British Journal of Cancer (1997) 76(1), 54–59

Prognostic significance of Ki-67 antigen and p53 protein expression in pancreatic duct carcinoma: a study of the monoclonal antibodies MIB-1 and DO-7 in formalin-fixed paraffin-embedded tumour material

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Summary Formalin-fixed paraffin-embedded material from 57 patients in whom curative resection of pancreatic carcinoma had been attempted was analysed by an immunohistochemical procedure to estimate proliferation and p53 protein expression. Using the monoclonal antibody (MAb) MIB-1, which recognizes a Ki-67 epitope, the proliferating cell index (PCI, percentage of immunoreactive tumour nuclei) and proliferating cell area (PCA, percentage of immunoreactive tumour nuclear area) were calculated using an interactive image analysis system and were compared with semiquantitative scoring of stainability. MAb DO-7, which recognizes both wild- and mutant-type p53 protein, was used to assess p53 expression in the same material. MIB-1 stainings were of high quality in 53 tumours. The median PCI was 29.7% (range 0.5–82.1%) and the median PCA was 10.6% (range 0.0–36.5%). There was a close correlation between PCI and PCA ($P < 0.0001$). PCI and PCA values were in conformity with the semiquantitative scoring ($P < 0.0001$). The p53 immunohistochemical stainings were successful in 48 tumours and the protein was expressed in 22 (46%). High PCI values (> 45%, $n = 14$) correlated with shorter survival time ($P < 0.01$). PCA ($P < 0.05$) and the expression of p53 protein ($P < 0.001$) were independent prognostic variables.

Keywords: pancreatic carcinoma; Ki-67 antigen; p53; immunohistochemistry; prognosis

Different epitopes of the Ki-67 antigen (Ki-67, Ki-S1, Ki-S5, MIB-1-3) have been used to estimate proliferation in various tumours (Kelleher et al, 1994). The proliferating rate has often been described as a proliferating cell index (PCI) (Pinder et al, 1995) or has been scored subjectively (Railo et al, 1993; Lam et al, 1996). The PCI may be calculated with or without the use of interactive image analysis systems (Pinder et al, 1995; Lam et al, 1996).

The more recently developed MIB antibodies exhibit a pattern of immunostaining in formalin-fixed paraffin-embedded material identical with that of Ki-67 antibodies in fresh or frozen material (McCormick et al, 1993) and correlate with other markers of proliferation (Weidner et al, 1994). Thus, expression of the Ki-67 antigen has been used in studies on archival tumour material and has been correlated with patient survival time (Railo et al, 1993; Pinder et al, 1995).

p53 alterations may be detected at the protein level by immunohistochemical staining procedures (IHC) and at the DNA or RNA level by direct sequence studies (Bodner et al, 1992; Berrozpe et al, 1994). The presence of p53 abnormalities correlates in some tumours with short survival time (Isola et al, 1992; Martin et al, 1992; Hamelin et al, 1994). Only a few studies have been published on the prognostic value of p53 alterations in pancreatic carcinoma, and the results are contradictory (DiGiuseppe et al, 1994; Lundin et al, 1996).

Received 29 August 1996
Revised 17 December 1996
Accepted 3 January 1997

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We assessed the proliferating activity in pancreatic carcinoma using IHC on formalin-fixed paraffin-embedded material using the MIB-1 antibody. PCI and the proliferating cell area (PCA) were calculated using interactive image analyses and compared with scoring. Immunostaining with the DO-7 antibody was performed to detect p53 alterations. The prognostic significance of PCI, PCA and p53 protein expression was analysed.

MATERIALS AND METHODS

Patient and tumour material

Tumour tissue was available from 57 patients with pancreatic carcinoma in whom curative pancreaticoduodenal resection had been attempted in the Department of Surgery, Stockholm Söder Hospital, between 1981 and 1992. It was not possible to evaluate MIB-1 IHC in four tumours. Thus, the series comprised 53 patients, 17 men with a median age of 65 years (range 45–83 years) and 36 women with a median age of 66 years (range 45–83 years). In 48 patients, staining of the tumours with the DO-7 antibody was of high quality. Complete clinical records were available in all cases. The female dominance is explained by the sex distribution of elderly people in the catchment area of the hospital.

