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Prognostic criteria in nonfunctioning pancreatic endocrine tumours

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Abstract To identify prognostic subgroups among nonfunctioning (nonsyndromic) pancreatic endocrine tumours, a series of 61 tumours were analysed systematically for macroscopic, histopathological and immunohistochemical variables potentially predictive of malignancy. High-grade nuclear atypia, elevated mitotic rate and multifocal necrosis allowed us to separate 5 poorly differentiated carcinomas from 56 well differentiated tumours. Among the latter, 29 well-differentiated carcinomas showing gross local invasion or metastases were identified. Vascular or perineural microinvasion, Ki67 proliferative index >2%, mitotic rate ≥2, size ≥4 cm, capsular penetration, nuclear atypia, lack of progesterone receptors and presence of calcitonin were among the variables correlated with malignancy. The first two were the most sensitive and specific. Their presence or absence was used in the 27 tumours lacking evidence of malignancy at the time of surgery to separate 11 cases with increased risk of malignancy (in 2 of which metastases developed during follow-up) from 16 cases with limited risk. The resulting four prognostic groups of nonfunctioning pancreatic endocrine tumours (limited- and increased-risk tumours, well-differentiated carcinomas and poorly differentiated carcinomas) showed distinct survival curves, which were significantly affected by vascular microinvasion, Ki67 proliferative index and metastases.

Key words Nonfunctioning pancreatic endocrine tumours · Prognostic factors · Ki67 proliferative index · Immunohistochemical profile

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Introduction

"Nonfunctioning" endocrine tumours (NFETs) of the pancreas are neoplasms that have an endocrine-type histology and express endocrine markers, but lack any association with a clinical syndrome caused by hormone hypersecretion [21, 34, 41]. It would perhaps be more appropriate to call them nonsyndromic tumours. Among these neoplasms, clinically relevant growths presenting with symptoms of an expanding mass or unexpectedly found at radiological, surgical or autopsy investigation should be distinguished from microscopic growths detected by systematic histological investigation on serial sections of pancreases removed during surgical operations or at autopsy [13]. The latter are benign, clinically indolent microadenomas, whereas the former, a large proportion of which proved to be malignant, are more or less aggressive and require surgical therapy [4, 21, 34]. Clinically relevant NFETs are relatively uncommon neoplasms accounting for 15–43% of surgically resected pancreatic endocrine tumours [4, 21]. With the exception of a few poorly differentiated carcinomas (neuroendocrine carcinomas) that are easily identified at conventional histopathological investigation, NFETs are well-differentiated neoplasms showing extensive reactivity for general endocrine markers and variable reactivity for hormonal products [24, 41].

The malignancy of NFETs cannot be easily predicted on the basis of histology alone, except for those poorly differentiated carcinomas that resemble small to intermediate cell carcinomas of the lung [7, 35, 41]. The only definite criteria of malignancy are gross invasion of adjacent organs (duodenum, spleen, retroperitoneum) and

metastases in regional lymph nodes, liver or other distant sites [7, 14, 24]. Several attempts have been made to overcome the difficulties in assessing a prognostic evaluation based on conventional histology alone. Tumour cell aneuploidy evaluated by flow cytometry DNA analysis [10], morphometric analysis [20], a percentage of Ag-NOR-rich cells higher than 5% [38], *Ha-ras* oncogene overexpression [17], lack of the progesterone receptor immunoreactivity normally found in some islet cells [47], presence of alpha-hCG immunoreactivity [16, 19],

Table 1 Clinico-pathological features of 61 nonfunctioning endocrine tumours (*Met* metastasis, *Gross inv.* gross local invasion, *Vasc. inv.* vascular microinvasion, *Neur. inv.* perineural microinvasion, *ne* not evaluated, *AFD* alive, free of disease, *PD* perioperative death, *L* lost to follow-up, *AWD* alive with disease, *DOD* died of disease, *DOC* died of other cause)

Case	Size (cm)	Met/ Gross inv.	Vasc. inv.	Neur. inv.	Mitoses	Ki67	Follow-up (months)
1	0.3	No	No	No	0	0	AFD (48)
2	0.5	No	No	No	0	0	AFD (12)
3	2	No	No	No	0	0	AFD (70)
4	3	No	No	No	0	0	AFD (49)
5	1	No	No	No	0	0.55	AFD (50)
6	2	No	No	No	0	2	AFD (36)
7	2.5	No	No	No	0	1.2	PD
8	1.7	No	No	No	1	1	AFD(6)
9	2.3	No	No	No	1	0.5	AFD (84)
10	4.5	No	No	No	1	2	AFD (36)
11	4.5	No	No	ne	0	1.35	L
12	6.5	No	No	No	1	1.7	AFD (12)
13	3	No	No	No	3	2	AFD(115)
14	1.9	No	No	No	3	0.85	AFD (66)
15	3	No	No	No	2	1.9	AFD (56)
16	10	No	No	ne	3	1.7	AFD(12)
17	5.5	No	Yes	ne	1	1	AFD (24)
18	2	No	Yes	No	1	1	AFD (36)
19	7	No	Yes	ne	0	2	Autoptic
20	3.5	No	No	Yes	1	2	AFD (79)
21	11	No	Yes	ne	1	2	AFD (40)
22	6.5	No	No	No	3	6.75	AFD(2)
23	3.8	Adrenal[a]	Yes	Yes	0	2	AWD(96)
24	2	No	Yes	Yes	5	6.7	AFD (10)
25	4.5	No	Yes	No	1	6.35	AFD(6)
26	10	No	Yes	Yes	7	2.7	AFD(120)
27	7	Liver[a]	Yes	No	3	6.75	DOD (94)
28	3.5	Choledochus	Yes	Yes	15	16.75	DOD (60)
29	3.5	Spleen	Yes	No	3	2	AFD(79)
30	15	Spleen	Yes	Yes	2	ne	AFD(77)
31	3.8	Node	Yes	Yes	12	4.5	DOD (67)
32	11	Node/liver	Yes	Yes	3	6.05	AWD(68)
33	8	Liver/adrenal	Yes	ne	1	7.9	DOD (24)
34	5.5	Node	Yes	ne	2	2.7	PD
35	3.8	Node	Yes	ne	10	17.4	AWD (29)
36	4	Duodenum	Yes	Yes	0	4.6	AFD(15)
37	4.5	Node	Yes	ne	6	11.8	AFD (3)
38	6	Liver/spleen	Yes	ne	0	3.2	AWD (24)
39	3	Node	Yes	Yes	1	10.8	AFD(40)
40	6.3	Node	Yes	Yes	2	6.4	AFD(27)
41	3	Node	Yes	Yes	2	0.25	AFD(43)
42	5	Liver	Yes	ne	1	5.15	DOD (134)
43	10	Liver	No	Yes	0	4.3	AWD (155)
44	6	Liver	Yes	Yes	11	12.6	DOD(3)
45	6	Liver	Yes	Yes	1	7	AWD(24)
46	ne	Liver	Yes	ne	2	7.7	L
47	ne	Liver	Yes	ne	3	2.5	DOD(16)
48	5.5	Spleen	No	ne	0	ne	DOC(6)
49	ne	Liver	ne	ne	0	5	DOD(20)
50	10	Duodenum/lung	ne	ne	0	5	DOD(3)
51	6	Spleen	Yes	Yes	5	ne	AFD (112)
52	2	Node	Yes	Yes	5	7	DOC (12)
53	ne	Node/liver	Yes	No	16	6.2	DOD(48)
54	ne	Node	Yes	Yes	1	ne	Autoptic
55	8	Node	Yes	Yes	3	3.1	AWD(32)
56	5	Node/liver	ne	ne	1	ne	AWD(29)
57	4.6	Node/liver	Yes	ne	8	18.8	DOD(2)
58	3.5	Node	Yes	Yes	30	27.33	DOD(3)
59	10	Node	Yes	ne	25	16.85	DOD(1)
60	4	Node/liver	Yes	Yes	27	27.85	DOD(9)
61	10	Liver	Yes	Yes	35	40.02	PD

