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Nuclear DNA content in 27 pancreatic endocrine tumours: correlation with malignancy, survival and expression of glycoprotein hormone alpha chain*

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Summary. Paraffin-embedded tissue from resection specimens of 14 functioning and 13 nonfunctioning pancreatic endocrine tumours (PET) was analysed for nuclear DNA content by image cytometry. Data on follow-up (mean 5.5 years) were available in all patients. DNA histograms with a diploid pattern were found in 13 (48%) tumours, while an aneuploid pattern was seen in the remaining 14 tumours (52%). Six (40%) of the diploid tumours and 9 (60%) of the aneuploid tumours were malignant. Survival was shorter in patients with malignant and aneuploid PET (mean 3.5 years, range 0.5–7) than in those with malignant and diploid PET (mean 5.7 years, range 3–8). Human chorionic gonadotropin-alpha was expressed in 3 of 12 benign PET, with 1 being aneuploid, and 6 of 15 malignant PET, with 4 being aneuploid. We conclude from these results that the ploidy pattern of PET allows no discrimination between benign and malignant tumours but may provide prognostic information on the aggressiveness of malignant PET.

Key words: Pancreatic endocrine tumours – Image cytometry – DNA content – Prognosis

Introduction

The prognosis of a number of commonly occurring malignancies seems to be related to their DNA ploidy status (Jakobsen et al. 1979; Tribukait et al. 1982; Wolley et al. 1982; Friedlander et al. 1984; Hedley et al. 1984; Tytor et al. 1987; Auer et al. 1989; Mellin 1990). Neuroendocrine tumours were originally thought to be diploid (Blondal et al. 1983; Wilander et al. 1985; Jones et al. 1988), but more recent investigations revealed aneuploidy in a considerable number of tumours (Bäckdahl et al. 1985; Hosaka et al. 1988; Joensuu and Klemi 1988; Kujari et al. 1988; Schröder et al. 1988; Cibas et al. 1990).

In pancreatic endocrine tumours (PET), as in so many other endocrine neoplasms, biological behaviour is notoriously difficult to predict (Klöppel and Heitz 1988; Solcia et al. 1989). Although some supplementary tests have been developed (Oeberg and Wide 1981; Heitz et al. 1983, 1987; Rüschoff et al. 1990), there is a need for further diagnostic procedures allowing us to assess the prognosis in these neoplasms more precisely.

Recently, three studies have evaluated the nuclear DNA content of PET. Stipa et al. (1987) measured the DNA content of 14 gastrinomas and 11 insulinomas by image cytometry (ICM). They suggested a prognostic role for this test although only 1 of 6 aneuploid insulinomas proved to be malignant. Alanen et al. (1990) used flow cytometry (FCM) for measuring the DNA content in 17 PET and found aneuploidy in 59%, but no clear correlation between ploidy status and clinical outcome. Graeme-Cook et al. (1990a) did a similar study in 14 insulinomas and also failed to find a relationship between DNA content and biological behaviour.

In all three studies the number of patients examined was relatively small and thus additional information is needed to settle the issue of the prognostic significance of DNA content in PET. In an investigation of 27 PET, we analysed the DNA ploidy by ICM retrospectively and correlated the data with malignancy, survival and immunoreactivity for the alpha subunit of human chorionogonadotropin (HCG-alpha).

Materials and methods

Formalin-fixed, paraffin-embedded tissue of surgical specimens of PET from 27 patients was collected from the files of surgical pathology of the Departments of Pathology, University of Hamburg, and the University of Zürich as well as various other Institutes of Pathology in the Federal Republic of Germany.

* Dedicated to Prof. G. Seifert on the occasion of his 70th birthday

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Table 1. Summary of clinical data, DNA ploidy status and HCG-alpha positivity in pancreatic endocrine tumours (PET)