There were five long-term survivors. Four patients are still alive after 48, 60, 64 and 126 months. One patient died after 80 months without a recurrence. Four patients died in the perioperative period and were censored in the survival analyses.

All tumours were located in the head of the pancreas. Tumour stage was assessed according to the post-surgical TNM system, and tumour differentiation and malignancy grading were estimated

(Hermanek and Sobin, 1987, pp 65–87). All original haematoxylin–eosin (H and E)-stained slides were re-examined and confirmed the diagnosis of ductal pancreatic carcinoma.

The IHC procedure

The avidin–biotin–peroxidase complex (ABC) technique was used. Sections (4 μm thick) were cut from the routinely processed formalin-fixed paraffin-embedded blocks and mounted on Superfrost glass slides. One section was H and E-stained and the histopathological diagnosis was verified. They were deparaffinized in xylene and rehydrated to distilled water through the conventional ethanol scale. To enhance immunoreactivity, the sections were microwave heated. The slides were immersed in 0.01 M sodium citrate buffer (pH 6.0), placed into plastic containers and incubated in a conventional domestic microwave oven, equipped with a rotation plate, at its maximal power until boiling. Then, at 70% of its maximal power, in three consecutive intervals of 5 min each, the buffer was replenished after each interval. After microwave irradiation, the sections were cooled down to room temperature for about 20 min. Subsequently, the slides were rinsed in distilled water and incubated with 0.6% hydrogen peroxide for 25 min and rinsed in distilled water. Thereafter, they were immersed in 1% bovine serum albumin (BSA) for 30 min and incubated overnight at 4°C with the primary monoclonal antibodies (MAbs). MIB-1 MAb (code no. 0505, Immunotech, Marseilles, France), recognizing an epitope of the Ki-67 antigen, was diluted 1:150 in 1% BSA in buffer and the DO-7 MAb (Dako, Glostrup, Denmark) reacting with both wild-type and mutant p53 protein in 1:50. The slides were rinsed in buffer and incubated with biotinylated horse anti-mouse antibody, diluted 1:200 in buffer, for 30 min. The buffer used was a mixture of phosphate-buffered saline (0.01 M) and Tris-buffer (0.05 M), in saline (0.15 M), pH 7.6. The sections were incubated with the avidin–biotin–peroxidase complex (Vectastain-ABC Kit, Vector Lab, Burlingam, CA, USA) for 30 min. They were rinsed in buffer and developed in 3-amino-9-ethyl-carbazol (AEC) (Sigma Chemicals, St Louis, MO, USA). The nuclei were counterstained with Meyer's haematoxylin. The slides were mounted with glycerol gelatine (Merck, Darmstadt, Germany). One slide with breast carcinoma known to contain MIB-1 reactive cells or cells showing p53 protein expression was included in each staining procedure as a control for immunoreactivity. One slide preparation in which the primary antibody had been replaced by 1% BSA in buffer was used as 'negative' control.

IHC evaluation

The MIB-1 immunoreactivity was evaluated by image analysis using the ACAS ICM System (Ahrens Cytometry Systems, Bargteheide, Germany) (Parrado et al, 1996). PCI (percentage of positively stained tumour nuclei) and PCA (percentage of positively stained tumour nuclear area) were calculated. The immunostaining analysis software module allowed automatic detection and evaluation of immunoreactive and counterstained nuclei. The system was equipped with a Leitz Orthoplan microscope, plan objective 40/0.95, a single-chip colour CCD (charge-coupled device) camera (JVC TK 870) and a colour-image frame grabber. In the immunostaining analysis routine of the system, 'positive' reddish-brown AEC tumour nuclei were detected and discriminated from 'negative' blue haematoxylin Meyer nuclei. The measuring sequence consisted of two general phases: one calibration phase and one measuring phase. During the calibration phase, the immunostained nuclei were defined by the user with respect to the colour shade of the positive staining (two or three test image fields). During the measuring phase, the 'positive' and 'negative' nuclei were detected automatically but were corrected interactively by adding or removing 'positive/negative' particles. This technique allowed exclusion of stromal, vascular, necrotic, haemorrhagic and other irrelevant areas, as well as the inclusion of appropriate corrections for superimposed nuclei ('doublets'), for multiple or giant nuclei and for too weakly stained nuclei.