[a] Metastases: absent at the time of surgery; appeared during follow-up

proliferating cell nuclear antigen (PCNA) [33] or Ki67 proliferative index and p53 overexpression [27] have all been suggested to be predictive of malignancy. In spite of some improvements, the malignant potential and prognosis of NFETs continue to be a matter of debate and their classification remains somewhat arbitrary.

To investigate these issues in more detail, we collected a series of 61 tumours. The questions we addressed were: whether there is heterogeneity among NFETs with regard to epithelial differentiation and endocrine markers expression; whether tumour diameter, microinvasive features, mitotic count and Ki67 proliferative index or p53 overexpression are useful in evaluating the malignancy of NFETs; and whether these variables have any prognostic significance in terms of patients survival The results suggest that NFETs are a group of heterogeneous tumours, which can be classified into those with limited or increased risk of malignancy, low-grade carcinomas and high-grade carcinomas.

Materials and methods

Tumours and methods of investigation

Sixty-one nonfunctioning pancreatic endocrine tumours were collected from the files of the Departments of Pathology of the University of Pavia (I and II Varese Medical Faculties) and Milan (San Raffaele Hospital), from November 1978 to December 1994. There were 59 tumours that were surgically resected, while 2 were found incidentally during autopsy (Table 1). Clinical information on age, tumour site and size, evidence of local invasion, type of surgical treatment performed, perioperative mortality, and evidence of recurrence or distant metastases were collected from patient charts or by contacting clinicians. Information on follow up was available for 57 of the 59 patients who were surgically treated. The average duration of follow up was 43 months (range 1–155 months).

Samples of primary tumours were fixed in formalin or Bouin's fluid and routinely processed to paraffin wax. Serial sections were stained with haematoxylin-eosin (H&E), alcian blue (1%, pH 2.5)–periodic acid–Schiff (AB-PAS) technique and Grimelius' silver impregnation. The histological architecture was evaluated according to Soga and Tazawa [40]: type A or solid nest, type B or trabecular, type C or tubulo-acinar and type D or solid-undifferentiated pattern. The nuclear atypia was coded as follows: absent (0) when the nuclei were regular, similar to those of normal islet cells, moderate (1+) when they showed a moderate increase of nuclear/cytoplasmic ratio, moderate pleomorphism, a coarsely granular chromatin and evident small to medium-sized nucleoli; and severe (2+) if they were markedly hyperchromatic, irregular in size and distorted. To assess the mitotic index, at least 20 microscopic fields in the tumour periphery of each case were examined under high power (×400) and the number of mitoses/10 HPF was counted.

A panel of immunohistochemical stains was applied to all 61 cases. Paraffin sections were deparaffinized, quenched with 3% hydrogen peroxide for 10 min and then incubated with primary antibodies (Table 2) at 4°C for 18–20 h, followed by the avidin–biotin complex (ABC) procedure according to Hsu et al. [18]. The sections stained for glucagon, somatostatin, S-100, p53, and progesterone and oestrogen receptors were pretreated for 10 min with 0.003% subtilisin (Sigma, P4789; protease type XXVII or Nagarse protease) in 0.05 M TRIS-buffered saline pH 7.4 or with 0.01 M citrate buffer pH 6 (2×5 min) in a microwave oven at 650 W. The sections stained for Ki67 (MIB1 antibody) were pretreated with 0.05% trypsin in 0.05 M TRIS-buffered saline, pH 7.4, before microwave treatment. Specificity controls consisted in absorption of each antiserum with 10–20 nM of its homologous antigen per ml of diluted antiserum, omission of the first layer, and use of control tissues with or without the pertinent antigen.

The percentage of Ki67-positive cells was determined by one observer (S.L.R.) with a microscope Leitz Laborlux S holding an objective PLAN100/1.25. A minimum of 2000 tumour cells in areas of the highest immunostaining was scored. The immunoreactivities for endocrine markers, progesterone receptors and p53 were semiquantitatively evaluated and expressed as a percentage of positive tumour cells.

Vascular and perineural microinvasion were evaluated in sections stained for CD31 and protein S-100, respectively. Vascular microinvasion was defined as the presence of neoplastic thrombi within CD31 positive endothelium-lined spaces present in tumour capsule and/or in fibrous septa. Neural microinvasion was assessed by the finding of tumour cells in the perineural space or within nerve fibres identified by S-100 protein immunostain.