Case no.	Age (years) at diagnosis	Sex	Syndrome	Principal hormone	Tumour size (cm)	Biology	Survival (years)		HCG-alpha positivity[c]
Diploid PET									
1	55	F	None	PP	10.0	Benign	2	alive	–
2	59	M	Insulinoma	Insulin	1.5	Benign	9	dead[a]	1
3	32	F	Gastrinoma	Gastrin	1.5	Benign	9	dead[a]	2
4	57	F	Insulinoma	Insulin	2.0	Benign	10	alive	–
5	66	F	Insulinoma	Insulin	2.0	Benign	10	alive	–
6	70	F	Insulinoma	Insulin	1.5	Benign	13	dead[a]	–
7	25	F	Insulinoma	Insulin	1.0	Benign	15	alive	–
8	64	M	None	None	2.5	Malignant$^{+/++}$	3	dead	–
9	41	M	None	None	8.0	Malignant^{+++}	4	alive	–
10	21	F	None	None	6.0	Malignant^{+++}	4	alive[b]	–
11	45	F	None	None	8.0	Malignant^{++}	6	dead[b]	1
12	51	F	Glucagonoma	Glucagon	8.0	Malignant^{+++}	8	dead[b]	–
13	40	M	None	PP	6.0	Malignant$^{+/+++}$	10	alive[b]	1
Aneuploid PET									
14	62	F	None	PP	2.0	Benign	0.5	dead[b]	1
15	26	F	None	None	8.0	Benign	2	alive	–
16	70	F	None	None	8.0	Benign	4	alive	–
17	66	F	None	PP	1.5	Benign	4	alive	–
18	56	F	Insulinoma	Insulin	1.5	Benign	8	alive	–
19	76	F	Insulinoma	Insulin	3.0	Malignant^{++}	0.5	dead	1
20	47	F	Glucagonoma	Glucagon	6.0	Malignant^{++}	0.5	alive	–
21	58	M	Insulinoma	Insulin	2.0	Malignant^{+++}	2	dead	–
22	62	F	Gastrinoma	Gastrin	9.0	Malignant^{+}	2	dead	1
23	42	F	None	PP	9.0	Malignant^{+++}	3	alive	–
24	44	M	None	None	3.5	Malignant^{++}	5	alive[b]	–
25	58	M	Gastrinoma	Gastrin	3.0	Malignant^{++}	5	alive	–
26	50	F	None	PP	6.0	Malignant^{++}	6	dead[b]	2
27	49	M	Insulinoma	Insulin	5.0	Malignant^{++}	7	dead[b]	1

Immunocytochemical examination; [a] died of intercurrent disease without evidence of residual tumour; [b] received chemotherapy; [c] immunoreactivity for HCG-alpha: 1, single cells, 2, >5%; $^{+}$ gross invasion; $^{++}$ metastasis at diagnosis; $^{+++}$ metastasis during follow-up; PP, pancreatic polypeptide

In all patients clinical information on symptomatology and follow-up was available. The most important clinical data are summarized on Table 1. There were 19 women and 8 men with a mean age of 51.5 and 51.6 years, respectively (range 21–76 years). The tumours comprised 14 functioning and 13 nonfunctioning PET. Among the functioning tumours were 9 insulinomas, 3 gastrinomas and 2 glucagonomas (Table 1). The tumours were classified as malignant if they infiltrated adjacent organs grossly, and/or if metastases were present or appeared during follow-up (0.5–15 years; mean 5.5 years). Microscopic tumour invasion of pancreatic parenchyma or peripancreatic fatty tissue was not considered to be a criterion for malignancy (Klöppel and Heitz 1988). All patients were followed until their death or to the closing date of this study on 1 July 1987. By this time 11 patients had died of PET, another 4 had died of intercurrent disease with no evidence of residual tumour, and 15 patients were still alive, of whom 11 had no clinical evidence of recurrent disease at repeated check-ups and 4 had tumour metastasis (Table 1).

For histological (haematoxylin and eosin, periodic acid-Schiff reagent) and immunocytochemical identification (Heitz et al. 1982) serial sections (3–4 μm) were cut from one block or (if the tumour exceeded 1.5 cm in diameter) from two blocks per specimen. Immunocytochemical staining was performed, using the unlabelled antibody enzyme technique or the avidin-biotin-complex technique. Commercially available antisera to insulin (1:5000 monoclonal; Biogenex, Dublin, Calif., USA), glucagon (1:2500 polyclonal; Milab, Malmö, Sweden), somatostatin (1:2000 polyclonal; Immunonuclear, Stillwater, Mich., USA), pancreatic polypeptide (1:10000 polyclonal; gift of Dr. R.E. Chance, Indianapolis, Ind., USA), gastrin G_{17} (1:3000 polyclonal; Paesel, Frankfurt, FRG), vasoactive intestinal polypeptide (1:5000 polylconal; Immunonuclear), serotonin (1:2000; polyclonal; gift of Dr. J.J. Verhofstad, Nijmegen, The Netherlands), HCG-alpha (1:5000 polyclonal; own source), and chromogranin-A (1:5000 monoclonal; Hybritech, La Jolla, Calif., USA) were used. Positive and negative controls were included systematically, as described previously (Heitz et al. 1982).