Calculations were based on 25 fields in each section within cancer cell areas. Fields were not selected at random to avoid areas with stroma, but the measurements were not performed specifically in the most proliferative areas to obtain an average estimation of the specimens.

The interactive measurement of MIB-1-stained nuclei was compared with conventional semiquantitative scoring. The scoring system was designed as follows: –, no staining; (+), clear staining in less than 20% of the nuclei; +, clear staining in up to 40%; ++, clear staining in up to 60%; and +++, more than 60% of the nuclei clearly stained.

The p53 immunoreactivity was only scored conventionally. As in the MIB-1 immunostainings, specimens with only occasional (< 1%) weakly immunoreactive nuclei were scored as negative (Scarpa et al, 1993). Negative cases were compared with those with positive scoring (+, ++ or +++) in the survival analysis.

Statistics

The log-rank test was used to assess differences in survival time between groups. Continuous factors were tested for influence on survival using the Cox bivariate regression. Factors remaining significant for survival in the bivariate analysis were tested in a Cox multivariate regression analysis. When comparing the PCI and PCA with conventional MIB-1 scoring and with p53 protein expression Student's unpaired *t*-test was used. The PCI was compared with PCA using simple regression analysis.

RESULTS

Tumour stage – histopathological grade

There were 27 stage I, eight stage II and 18 stage III tumours. The median size was 25 mm (range 10–70 mm). There were five well-differentiated, 18 moderately differentiated and 30 poorly differentiated tumours.

MIB-1 immunoreactivity (Ki-67 antigen)

A median of 384 nuclei (range 163–557) were evaluated in the 53 specimens with MIB-1 immunostainings (Figure 1). The median PCI value was 29.7% (range 0.5–82.1%). There was no correlation between PCI and tumour grade. According to scoring, there were two specimens without immunoreactivity, 22 scored as (+), 14 as + and 15 specimens as ++. Tumour specimens classified as –/(+) had significantly lower PCI values (15.6 ± 11.3%, mean ± s.d.) than those scored as +/++ (42.9 ± 18.1%, mean ± s.d.) ($P < 0.0001$). Thus, there was good agreement between the two methods. The median PCA was 10.6% (range 0.0–36.5%) and the PCA correlated well with the PCI ($P < 0.0001$). Also, with respect to the PCA,

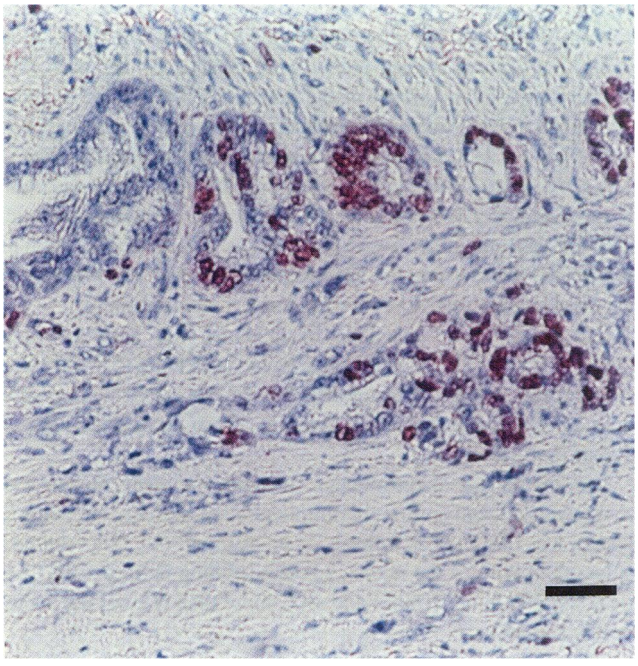

Figure 1 Ki-67 immunoreactivity in pancreatic carcinoma using the MIB-1 MAb. Immunoreactive nuclei have a distinct reddish-brown colour in contrast to the blue haematoxylin nuclei (horizontal bar = 50 μm)

