate the role as well as the prognostic significance of different cell cycle and proliferation markers, namely p21, p27, p53 and Ki-67, in pancreatic carcinogenesis.

Methods: We analysed the expression of p21, p27, p53 and Ki-67, in 210 ductal pancreatic adenocarcinomas, 40 PanIN-3 cases and 40 normal controls combined in a tissue microarray. The results were correlated with clinicopathological and follow-up data.

Results: Our study revealed a differential p27, p21, p53, and Ki-67 expression between ductal adenocarcinoma, PanIN-3 and normal pancreas. p27 expression progressively decreased from normal pancreas to PanIN and to pancreatic cancer. Decreased p27 and increased p53 expression showed a significant association with the T stage. A Ki-67 > 5% correlated with reduced survival.

Conclusions: In pancreatic cancer, loss of p27 and increased p53 expression is associated with a more aggressive phenotype. p27 may play an important role in pancreatic carcinogenesis. A Ki-67 > 5% independently predicted poor outcome.

Key words: Pancreatic cancer, PanIN, prognosis, tissue microarray, immunohistochemistry.

INTRODUCTION

Pancreatic adenocarcinoma is the fourth leading cause of cancer death and has dismal prognosis.¹ Clinicopathological parameters like tumour size, clinical stage, nodal metastases and evidence of blood vessel or lymphatic invasion have been proven to be reliable prognostic determinants in patients with pancreatic cancer.^{2–6} However, earlier detection of pancreatic carcinoma would help to improve patient outcome. Moreover, the identification of reliable and

reproducible prognostic markers would enable the stratification of patients into different groups, and would eventually provide a guide in developing new therapeutic modalities.

Pancreatic cancer follows a multistep progression model through non-invasive precursor lesions,^{7–10} classified into four groups of pancreatic intraepithelial neoplasias (PanINs): PanIN-1A, -1B, -2 and -3.^{10,11} PanIN-3 demonstrates severe epithelial dysplasia and is most likely to progress to invasive carcinoma.¹² Comparative molecular analysis has shown that PanINs harbour the same genetic alterations as invasive carcinomas, however not with the same frequency.^{13,14}

Loss of cell cycle control is taking place during the neoplastic process and is implicated in development and progression of most malignancies.¹⁵ Cell cycle arrest after DNA damage is partly mediated through the transcriptional activation of p21 by the p53 tumour suppressor gene.¹⁶ The expression level of p27, an inhibitor of cyclin-dependent kinases in mammalian cells, has been implicated in tumour progression and has proved to be a prognostic predictor for several human cancers.^{17–20} Cell cycle-associated markers, including p21, p27 and p53, have been evaluated in pancreatic cancer without conclusive results regarding prognostic significance or correlation to precursor lesions.^{21–28} Studies analysing the proliferation rate of pancreatic adenocarcinomas have also failed to produce consistent conclusions regarding prognosis.^{29–31}

Our objective was to investigate the role of the cell cycle and proliferation markers p21, p27, p53 and Ki-67 in pancreatic carcinoma. For this purpose we constructed a tissue microarray containing 210 pancreatic cancer cases from resection specimens, as well as 40 PanIN-3 cases and 40 normal controls. The immunohistochemical expression was correlated with clinicopathological variables.

MATERIALS AND METHODS

Patients and specimens

Tumour and control specimens were retrieved from the archives of the Institute of Pathology, University of Bern, Switzerland. Histologies other

than ductal carcinoma were excluded. Tumours were restaged according to the American Joint Committee on Cancer (AJCC) Staging Manual (6th edition). Representative areas were selected for construction of the tissue microarray (TMA). The TMA consisted of 210 cases of pancreatic adenocarcinoma from resection specimens, 40 PanIN-3 cases and 40 normal controls. Normal pancreatic tissue and PanINs were selected from areas distant to carcinoma. PanIN-3 cases included in the study had mostly a low papillary and occasionally a mixed low papillary and flat pattern. Lesions with tubular pattern (thought to represent intraductal cancerisation) were excluded from the study on the basis of previous reports.^{32,33} The 210 patients comprised 110 males and 100 females. The mean age of the patients was 66.5 years (range 20–92). The study was approved by the ethical committee of the University of Bern.