Nuclear DNA content was measured on Feulgen-stained, 7-μm-thick sections from blocks containing tissue from the tumour and surrounding normal exocrine pancreatic tissue. For ICM an automatic scanning integration system was used, consisting of a MPV cytophotometer combined with a Leitz Orthoplan microscope. The scanning steps were 0.5 μm wide. Absorption measurements were made using an interferential filter centered at 587 nm. One hundred normal cells and 100 tumour cells were analysed from each section. The DNA content of nuclei of normal pancreatic exocrine cells served as control, and a diploid value (2c) was therefore defined as the median DNA value (P50) of the control cells (Moberger et al. 1984). A histogram was classified as aneuploid if 50% of the tumour cells exceeded the 90th percentile (P90) of control cells, and/or if more than 3% of the examined tumour cells exceeded the $2 \times$ P90 value (Table 2).

Survival curves were calculated according to the method of Kaplan-Meier for two groups of patients, one group with diploid PET and the other with aneuploid PET. Patients (including those with cause of death unrelated to tumour) still alive on 1 July 1987 were considered as censored observations. The log-rank test and

Table 2. DNA values in pancreatic endocrine tumours

Case no.	DNA ploidy status		
	P90	2 × P90	Category
1	23%	0%	Diploid
2	30%	2%	Diploid
3	29%	0%	Diploid
4	16%	0%	Diploid
5	26%	1%	Diploid
6	26%	1%	Diploid
7	42%	2%	Diploid
8	29%	2%	Diploid
9	43%	2%	Diploid
10	15%	0%	Diploid
11	10%	0%	Diploid
12	14%	1%	Diploid
13	37%	0%	Diploid
14	40%	6%	Aneuploid
15	68%	31%	Aneuploid
16	79%	7%	Aneuploid
17	95%	1%	Aneuploid
18	77%	10%	Aneuploid
19	72%	12%	Aneuploid
20	100%	8%	Aneuploid
21	64%	4%	Aneuploid
22	53%	2%	Aneuploid
23	40%	9%	Aneuploid
24	96%	45%	Aneuploid
25	46%	3%	Aneuploid
26	56%	2%	Aneuploid
27	87%	23%	Aneuploid

P90, 90 percentile

Gehan's test were used to compare survival in the two groups (Miller 1981).

Results

Twelve of 27 patients with PET were considered to have benign tumours, because they showed neither grossly invasive tumours or metastasis at diagnosis, nor had tumour recurrence or clinical evidence for metastasis during follow-up (range 0.5–15 years; mean 7.3 years). Fifteen patients were found to have malignant PET as evidenced by gross invasion of adjacent organs, presence of metastases at the time of diagnosis, and/or development of metastasis during follow-up (Table 1). The metastases which occurred during follow-up were detected after 2–6 years (mean 4.2 years).

All 27 PET were suitable for DNA analysis. Thirteen PET (48%) were found to be diploid, and 14 (52%) were aneuploid (Table 1). Typical ICM histograms of PET with a diploid or aneuploid pattern are shown in Fig. 1. Five of the 12 benign (7 functioning, 5 nonfunctioning) tumours (41%), and 9 of the 15 malignant (7 functioning, 8 nonfunctioning) PET (60%) were aneuploid (Table 2). Six of 7 (85%) malignant functioning PET, and 3 of 8 (37%) malignant nonfunctioning PET were aneuploid. Among the 14 functioning PET there were 9 insulinomas, 4 of which were aneuploid (1 benign, 3 malignant). Among the 6 tumours that only developed metastases during follow-up, there were 4 diploid PET and 2 aneuploid PET.

Figure 2 shows the actuarial survival of all patients by ploidy of the tumour. The difference in survival between diploid PET and aneuploid PET in this censored estimate was statistically significant ($P<0.01$). Of the 6 patients with malignant diploid PET, 3 died from tumour after disease duration of 3, 6 and 8 years, respectively (mean 5.7 years), while the remaining 3 were still alive with metastasis after 4, 4, and 10 years, respectively (mean 6 years). Five of the 9 patients with malignant aneuploid PET died. Their survival times varied between 0.5 and 7 years (mean 3.5 years). For the 4 survivors, the follow-up times ranged from 0.5 to 5 years (mean 4.4 years). Patients who received chemotherapy had the longest survival times within the respective groups (see Table 1).

Of the 12 patients with benign PET, 4 had died of causes unrelated to tumour disease (Table 1). The remaining 8 patients survived, with follow-up times of from 2 to 10 years (mean 9 years) for the diploid PET, and from 2 to 8 years (mean 4.5 years) for the aneuploid PET.