Table 2 Antibodies and antisera used

Antibodies/antisera	Clone/code	Dilution	Source
Neuron specific enolase (NSE)	A589	1:200	Dako, Copenhagen, Denmark
Chromogranin A (CgA)	Phe5	1:50	Enzo Diagnostics, New York, USA
Chromogranin B (CgB)	B11	1:4000	Dr. Siccardi, University of Milan, Italy
Insulin (Ins)	AE9D6	1:200	Biogenex Laboratories, San Ramon, USA
Glucagon (Gluc)	B31-1	1:1250	Milab, Malmö, Sweden
Pancreatic polypeptide (PP)	CA08327	1:4000	Cambridge Research Biochemicals, Cambridge, UK
Somatostatin (Som)	A566	1:500	Dako
Serotonin (5HT)	YC5	1:50	Biogenesis, Bournemouth, UK
Substance P (Sub P)	B45-1	1:640	Milab
C-terminus gastrin-CCK-cerulein (Gastr)	B4	1:10000	Farmitalia, Milan, Italy
α-Human chorionic gonadotropin (αhCG)	5E8	1:5000	Dr.Ghielmi, University of Brescia, Italy
Vasoactive intestinal peptide (VIP)	B34-1	1:12000	Milab
Calcitonin (Cal)	SILVIA	undiluted	Ortho Diagnostic System, Raritan, N.J., USA
Neurotensin (NT)	B44-1	1:6000	Milab
Ki67	MIB1	1:50	Immunotech, Marseille, France
p53	D07	1:100	Dako
Progesterone receptors (PgR)	1A6	1:20	Novo Castra, New Castle, UK
Oestrogen receptors (ER)	1D5	1:100	Dako
CD31	M823	1:20	Dako
S100 protein	Z311	1:400	Dako

Statistical analysis

To compare the immunohistochemical or clinico-morphological variables among different classes of tumours the Fisher exact test for categorical data has been adopted.

The identification of morphological and immunohistochemical variables predicting malignancy at surgery among well-differentiated endocrine tumours was performed by logistic regression modelling. For each variable, the malignant versus nonmalignant odds ratio (OR) with its confidence interval and the observed *P*-value were calculated, and also the percentages of benign and malignant cases correctly predicted by the model [48]. Variables with a high predictive capability (>70%) were used to classify the nonmetastatic or non-locally invasive cases into limited- and increased-risk groups, according to their absence or presence (singly or as a combination).

Survival analysis has been performed by considering a proportional hazard for each variable. Log rank test has been computed to identify the univariate predictors.

In both logistic and Cox analysis, variables with a *P*-value ≤0.05 were included in a multivariate logistic or Cox model; however, owing to the high multicollinearity between variables in this particular data set, the parameters of these models could not be estimated (presence of a singular matrix).

The incidence of metastases arising after surgery (number of patients with metastases per 1000 persons per unit time of observation) was computed in the different groups and compared by the mean Chi-square test for unequal rates. Maximum-likelihood 95% confidence intervals (95% CI) were calculated for rates.

All computations were performed with STATISTICA 5.0 (Statsoft) and Egret (Serc & Cytel).

Results

As detailed in Table 1, 34 of the 61 pancreatic endocrine tumours investigated showed lymph node, adrenal, lung and/or liver metastases or direct macroscopic invasion of duodenal wall, spleen and gall bladder at the time of surgery and were diagnosed as carcinomas. In 5 of the 34 cases with proven malignancy, tumour cells were small to intermediate in size, with dense chromatin pattern, relatively scanty cytoplasm, frequent mitoses and focal necrosis. Such tumours were classified as poorly differentiated carcinomas and distinguished from the 56 well-differentiated tumours (29 with and 27 without evidence of malignancy) whose tumour cells resembled islet cells to a greater or lesser degree.

Of 17 clinico-morphological (sex, age, site, size, presence of local symptoms, histological pattern, capsular penetration, nuclear atypia, tumour necrosis, vascular microinvasion, perineural microinvasion and mitoses) and histochemical (calcitonin, alpha-hCG, Ki67, progesterone receptor and p53 immunoreactivities) variables investigated, 8 correlated significantly ($P<0.05$) with malignancy demonstrated by metastases and/or gross local invasion in the 56 well-differentiated tumours analysed by the univariate logistic regression model (Table 3). Among these 8 variables, Ki67 proliferative index >2% and vascular and/or perineural microinvasion resulted the most sensitive and specific. The absence or presence of one or both of these has been taken as a basis for the classification of the 27 well-differentiated, nonmetastatic, non-locally invasive tumours. We classified 16 tumours showing neither of these 2 variables as limited-risk tumours (LRTs) and distinguished them from tumours showing one or both (11 increased-risk tumours or IRTs; Table 4).

Table 4 Distribution of tumours in relation to the most predictive variables at univariate analysis (*WDTs* 49 well-differentiated tumours with the variables available, *LRTs* 16 limited-risk tumours, *IRTs* 11 increased risk tumours)

Vascular and/or perineural invasion	Ki67 >2%	WDTs	LRTs	IRTs
No	No	16	16	0
Yes	No	8	0	6
No	Yes	1	0	1
Yes	Yes	24	0	4

During follow up, 2 of the 10 patients with IRTs, in whom follow up amounted to 433 person-months, compared with none of the 14 with LRTs, with follow up of 641 person-months, developed metastases (36 and 80 months after operation, respectively). This gives metastases rates of 4.6 per 1000 person-months (95% CI=0.8–14.3) and 0 per 1000 person-months (95% CI=0–2.9), respectively ($P=0.08$). The first of the 2 patients died of her tumour 94 months after the operation.

All the 14 patients with LRTs followed up after surgery for a mean of 46.5 months were alive and apparently free of tumour. In contrast, 1 of 10 (10%) patients with IRTs, 9 of 26 (34.6%) with well-differentiated carcinomas (WDCs) and all 4 with poorly differentiated carcinomas (PDCs) who were followed after surgery for a mean of 50.7, 44.2, and 3.7 months, respectively, died of their tumour after a mean survival of 94, 41.6 and 3.7 months, respectively. In addition, 1 of the 10 (10%) patients with IRTs and 7 of the 26 (26.9%) patients with WDCs were living with signs of locally recurrent tumour

Table 3 Variables statistically significant in predicting malignancy of well-differentiated nonfunctioning endocrine tumours in univariate logistic regression model (OR_{log} logistic odd ratio, *CI* confidence interval)

[a] With >3 mitoses 92.3% of nonmalignant and 24.2% of malignant cases were correctly predicted