Assessment of behaviour

Medical charts were available from 77 of the 210 patients. Of these, 60 (78%) died of the disease, and seven (9%) were alive with recurrent/metastatic disease. An additional 10 patients (13%) were alive without disease. Median follow-up was 16 months. The characteristics of carcinomas with survival information are shown in Table 1.

Construction of TMA

A TMA of 210 unselected pancreatic carcinoma specimens was constructed as described previously.³⁴ Briefly, formalin-fixed, paraffin-embedded tissue blocks of pancreatectomies were retrieved from the archives of the Institute of Pathology, University of Bern. Tissue cylinders with a diameter of 0.6 mm were punched from morphologically representative tissue areas of each 'donor' tissue block and transferred into one recipient paraffin block using a homemade semiautomated tissue arrayer. Each patient had three to four tissue punches taken from the donor blocks and brought into the recipient block.

Immunohistochemistry

Five µm TMA sections were dewaxed and rehydrated. After peroxidase blocking sections were incubated with 10% normal goat serum (Dako, USA) for 20 min and then with primary antibody for 60 min at room temperature (Ki-67, 1:100, Dako; p53, 1:200, Dako; p21, 1:20, Novocastra,

England; p27, 1:100, Dako). Subsequently, sections were incubated with peroxidase-labelled secondary antibody (Dako) for 30 min. DAB was used as chromogen. Sections were counterstained with haematoxylin. The primary antibody was omitted for negative controls. As positive control, a TMA with various normal tissue samples was stained in parallel.

Immunohistochemical evaluation

Slides were screened semi-quantitatively for the percentage of positivity and the intensity of the signal for p21, p27, p53 and Ki-67 staining (all nuclear). At least 100 cells were counted for each punch. The percentage of positive cells per number of cells counted was assessed in 10 groups from 0 (0–9%) to 9 (91–100%). Intensity of the signal was graded semiquantitatively in four groups from 0 (no positivity) to 3 (strong positivity). A case was considered positive if belonging at least to group 1 for the percentage (i.e., ≥10%) and intensity on the basis of previous reports.^{35,36} In PanINs and normal controls the epithelial cells of ductal structures were evaluated.

Statistical analysis

Selection of cut-off scores

The selection of clinically important cut-off scores to classify protein marker expression as 'positive/over-expressed' was based on receiver operating characteristic (ROC) curve analysis, a well-established cut-point determination approach.³⁷ At each immunohistochemical protein expression score, the sensitivity and false positive rate (1–specificity) for survival was obtained and plotted, thus generating the ROC curve. From this curve, the threshold value best classifying survivors and non-survivors can be selected using the (0,1)-criterion, to identify the protein expression score which maximises both sensitivity and specificity for survival. Additionally, to determine the reliability of the selected cut-off scores and to obtain 95% confidence intervals (CI) for the area under the ROC curve, the data were re-sampled with replacement 200 times (200 bootstrapped replications of the data). Finally, the most frequently obtained immunohistochemical score after re-sampling was selected as the final cut-off with values above the threshold considered as 'positive' or 'over-expressed' and those below as 'negative' or 'loss'.

Statistical tests

The Chi-square and Kruskal-Wallis tests were used to study the relationship between expression of different markers and histological subgroups. Univariate survival analysis was carried out by the Kaplan-Meier method and log-rank test. The assumption of proportional hazards was verified by analysing the correlation of Schoenfeld residuals and the ranks of individual failure times and Cox proportional hazards regression was performed for multivariable analysis. Hazard ratios (HR) and 95%CI were used to determine the effect of each variable on survival time. In addition, logistic regression was performed in univariate and multivariable settings to determine the association of protein marker expression and their independent effect on outcome. The odds ratios (OR) and 95%CI were evaluated. A Bonferroni correction for multiple comparisons was performed. $p \leq 0.01$ (two-sided) were required for the association to be statistically significant. All analyses were carried out using SAS (V9; The SAS Institute, USA).