Immunoreactivity for HCG-alpha was found in 9 of 27 PET, 3 of which were benign (2 functioning) and 6 were malignant (3 functioning). Diploid and aneuploid PET had a similar HCG-alpha positivity rate (31% versus 36%). Four of the 6 malignant PET expressing HCG-alpha were aneuploid.

Discussion

PET are rare neoplasms and, in addition, require a long follow-up to assess their biological behaviour, since metastases may appear years after resection of the primary. Studies dealing with these tumours are therefore generally hampered by the fact that the number of available cases with appropriate follow-up is so small that all statistical evaluations have to be interpreted with great caution. As our study was also based on a relatively small number of patients, we had to restrict statistical analysis to a comparison of the survival data between patients with diploid PET and those with aneuploid PET, irrespective of whether the tumours were found to be benign or malignant. Because of this small number of patients, it was not possible to compare the patients with malignant PET with respect to survival and DNA ploidy statistically. However, despite this limitation, we believe that the data of our cytometric investigation of 27 PET, which partly confirm and partly extend the observations of others (Stipa et al. 1987; Alanen et al. 1990; Graeme-Cook et al. 1990a), may further the understanding of the relationship between biology and DNA status in PET.

In essence, we found that DNA diploidy was significantly correlated with survival, but we failed to reveal aneuploidy (present in about half the tumours) as a helpful adjunct in predicting malignancy.