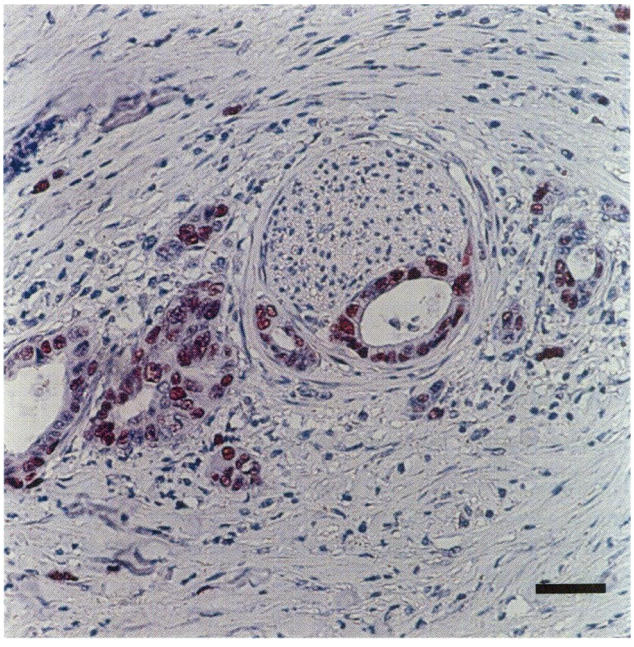

Figure 2 p53 immunoreactivity in pancreatic carcinoma using the DO-7 MAb. Perineural invasion is seen. The colour is clearly reddish-brown in the nuclei immunoreactive for p53 protein expression (horizontal bar = 50 μm)

tumours scored as –/(+) had lower values (7.1 ± 6.9%, mean ± s.d.) than those scored as +/++ (18.7 ± 9.8%, mean ± s.d.) ($P < 0.0001$).

p53 immunoreactivity (DO-7)

Evaluation of the p53 stainings was possible in 48 cases (Figure 2). There were 22 tumour specimens (46%) that were immunoreactive