RESULTS

Immunohistochemical findings are summarised in Tables 2–6 (Fig. 1 and 2). Positivity rates in different TMA samples from the same patients were reproducible.

Pancreatic carcinomas versus normal controls

In comparison with normal tissue, pancreatic carcinomas expressed p53 and p21 significantly more frequently. The expression of p27 was significantly decreased in carcinomas compared with normal tissue. In more detail, mean p53 protein expression was 29.4 ± 30.1 in carcinomas com-

Table 1 Characteristics of carcinomas with survival information ($n = 77$)

Clinicopathological features	Frequency, n (%)
Diagnosis	
Ductal carcinoma	77 (100.0)
Sex	
Female	33 (42.9)
Male	44 (57.1)
Tumour grade	
G1	16 (20.8)
G2	42 (54.6)
G3	19 (24.7)
pT stage	
pT1	3 (4.1)
pT2	12 (16.2)
pT3	52 (70.3)
pT4	7 (9.5)
pN stage	
pN0	27 (38.0)
pN1	43 (60.6)
pN2	1 (1.4)
Metastasis	
Absence	72 (93.5)
Presence	5 (6.5)
Tumour diameter	
Mean $\pm$ SD	3.14 $\pm$ 1.4
Survival time (months)	
Median (95%CI)	13.0 (11.0–16.0)

pared to 4.8 ± 2.6 in normal tissue ($p < 0.001$). For p21 and p27, mean protein expression in carcinomas was found to be 8.1 ± 10.3 and 2.6 ± 6.4 , respectively, while in normal tissue it was 1.9 ± 3.1 and 17.2 ± 16.6 , respectively ($p < 0.001$). Mean Ki-67 labelling index was significantly higher in carcinomas (6.7 ± 5.6) than in normal controls (1.0 ± 0 ; $p < 0.001$).

Pancreatic carcinoma versus PanIN-3 lesions

Mean expression of p53 (29.4 ± 30.1) and p21 (8.1 ± 10.3) was significantly higher in carcinomas compared with

Table 2 Mean protein expression (%) differences in carcinoma and PanIN

Marker	Ductal carcinoma	PanIN-3	<i>p</i> value
Ki-67	6.7 ± 5.6	2.1 ± 0.5	<0.001
p53	29.4 ± 30.1	7.8 ± 11.8	<0.001
p21	8.1 ± 10.3	2.4 ± 3.8	<0.001
p27	2.6 ± 6.4	7.9 ± 9.4	<0.001

Table 3 Mean protein expression (%) differences in carcinoma and normal

Marker	Ductal carcinoma	Normal	<i>p</i> value
Ki-67	6.7 ± 5.6	1.0 ± 0	<0.001
p53	29.4 ± 30.1	4.8 ± 2.6	<0.001
p21	8.1 ± 10.3	1.9 ± 3.1	<0.001
p27	2.6 ± 6.4	17.2 ± 16.6	<0.001

Table 4 Mean protein expression (%) differences in normal and PanIN

Marker	PanIN-3	Normal	<i>p</i> value
Ki-67	2.1 ± 0.5	1.0 ± 0	<0.001
p53	7.8 ± 11.8	4.8 ± 2.6	0.772
p21	2.4 ± 3.8	1.9 ± 3.1	0.667
p27	7.9 ± 9.4	17.2 ± 16.6	0.002

Table 5 Association of marker expression and survival time

Marker	ROC-derived cut-off score	Negative <i>n</i> (%)	Positive <i>n</i> (%)	Hazard ratio (95%CI)	<i>p</i> value
Ki-67	5%	47 (65.3)	25 (34.7)	2.88 (1.7–5.0)	<0.001
p53	20%	48 (67.6)	23 (32.4)	1.33 (0.8–2.3)	0.315
p21	5%	52 (72.2)	20 (27.8)	1.62 (0.9–2.9)	0.1
p27	10%	42 (57.5)	31 (42.5)	1.21 (0.7–2.0)	0.483