Variable	OR_{log}	95%CI	*P*-value	% non malignant correctly predicted	% malignant correctly predicted
Diameter (≥4cm)	3.6	1.2-11.2	0.02	59.2	71.5
Vascular invasion	33.4	6.2-179.8	<0.001	72.0	92.9
Perineurat invasion	32	5.6-181.6	<0.001	85.7	84.2
Mitoses (≥2)[a]	3.03	1.1-9.8	0.036	73.1	54.5
Capsular penetration	5.6	2.3-23.5	0.02	40	89
Ki67(>2%)	51.1	9.2-282.3	<0.001	87	88
Nuclear atypia	2.3	1.0-5.3	0.044	52	76
PgR	0.15	0.04-0.6	0.007	53	86

Table 5 Summary of clinical and follow-up data of tumour groups with different probability of malignancy (*WDCs* well-differentiated carcinomas, *PDCs* poorly differentiated carcinomas, *LN* lymph nodes, *DOD* died of disease, *AWD* alive with disease)

	Age (years)		Male/female ratio	Local symptoms	Metastases		Mean length of follow-up (months)	DOD (months)	AWD (months)	Mean survival of DOD
	Mean	SD			LN	Distant				
LRTs	53.2	15.2	2/14	1/14	No	No	46.5	No	No	
IRTs	56.2	12.9	7/4	4/7	No	No[a]	50.7	1	1	94
WDCs	49.3	16.1	10/18	13/19	13	13	44.2	9	7	41.6
PDCs	52.8	19.8	2/3	3/3	3	3	3.7	4	0	3.7

[a] In 2 tumours metastases: absent at the time of surgery, appeared during follow-up

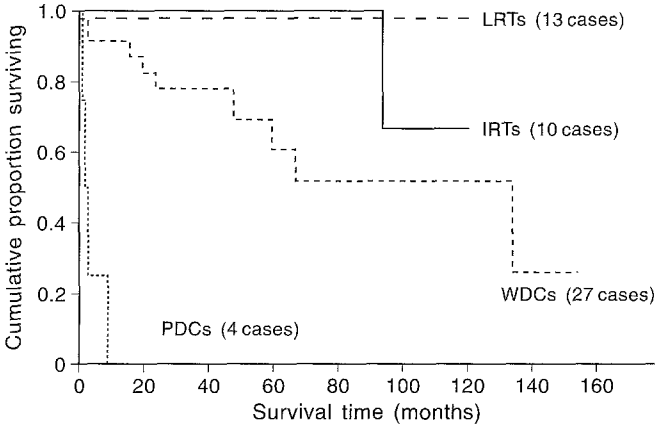

Fig. 1 Influence of tumour prognostic subtype on survival rate of 54 nonfunctioning pancreatic endocrine tumours. Univariate analysis P=0.0001 (*LRTs* limited risk tumours, *IRTs* increased risk tumours, *WDCs* well-differentiated carcinomas, *PDCs* poorly differentiated carcinomas)

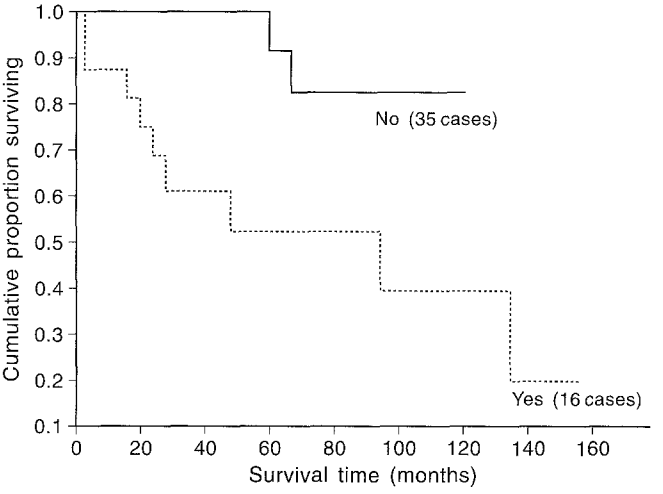

Fig. 2 Influence of distant metastases on survival rate in 51 well-differentiated endocrine tumours. Univariate analysis P=0.002

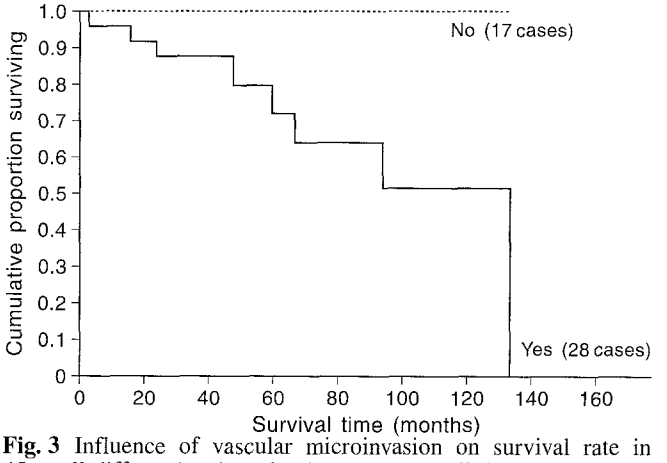

Fig. 3 Influence of vascular microinvasion on survival rate in 45 well-differentiated endocrine tumours. Univariate analysis P= 0.025

or metastases when last seen (Table 5). A significant difference in survival rate was found among tumour subsets with different prognostic evaluation (P=0.0001), with IRTs, WDCs and PDCs showing increasingly poor survival (Fig. 1).

For the 50 well-differentiated endocrine tumours with follow up, the following clinico-pathological variables were entered in a univariate analysis to identify predictors for patient survival: sex, age (dichotomized at 60 years), tumour location (head versus body-tail), type of surgery (enucleation versus resection), tumour size (<4 cm versus ≥4 cm), histological pattern, capsular penetration, gross local invasion, lymph node and distant metastases, nuclear atypia, vascular and perineural microinvasion, mitotic index (<2 versus ≥2 per 10 HPF), Ki67 index (≤2% versus >2%), progesterone receptors and alpha-hCG immunoreactivity. Of these 17 clinico-pathological variables, 4 were found to have a significant influence on survival: capsular penetration (P=0.049), distant metastases (P=0.002), vascular microinvasion (P=0.025) and Ki67 proliferative index (P=0.002). Multivariate analysis, performed using the Cox test, failed to identify an independent variable in predicting prognosis. Survival curves were calculated for each of the 4 significant variables (Figs. 2–4).