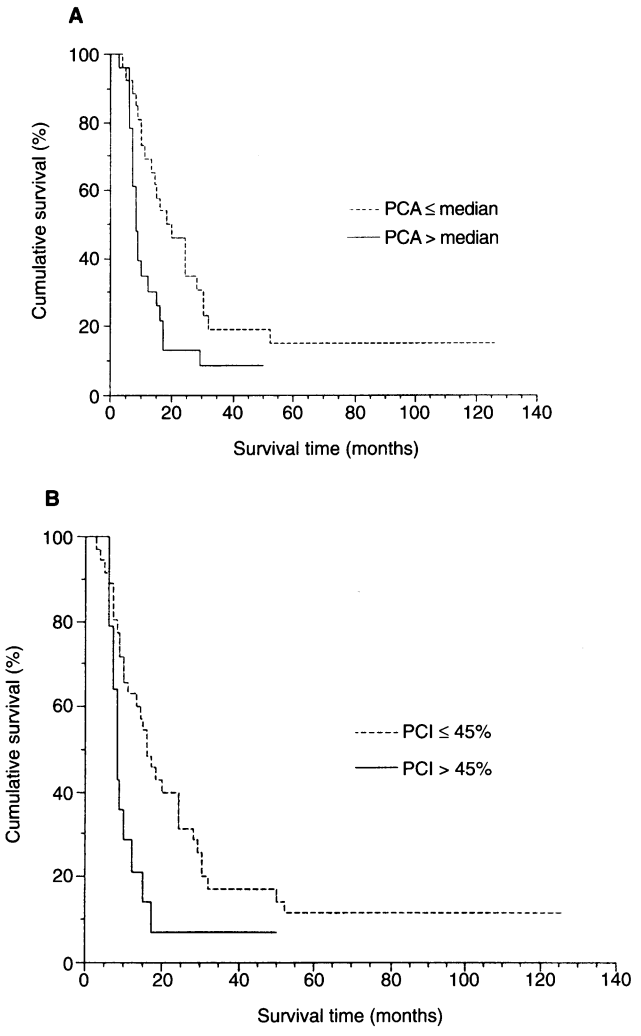

Figure 3 Patient survival time in relation to proliferating cell area (PCA) and proliferating cell index (PCI) in pancreatic carcinoma ($n = 53$). Proliferating cell area (PCA) values above the median were associated with shorter survival ($P < 0.01$) (**A**). Patients with low PCI values (≤ 45%) had longer survival time than those with PCI > 45% ($P < 0.05$) (**B**)

for p53 protein expression. Three specimens were classified as +, 13 as ++ and six as +++. No p53 protein expression was found in 22 tumours and, in four stained nuclei, was found only occasionally (< 1%). Thus, 26 tumours (54%) were classified as p53 negative.

Survival analysis

There was a relation between tumour size ($P < 0.01$) and survival time, but tumour stage and tumour grade had no influence on the prognosis. The median overall survival time was 13 months.

Patients with PCA values below the median (10.6%) had a better prognosis than patients with values above the median ($P < 0.01$) (Figure 3A), whereas patients with tumours with a PCI below the median (29.7%) did not survive longer than those with a PCI above the median. However, if a higher PCI level was chosen as cut-off when comparing survival time, patients with PCI values ≤ 45% ($n = 14$) had a longer survival time than those with PCI values > 45% ($n = 39$) ($P < 0.01$) (Figure 3B). Correspondingly, patients with tumours scored as –/(+) ($n = 24$) survived longer than

Table 1 Factors influencing survival time in 53 patients resected for pancreatic carcinoma determined by Cox bivariate regression analysis with respect to disease-specific survival

Factor	RH	CI	P
Age (years)[a]	1.15	0.83–1.60	0.395
Sex (female vs male) (0/1)	1.49	0.80–2.76	0.202
Size (mm)[a]	1.67	1.21–2.31	0.002
Stage (I vs II or III) (0/1)	1.65	0.90–3.03	0.102
Grade (well vs moderate or poor) (0/1)	2.24	0.68–7.30	0.172
PCI (≤ 45% vs > 45%) (0/1)	2.18	1.11–4.30	0.021
PCA (≤ median vs > median) (0/1)	2.24	1.21–4.13	0.009
MIB-1 score (– or (+) vs + or ++) (0/1)	1.79	0.97–3.28	0.058
p53 (negative vs positive) (0/1)	2.71	1.41–5.19	0.002

[a]Continuous, per 10-unit increments. 0/1 represents coding for the two patient groups; underlined groups represent a longer survival time. PCI, proliferating cell index; PCA, proliferating cell area. p53 (48 events). RH, relative hazards; CI, 95% confidence intervals.

Table 2 Evaluation of prognostic factors in 53 patients resected for pancreatic carcinoma by Cox multivariate regression analysis with respect to disease-specific survival

Factor	RH	CI	P
p53 (negative vs positive) (0/1)	3.26	1.65–6.45	< 0.001
Sex (female vs male) (0/1)	2.45	1.21–4.96	0.013
PCA (≤ median vs > median) (0/1)	2.08	1.08–3.99	0.028

0/1 represents coding for the two patient groups; underlined groups represent a longer survival time. PCA, proliferating cell area; RH, relative hazards; CI, 95% confidence intervals. p53 (48 events).