Table 6 Multivariable analysis of Ki-67 expression

Features	<i>p</i> value	Hazard ratio (95%CI)
Ki-67	<0.001	3.63 (1.9–6.9)
pT	0.325	1.31 (0.8–2.3)
pN	0.085	1.8 (0.9–3.5)
Tumour grade	0.391	1.24 (0.8–2.0)

PanIN-3 lesions, where mean protein expression of the above markers was 7.8 ± 11.8 and 2.4 ± 3.8 , respectively ($p < 0.001$). Mean expression of p27 was found to be higher in PanIN-3 lesions (7.9 ± 9.4) compared with carcinomas (2.6 ± 6.4 ; $p < 0.001$). Mean Ki-67 labelling index was significantly higher in carcinomas (6.7 ± 5.6) compared with PanINs (2.1 ± 0.5 ; $p < 0.001$).

PanIN-3 lesions versus normal controls

Mean p27 protein expression was significantly higher in normal tissue (17.2 ± 16.6) compared with PanIN-3 lesions (7.9 ± 9.4 ; $p = 0.002$). Mean Ki-67 labelling index was significantly higher in PanIN-3 lesions (2.1 ± 0.5) compared to normal controls (1.0 ± 0 ; $p < 0.001$). Mean protein expression of p53 and p21 did not differ significantly between PanINs and normal controls ($p > 0.05$).

Association of marker expression and clinicopathological features of the tumours

Increased expression of p53 [OR = 3.49 (1.3–9.5)] and loss of p27 [OR = 3.38 (1.1–10.1)] was significantly higher in late (T3 and T4) compared with early (T1 and T2) T stage ($p = 0.015$ and $p = 0.029$, respectively). The difference in p53 expression between early and late T stage remained significant after adjustment for multiple comparisons. p21 protein expression and the Ki-67 labelling index did not show any significant association with T stage. No association was noted between protein expression and tumour grade or lymph node status.

Prognostic significance

Median survival time for patients with Ki-67 labelling index of $>5\%$ (Ki-67 positive) was 9.0 (6.0–12.0 months) and for patients with Ki-67 labelling index of $<5\%$ (Ki-67 negative) was 17.0 (13.0–24.0) months ($p < 0.001$). The 12-month survival rate was 65.8% (95%CI 50–77) and 28% (95%CI 12–46) for patients with negative and positive Ki-67 tumours, respectively (Fig. 3). Adjusting for the prognostic effects of T stage, N stage and tumour grade in multivariable analysis, Ki-67 positivity maintained a significant and adverse effect on survival time [$p < 0.001$; HR (95%CI) 3.63 (1.9–6.9)].

Protein expression of the other markers (p53, p16, p21 and p27) did not show any correlation with survival.

DISCUSSION

A major finding of this study was the significantly differential mean protein expression of the examined cell cycle-associated biomarkers between pancreatic carcinoma, PanIN-3 lesions and normal pancreatic tissue.

In the present study we used ROC curve analysis, an already established method in clinical oncology,³⁷ in determining clinically important cut-off scores for the selected tumour markers. This was based on previous results³⁸ showing that ROC analysis can be used as an alternative method in the selection and validation of cut-off scores for immunohistochemical tumour positivity and that the evaluation of immunoreactivity using percentage of positive tumour cells is a reproducible scoring method with a strong inter-observer agreement.

Fig. 1 Examples of nuclear immunohistochemical detection of p27 in (A) normal pancreatic tissue (B) PanIN-3 and (C) pancreatic adenocarcinoma.

Fig. 2 Nuclear immunoreactivity of (A) p53, (B) p21, and (C) Ki-67 in ductal pancreatic adenocarcinomas. Typical examples are shown.