To analyse the clinico-pathological profile (Tables 5–7) of different tumour subsets, we grouped the 2 cases with malignancy manifested only during follow-up together with the well-differentiated carcinomas. Thus, in addition to tumours showing limited (16 LRTs, 26% of all cases) and increased risk of malignancy (9 IRTs, 15%) though actual proof was lacking, 31 well-differentiated carcinomas (WDCs, 51%) and 5 poorly differentiated carcinomas (PDCs, 8%) were considered.

Distinctive patterns of LRTs included preference for female sex, and body-tail of pancreas, size ≤3 cm and type B (trabecular) structure (Fig. 5), lack of local symp-

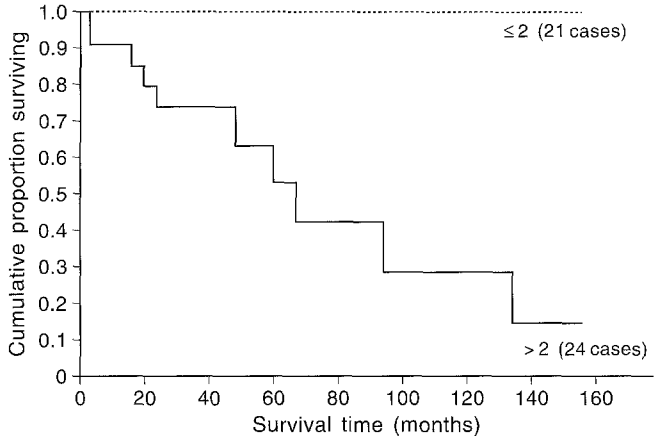

Fig. 4 Influence of Ki67 proliferative index on survival rate in 45 well-differentiated endocrine tumours. Univariate analysis P=0.002

Table 6 Pathological findings in the 61 tumours by prognostic group

		LRTs	IRTs	WDCs	PDCs
Site	Head	4	7	15	3
	Body-tail	12	2	14	1
Size (cm)	Mean	3	5.7	6	6.4
	Range	0.3-10	2–11	2–15	3.5-10
Histological pattern	A	0	1	2	0
	B	14	6	20	0
	C	0	0	0	0
	D	2	2	9	5
Vascular invasion		0/16	7/9	26/28	5/5
Gross local invasion		0/16	0/6	8/31	0/5
Capsular penetration		9/16	7/9	25/29	3/3
Perineural invasion		0/14	3/6	16/19	3/3
Nuclear atypia	0	3	2	0	0
	1+	13	8	31	0
	2+	0	0	0	5
Mitoses	Mean	0.9	2.2	3.6	25
	Range	0–3	0–7	0–16	8–35
Necrosis		0/16	0/9	2/31	4/5

toms (P=0.0006 versus the other well-differentiated tumours), absence of vascular and/or perineural microinvasion with or without apparent capsular penetration (Fig. 5), absent to moderate nuclear atypia, 0–3 mitotic

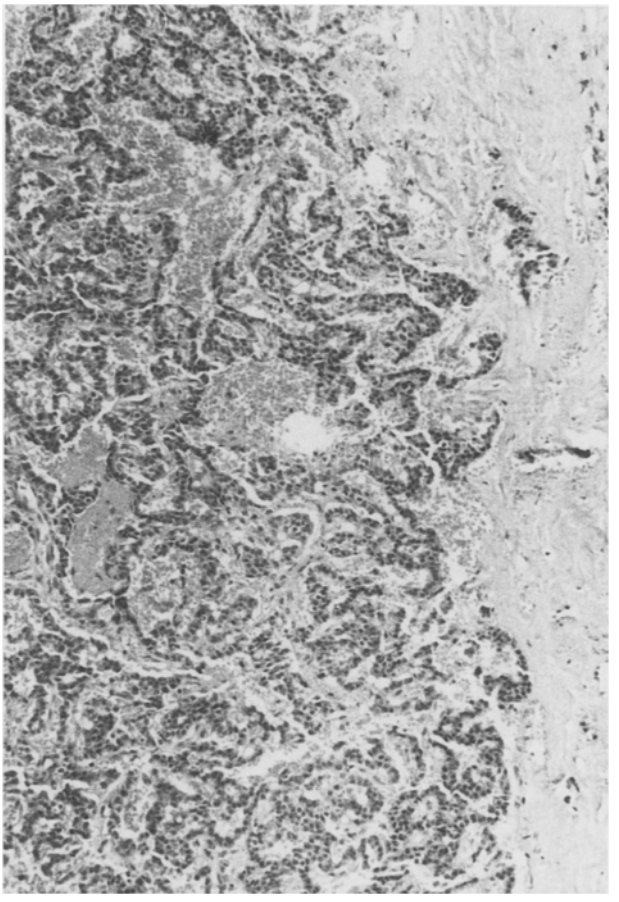

Fig. 5 Limited-risk tumour showing a type-B histological pattern and capsular penetration. H&E, ×100

Table 7 Histochemical and immunohistochemical findings of tumour groups [*S* staining, *MP* mean percentage of positive tumour cells in reactive tumours, ( ) percentage of positive tumours]

Marker or stain	LRTs		IRTs		WDCs		PDCs	
	S	MP	S	MP	S	MP	S	MP
GRIM	15/15 (100)	74.3	6/6 (100)	60	25/30 (83.3)	51.8	3/5 (60)	28.6
NSE	13/13 (100)	95.3	9/9 (100)	80	30/30 (100)	86.6	4/4 (100)	80
CgA	11/11 (100)	59	5/5 (100)	58	16/20 (80)	44	4/4 (100)	40.2
CgB	14/14 (100)	80	7/7 (100)	52.8	25/27 (92.6)	56	4/4 (100)	30
Ins	1/16 (6.2)	2	2/9 (22.2)	4	6/31 (19.3)	2	0/5	
Gluc	12/16 (75)	41.8	1/9 (11.1)	5	5/31 (16.1)	5.1	0/5	
Som	1/16 (6.2)	2	2/9 (22.2)	17.5	5/31 (16.1)	28.2	1/5 (20)	40
PP	9/16 (56.3)	32	3/8 (37.5)	61.6	9/31 (29)	24.2	0/5	
5HT	4/15 (26.6)	31.2	1/9 (11.1)	5	2/30 (6.6)	7.5	0/5	
Gastr	0/11		0/5		0/17		0/4	
Cal	0/15		2/9 (22.2)	8.5	7/29 (24.1)	12.1	2/3 (66.6)	3
NT	1/11 (9)	2	2/6 (33.3)	15	1/29 (3.4)	1	0/3	
αhCG	6/15 (40)	5.1	4/9 (44.4)	18	8/30 (26.6)	7.5	2/5 (40)	20
PgR	7/11 (63.6)	28.7	0/3		3/26 (11.5)	36.6	0/3	
ER	0/9		0/2		0/11		0/1	
Ki67	12/16 (75)	1.4	9/9 (100)	3.4	26/26 (100)	6.4	5/5 (100)	26.1
p53	0/15		1/7 (14.2)	3	9/30 (30)	1.8	3/5 (60)	5.3