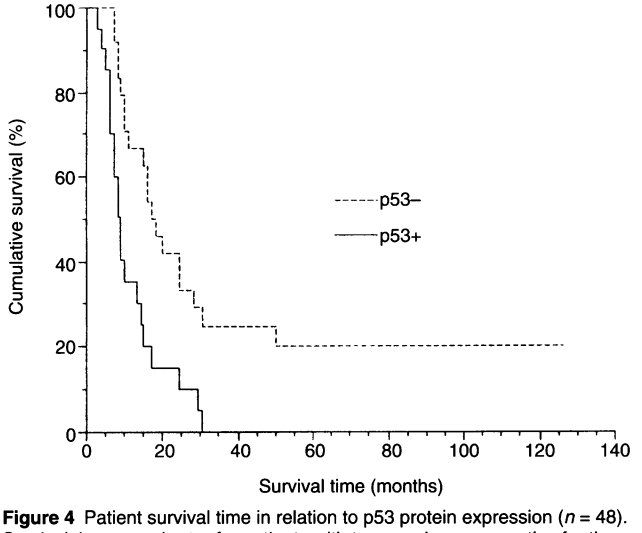

Figure 4 Patient survival time in relation to p53 protein expression ($n = 48$). Survival time was shorter for patients with tumours immunoreactive for the p53 protein ($P < 0.01$)

those with +/++ tumours ($P = 0.05$). The prognostic variables tested in the bivariate analysis are shown in Table 1. The PCA was a stronger predictor of survival than the PCI and had an independent prognostic value ($P < 0.01$) (Table 2).

Patients classified as negative for p53 protein expression ($n = 26$) had longer survival time than those with positive p53 scores ($n = 22$) ($P < 0.01$) (Figure 4). Positive p53 immunostaining

Table 3 Patient survival time – distribution according to prognostic variables

Prognostic variable	Median survival time (months)
PCI ≤ 45%	16.0
PCI > 45%	8.0
PCI ≤ median value	18.0
PCA > median value	8.0
MIB-1 scoring (–/(+))	16.5
MIB-1 scoring (+/++)	8.0
p53 negative	16.0
p53 positive	8.7
Female	14.5
Male	9.0

PCI, proliferating cell index; PCA, proliferating cell area. p53 (48 events).

was the most pronounced independent prognosticator of short survival time ($P < 0.001$) (Table 2). The median survival time in relation to prognostic variables is shown in Table 3.

The PCI was not higher in tumours with p53 protein expression (29.5 ± 21.5%, mean ± s.d.) than in those negative for the protein (29.8 ± 19.7%, mean ± s.d.).

Tumour characteristics in the five long-term survivors, of whom four were women, are shown in Table 4. All five long-term survivors had stage I tumours of 30 mm or less in size and were classified as p53 negative, but the MIB-1 pattern was varying.

DISCUSSION

Immunohistochemical staining with the MIB-1 antibody has been performed in various tumours but not in pancreatic neoplasms (Pinder et al, 1995; Lam et al, 1996; Parrado et al, 1996). The present study shows that the MIB-1 antibody is also highly effective in archival material from pancreatic carcinoma.

The proliferating activity of the tumour cells was evaluated by interactive image analysis and expressed as PCI and PCA. While PCI is frequently used to estimate the proliferating rate (Lam et al, 1996; Parrado et al, 1996), estimation of PCA has been used less often (Pinder et al, 1995; Parrado et al, 1996). Lundin et al (1995) used polyclonal Ki-67 antibodies in pancreatic carcinoma and found a median PCI value (26%) similar to that in the present series. In comparison to image analysis, semiquantitative scoring is less complicated. In the present study, the two techniques were compared and estimation of the proliferating activity seemed to be in accordance. Using image analysis, the PCA may also be calculated and there was a close correlation between PCI and PCA.

In the present series, only PCI levels exceeding 45% were related to short survival time. This is in agreement with Lundin et al (1995) who found that Ki-67 labelling exceeding 50% of the nuclei was associated with a poor outcome. Interestingly, we found that the PCA was an independent and stronger prognosticator than PCI. In comparison, studies of morphometric variables in pancreatic carcinoma have demonstrated that the area and variation in the area of tumour cells have a prognostic value in pancreatic carcinoma (Linder et al, 1995).