Fig. 3 (A) Receiver operating characteristic (ROC) curve for Ki-67 and survival in patients with pancreatic cancer. The area under the ROC curve (AUC) is 0.77. The 5% cut-off score for Ki-67 positivity was selected using the (0,1)-criterion. Arrow indicates point on ROC curve with shortest distance to coordinate (0,1). (B) Kaplan-Meier survival curve highlighting the significantly worse survival of patients with Ki-67 positive tumours compared to Ki-67 negative cases. CI, confidence interval. Circles indicate censored patients.

Mean p27 protein expression progressively decreased from normal pancreas to PanIN-3 lesions and to pancreatic cancer. Moreover, p27 showed a negative correlation with the T stage of the tumours. This finding suggests that loss of p27 is associated with an aggressive phenotype in pancreatic cancer. p27 is an important cyclin-dependent kinase inhibitor, promoting G₁ arrest³⁹ and its loss of expression has been linked to worse prognosis in many cancers.^{17–19,40} The functions of p27 in apoptotic process remain unclear with some studies reporting a pro-apoptotic and others an anti-apoptotic effect, suggesting that the survival effects of p27 may be cell-type specific or that p27 has other targets besides

cyclin dependent kinase.^{41,42} In pancreatic cancer, p27 immunohistochemical results have been contradictory regarding association with patient survival or frequency of p27 expression.^{26–28} Our results of progressively decreasing mean p27 expression from normal pancreatic tissue to PanIN and to invasive carcinoma suggest that p27 may be a key protein in controlling the neoplastic transformation and disease progression in pancreatic cancer.

p53 gene is found to be mutated or inactivated in 55–75% of pancreatic cancers and p53 mutations have been proved to have a very good correlation to p53 protein overexpression.^{43–44} Loss of normal p53 function leads to

deregulation of G₁/S cell cycle checkpoint and to impaired apoptosis in the majority of pancreatic cancers. The increased p53 nuclear accumulation observed in pancreatic adenocarcinomas in our study supports the observation that p53 inactivation is an important genetic event in pancreatic cancer progression.^{45,46}

p53 inhibits cell proliferation after DNA damage by increasing intracellular levels of p21.^{47,48} p21 prevents pRb phosphorylation by inhibiting activation of cyclin E/cdk2 complexes.¹⁶ Putative p21 mutations have been reported in pancreatic cancer.⁴⁷ Our findings are in agreement with previous studies, where p21 overexpression has been reported in a significant number of pancreatic carcinomas⁴⁸ and was demonstrated to play a role in the development of PanINs found in association with pancreatic adenocarcinoma.⁴⁹

Regarding prognosis, our findings show that a Ki-67 labelling index >5% was associated with reduced survival. This impact remained significant also in multivariable analysis indicating that Ki-67 may have an independent prognostic role in pancreatic cancer. Moreover, mean Ki-67 labelling index was significantly higher in pancreatic carcinoma compared to PanIN or normal pancreas, and in PanIN compared to normal controls. Previous results on prognostic significance of Ki-67 have been contradictory with most studies not revealing any association with survival.^{29–31} Some of these differences may be due to different numbers of tumours examined and/or to different statistical methods used. The TMA technique could also raise concerns as to a possible limitation: sampling of large, heterogeneous tumours. However, previous studies have shown comparable results between entire tissue sections and TMA cores and were able to reproduce numerous clinicopathological associations previously reported using whole tissue sections.^{50–52}

An interesting finding of this study was the demonstration of significant differences in the mean protein expression of the cell cycle-associated biomarkers p27, p21, and p53, and mean Ki-67 labelling index, between pancreatic adenocarcinoma, its precursor lesions (PanINs) and normal pancreatic tissue. A progressive reduction of p27 expression between normal pancreatic tissue, PanINs and ductal pancreatic adenocarcinoma could be demonstrated. Decreased p27 and increased p53 expression showed a significant association with the T stage of the tumours. These findings suggest that the above proteins, and especially p27, may play an important role in pancreatic carcinogenesis and are associated with an aggressive phenotype in pancreatic cancer. Finally, a prognostic role was identified for Ki-67, since a Ki-67 labelling index of more than 5% independently predicted poor outcome.

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