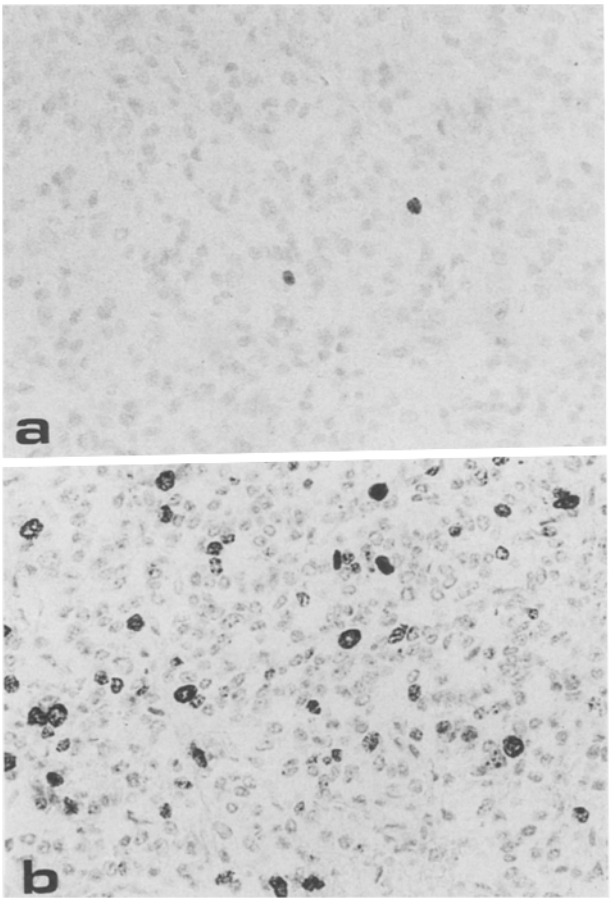

Fig. 6 Ki67 immunostain in **a** a limited-risk tumour and **b** a well-differentiated endocrine carcinoma. ABC technique with haematoxylin counterstain, ×200

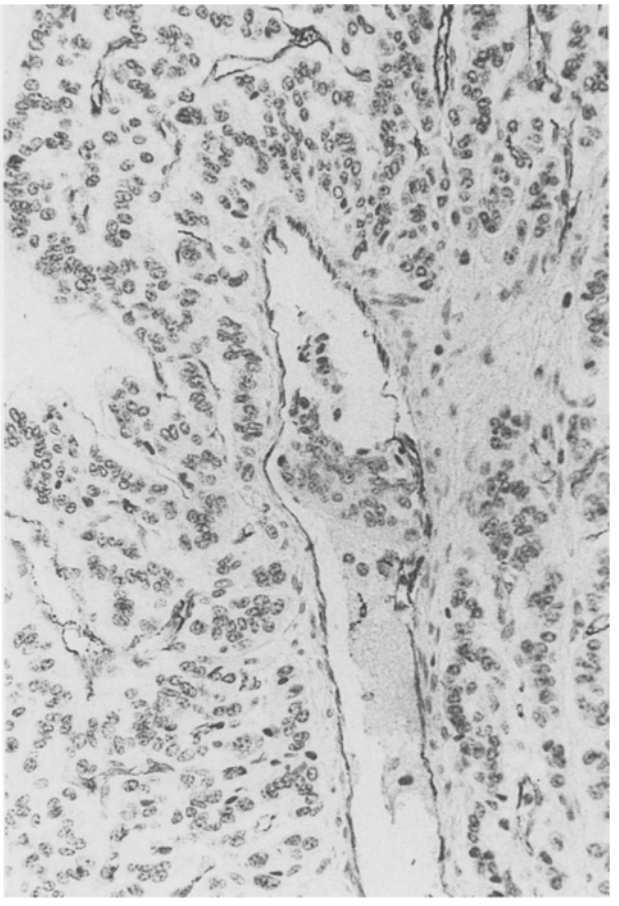

Fig. 7 Well differentiated endocrine carcinoma. Neoplastic thrombus within a vessel with CD31 positive endothelium. ABC technique with haematoxylin counterstain, ×250

index, 0–2% Ki67 expression (Fig. 6a), and absence of necrosis and p53 immunostaining (Tables 1, 5–7).

All cases investigated showed wide expression of general endocrine markers, either cytosolic, such as neuron-specific enolase (NSE) or granular, such as Grimelius' silver and chromogranins. In addition, 14 of 16 cases (87.5%) showed 5–100% of tumour cells positive for one or more of the hormonal substances investigated, with a marked preference for hormones normally found in the pancreas (expressed in 13 cases by a mean of 56% of tumour cells), with special reference to glucagon and pancreatic polypeptide (sometimes coexpressed by the same tumours) and serotonin. Inappropriate hormones not normally found in the pancreas were either absent, as in the cases of gastrin and calcitonin, or only expressed by a few (<5%) cells in isolated cases, such as vasoactive intestinal peptide (VIP) and neurotensin (NT) or by a restricted minority of cells (up to 10%) of several cases, such as human chorionic gonadotropin (hCG). Glucagon expression was found to be significantly more frequent in LRTs than in the other tumours as a whole (P=0.00001), the remaining well-differentiated tumours (P=0.00003), well-differentiated carcinomas (P=0.0001) or even IRTs (P=0.003), while calcitonin was expressed significantly less in LRTs than in all other tumours (P=0.02), the remaining well-differentiated tumours (P=0.02) or the well-differentiated carcinomas (P=0.04).