Paraffin sections, frozen material and carcinoma cell lines have been investigated for p53 abnormalities in pancreatic carcinoma (Barton et al, 1991; Ruggeri et al, 1992; Berrozpe et al, 1994). IHC has shown the same p53 protein pattern in frozen sections as in paraffin ones in pancreatic carcinoma (van dem Berg et al, 1993). Although there are sources of error in determination

Table 4 Pattern of prognostic variables in five long-term survivors (48–126 months) resected for pancreatic carcinoma

Case	Tumour stage	Tumour grade	Tumour size (mm)	PCI (%)	PCA (%)	p53 classification	Sex
1	I	Well	30	9.8	4.1	Negative	Female
2	I	Poor	24	0.5	0.0	Negative	Male
3	I	Well	23	15.7	6.5	Negative	Female
4	I	Moderate	11	33.0	8.9	Negative	Female
5	I	Moderate	20	82.0	36.0	Negative	Female

PCI, proliferating cell index; PCA, proliferating cell area.

of p53 alterations, there is in general a correspondence between IHC results and the findings obtained using molecular biological techniques (Barton et al, 1991; Ruggeri et al, 1992; Kalthoff et al, 1993). The percentage of tumours with p53 alterations in the present study is within the range described in the literature (20–75%), as estimated by IHC (Barton et al, 1991; van den Berg et al, 1993) or molecular biological techniques (Kalthoff et al, 1993; Scarpa et al, 1993; Pellegata et al, 1994). p53 immunoreactive cells have also been detected in peritumoral inflamed tissue specimens (Kalthoff et al, 1993). This phenomenon was not observed in the present study nor in the series of DiGuiseppe et al (1994). Extreme antigen enhancement in IHC using the DO-7 MAb has been shown to yield immunoreactivity in normal cells, stromal cells and in tumour cells with *p53* gene abnormalities precluding expression of the protein (Baas et al, 1996). In the present series, however, there was only immunoreactivity in tumour nuclei.

Although there are some studies of p53 alterations in pancreatic carcinoma, only a few discuss the impact on survival (DiGuiseppe et al, 1994; Lundin et al, 1996). As in the present study, the IHC technique was used. In the series of DiGuiseppe et al (1994), 26 of 48 tumours were immunoreactive and the difference in survival time between 'p53-positive' and 'p53-negative' patients (10 months and 20 months respectively) reached borderline significance. Lundin et al (1996) also used the DO-7 antibody but found no relation to survival, however patients with tumours expressing less than 20% of p53 immunoreactive nuclei were compared with those with 20% or more in the survival analyses. In the present study with a different cut-off level (1%), immunoreactivity for the p53 protein was the strongest independent prognosticator.

In the present study, MIB-1, PCI or PCA was not increased in p53 immunoreactive tumours. Similar findings have been made in lung carcinoma (Mørkve et al, 1992). In comparison, in one immunohistochemical study of pancreatic carcinoma, the proliferating index (percentage of cells > G_1 G_0) was not associated with p53 immunoreactivity (DiGuiseppe et al, 1994). It has been suggested that Ki-67 is not involved in the same steps of cell proliferation as p53, and therefore a similar staining pattern could not be expected (Thompson et al, 1992).

In conclusion, immunohistochemical staining by the MIB-1 antibody is feasible in paraffin sections of pancreatic carcinoma. The pattern of expression is clear and is possible to evaluate by conventional scoring. Alternatively, it is possible to calculate the PCI and PCA using an interactive image analysis system. In the majority of tumours, expression of p53 protein can be evaluated using the immunohistochemical technique. Although the number of patients in the present series is limited, highly proliferative pancreatic tumours or those expressing the p53 protein were associated with shorter survival time.

ACKNOWLEDGEMENTS

This work was supported by grants from Swedish Medical Research Council (project no. 102), the Swedish Cancer Society (project no. 2841), the Cancer Society of Stockholm, the King Gustav V Jubilee Fund, Stockholm, the Research Funds of the Faculty of Medicine at the Karolinska Institute, Stockholm, and the Swedish Society of Medicine (the Bengt Ihre Fund). A visiting scientist research fellowship was received by CP both from the Swedish Institute in Stockholm and from the Ministry of Education, Spain. We would also like to thank Mr Bo Nilsson, Department of Cancer Epidemiology and Biostatistics, Karolinska Hospital, Stockholm, for performing the statistical calculations.

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