As outlined in Tables 1, 5, 6, and 7, the two groups of well-differentiated tumours (IRTs and WDCs) showed no relevant difference in terms of size, site of occurrence, frequency of associated symptoms (abdominal pain, icterus), histological pattern, vascular (Fig. 7) and/or perineural microinvasion (Fig. 8), nuclear atypia, absence of necrosis and wide expression of general endocrine markers coupled with poor hormonal expression. Apart from the presence or absence of gross local invasion and metastases (the two criteria used to distinguish WDCs from IRTs) the two tumour groups differed only by a moderate excess of Ki67 expression (Fig. 6b) and mitoses among WDCs. Local invasion was found in the spleen (5 cases), duodenal wall (2 cases), gall bladder (1 case) and adrenal gland (1 case). There were 13 WDCs showing lymph node metastases, 9 of which were located in the head of the pancreas, while among 15 cases showing distant metastases, 7 were in the body-tail, 6 were in the head and 2 were not precisely located or involved both sites.

Poorly differentiated carcinomas (PDCs) differed

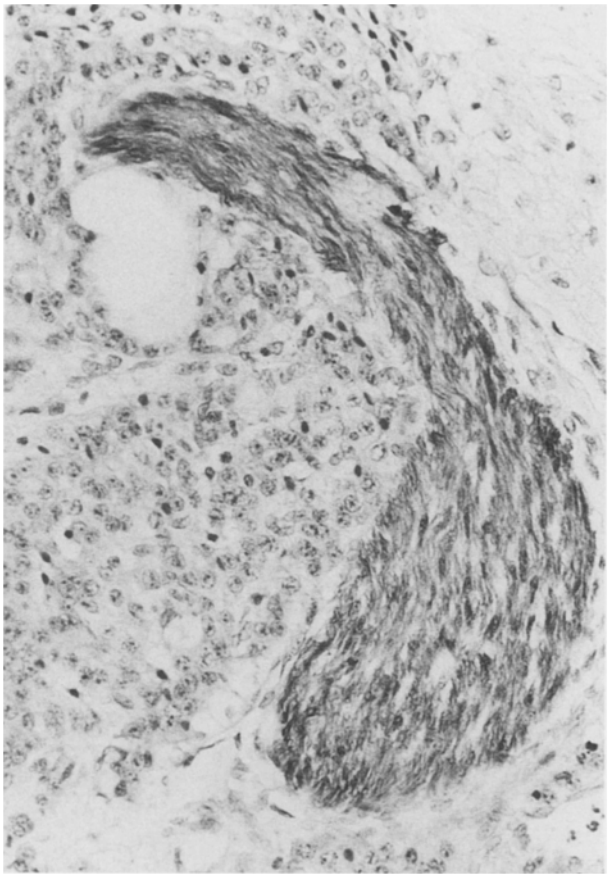

Fig. 8 Perineural microinvasion of a S-100 positive nerve in intrapancreatic connective tissue. ABC technique with haematoxylin counterstain, ×200

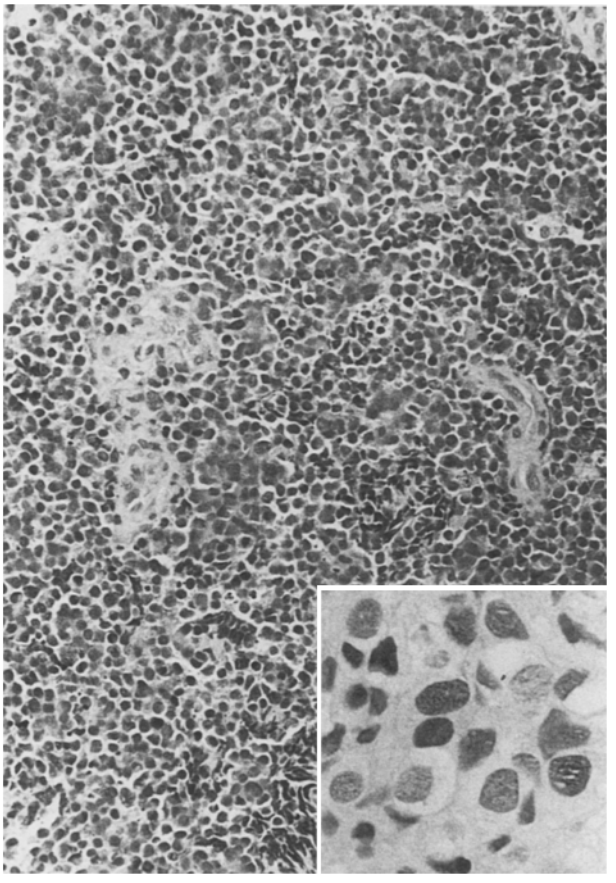

Fig. 9 Poorly differentiated endocrine carcinoma showing a solid growth pattern, severe nuclear atypia and p53 nuclear immunoreactivity (*inset*). ×200

from WDCs mainly in grade 2 nuclear atypia, high mitotic index (>20 in 4 of 5 PDCs versus none of the WDCs, P=0.00008), Ki67 expression (>18 in 4 of 5 PDCs versus none of WDCs, P=0.0001), presence of necrosis (4 of 5 PDCs versus 2 of 31 WDCs, P=0.001), a dominant type D histological pattern (Fig. 9) in all 5 PDCs versus 9 of the 31 WDCs (P=0.005), and p53 nuclear immunostaining (Fig. 9) of 0.5–10% of cells in 3 of 5 PDCs versus 9 of 30 WDCs. General endocrine markers such as NSE and chromogranins were still widely expressed; however, reactivity for Grimelius' silver was poor or absent in 4 of the 5 cases. Among pancreatic hormones, only somatostatin was detected in a single case, while, among inappropriate hormones, two cases showed alpha-hCG and 2 other cases calcitonin.

Discussion

Most NFETs (nonsyndromic endocrine tumours) of the pancreas are composed of well-differentiated cells arranged in trabecular or more solid aggregates and showing widespread reactivity for general endocrine markers. Their hormonal pattern is characterized by unreactivity for gastrin, poor reactivity for insulin and more substantial reactivity for pancreatic polypeptide, glucagon and other hormones known to have less dramatic clinical implications. These findings, which confirm and extend those of previous reports [15, 29, 41] may explain in part the failure of such tumours to develop a hyperfunctional syndrome. In fact, most insulin- or gastrin-producing tumours are known to develop a pertinent hyperfunctional syndrome, whereas only a restricted minority of those producing glucagon, somatostatin, serotonin or pancreatic polypeptide have been shown to cause a hyperfunctional syndrome, even when an excess of such hormones is detected in blood [15, 29, 39, 41, 45, 49].

The difficulty in predicting behaviour of well-differentiated pancreatic endocrine tumours on the basis of tumour histopathology, with special reference to cellular atypia and local microscopic infiltration, has been outlined by many investigators [11, 24, 30]. This led to the rather depressing conclusion that for such tumours metastases and gross local invasion are the only reliable evidence of malignancy. Among the difficulties inherent in malignancy prediction in pancreatic endocrine tumours is the fact that, as shown by clinical studies, the type of associated hyperfunctional syndrome may have a major

influence on tumour behaviour [5, 15, 28, 42, 44]. Although its independence of such morphological variables as tumour size, vascular microinvasion or proliferative markers remains to be determined, the functional syndrome is likely to be a major confounding factor in morphological studies aiming to predict tumour behaviour. To overcome this difficulty we conducted a systematic analysis of histopathological and histochemical parameters potentially predictive of tumour malignancy in a large series of NFETs.

Univariate analysis showed that 8 morphological variables were significantly related to malignancy, assessed on the basis of metastases or direct gross invasion: tumour diameter, vascular microinvasion, perineural microinvasion, capsular penetration, mitoses, Ki67 expression, nuclear atypia, progesterone receptor immunoreactivity and, in addition, the combination of vascular and/or perineural microinvasion. Among these, vascular and/or perineural microinvasion and Ki67 proliferative index above 2% were the most sensitive and specific variables. These findings are consistent with the role already established for the same factors as malignancy predictors in endocrine tumours of other tissues, such as thyroid [37, 46], parathyroid [1], pituitary [23, 25, 26], adrenal medulla [22] and lung [9].

Absence or presence of one or both of these variables was used to classify the 27 tumours lacking metastases or local invasion at the time of surgery into tumours with limited and increased risk of malignancy. Some indirect validation of this classification is provided by the mid-term clinical outcome of the patients in this series. In fact, subsequent follow up showed development of metastases, despite apparently complete surgical resection of the primary tumour, in 2 out of 10 patients with IRTs, as against none of 14 with LRTs. Although the difference failed to reach statistical significance (P=0.08), probably because of the limited number of events involved and a relatively short follow up for such low-grade tumour, the results tend to support the usefulness of these observations in the preduction of malignancy. This conclusion is in keeping with the capacity shown by two factors (vascular microinvasion and Ki67 proliferative index) to predict patients survival among the whole group of well differentiated endocrine tumours. In addition, a significant correlation was found between patient survival and tumour prognostic subtypes constructed according to these criteria.

The present study confirms the widely held opinion that moderate cytological atypia and tumour penetration through an often incomplete tumour capsule, without proven invasion of capsular vessels, are of no pratical value for the diagnosis of pancreatic endocrine tumours. The relatively low predictive value of these and two other factors, tumour size [20] and progesterone receptor expression [47], was characterized by poor specificity, which rendered them unsuitable for appropriate diagnosis of individual tumours. Another proliferative index, PCNA, which has been proposed as useful to assess the malignancy of pancreatic endocrine tumours [33], has been shown to be less reliable than Ki67 in other tumours, such as those of the adrenal cortex [12], a conclusion confirmed by a preliminary comparison of the two markers in 21 of our pancreatic endocrine tumours, which led us to dismiss PCNA in favour of Ki67.

Flow cytometry, recommended by Donow et al. [10], has the disadvantage of being poorly discriminative between benign and low-grade-malignancy tumours, the majority of which are near-diploid, in addition to requiring a highly specialized technology not universally available in pathology departments. The finding of more than 5% cells rich in silver nucleolar organizer regions (AgNORs), proposed by Rüschoff et al. [38] as a way of discriminating between benign and malignant pancreatic endocrine tumours, produced conflicting results when applied to the study of other endocrine tumours, such as pituitary adenomas [31, 43], and thyroid neoplasms [32]. Although this technique was highly predictive of malignancy, the sensitivity in detecting benign endocrine tumours of the pancreas was only 64% [38].

The alpha chain of human chorionic gonadotropin, proposed as a malignancy marker in both patient serum [19] and tumour tissue [16] investigations, proved ineffective in our nonfunctioning tumours, as were other inappropriate hormones, such as VIP and neurotensin. Our results are in accordance with those obtained for insulinomas [3], or gastroduodenal [6, 36] and pulmonary [2] endocrine tumours. From our study calcitonin appears to be a specific (100% specificity) though insensitive (31% sensitivity) marker of malignancy among NFETs of the pancreas.

By combining a few easily assessed histopathological variables with appropriate evaluation of the neoplastic disease at the time of surgery, it has been possible to separate NFETs of the pancreas into four groups with fairly distinct prognostic and clinico-pathological profiles. Indeed, in our series LRTs and IRTs were distinguished from WDCs of proven low-grade malignancy and PDCs of high-grade malignancy. These findings substantiate the classification guidelines for pancreatic endocrine tumours given in a recent paper [7], with special reference to the criteria for separation among tumours lacking proof of malignancy, those with limited risk (which on further investigation may well prove to be essentially benign) from potentially malignant "uncertain behaviour" tumours, here identified as IRTs.

Preference for female sex, body-tail localization, size less than 3 cm, lack of symptoms of an expanding mass, histopathological structure of trabecular type, expression of glucagon-related peptides and lack of calcitonin are among the characteristics of LRTs. Most clinico-pathological features of WDCs and IRTs overlap, so that discrimination of the two groups is based essentially on the presence or absence of metastases and gross local invasion. However, PDCs, with their high-grade nuclear atypia, elevated mitotic rate, multifocal necrosis, and solid to diffuse histological structure, can easily be distin-

guished by histology alone from all types of well-differentiated endocrine tumours. Histopathology apart, the main difference between well and poorly differentiated carcinomas is based on their overall survival rate (55.1% versus zero, respectively) and the mean survival time of patients dead of disease (41.6 versus 3.7 months). These differences are in keeping with those observed for well and poorly differentiated endocrine carcinomas in other sites, such as the lung [2], stomach [36] and intestine [8].

It is hoped that the application of such criteria as those described in this paper for the study of nonfunctioning tumours to larger series of pancreatic endocrine tumours, involving both functioning and nonfunctioning cases, will make it possible to develop useful guidelines for the histopathological evaluation of such tumours.

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