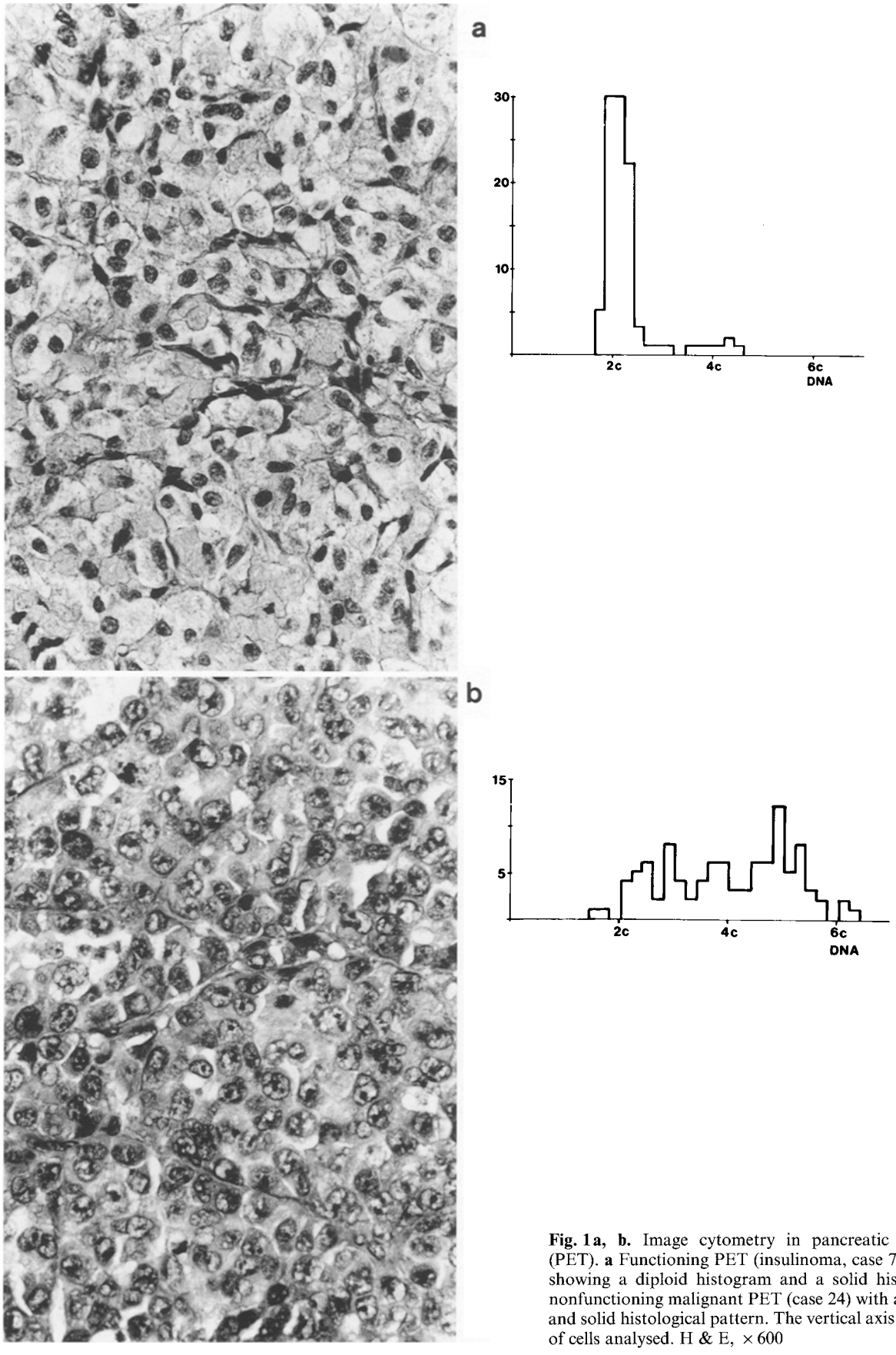

Fig. 1a, b. Image cytometry in pancreatic endocrine tumours (PET). **a** Functioning PET (insulinoma, case 7) without metastasis showing a diploid histogram and a solid histological pattern; **b** nonfunctioning malignant PET (case 24) with aneuploid histogram and solid histological pattern. The vertical axis gives the percentage of cells analysed. H & E, $\times 600$

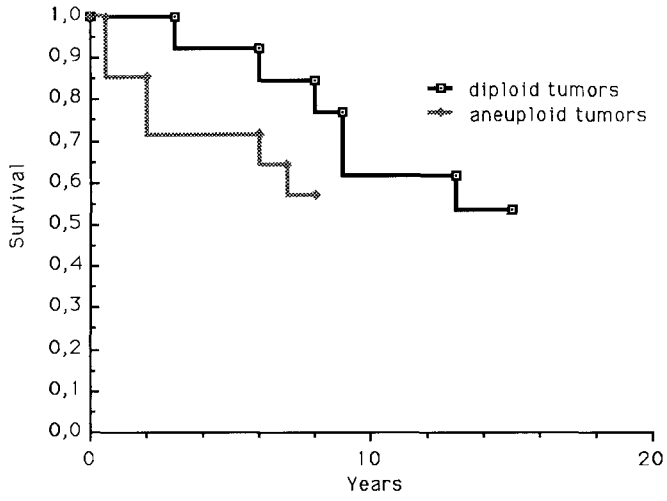

Fig. 2. Probability of survival of 27 patients with PET grouped by DNA ploidy. Thirteen PET were diploid, and 14 anaeuploid

The high rate of aneuploidy (52%) found in our series of PET compares well with that reported by Alanen et al. (1990) and Graeme-Cook et al. (1990a) applying FCM, and Stipa et al. (1987) using ICM. It seems therefore that PET, although they are usually slowly growing, well-differentiated neoplasms, show a high rate of aneuploidy. Recently, similar findings have also been reported in various endocrine tumours outside the pancreas (Bäckdahl et al. 1985; Hosaka et al. 1988; Joensuu and Klemi 1988; Jones et al. 1988; Kujari et al. 1988; Schröder et al. 1988; Cibas et al. 1990).

Aneuploidy by itself was no indicator for malignancy in PET, and could not predict whether a tumour that showed no secondaries at the time of diagnosis would later metastasize. Malignant behaviour occurred both in aneuploid (63%) and diploid PET (46%). However, if only the 7 malignant functioning PET, and within this group, the insulinomas were considered selectively, it was found that 85% of the malignant functioning PET and all 3 malignant insulinomas were aneuploid. It is therefore possible that functioning PET may differ in their relationship between DNA content and malignancy from nonfunctioning PET. This issue remains to be elucidated by further studies.

Analysis of the relationship between survival and DNA status revealed that diploid PET were associated with longer survival than aneuploid PET. This seems also to be true if the analysis is confined to patients with malignant PET, although this assumption could not be tested statistically. The mean survival times of patients with malignant diploid PET, who had died or were still alive, were 5.7 and 6 years, respectively, while the comparable survival figures in patients with malignant aneuploid PET were 3.5 and 4.4 years. These data may somehow be biased by the effect of chemotherapy, which was given to 4 patients with malignant diploid tumours and 3 patients with malignant aneuploid tumours. However, since in these patients the longest survival times were still associated with diploid PET, it appears that even after chemotherapy the difference in survival between patients with diploid and those with aneuploid tumours may remain. There is thus reason to believe that malignant diploid PET behave less aggressively than their aneuploid counterparts. This implies that the DNA content may be a useful prognostic indicator in malignant PET.

Immunocytochemical demonstration of HCG-alpha has been shown to indicate malignancy in functioning PET (Heitz et al. 1983, 1987). However, with increasing experience with this marker, HCG-alpha positivity has also been reported in benign PET, particularly insulinomas (Bordi et al. 1988; Graeme-Cook et al. 1990b). In this series we observed 9 PET immunoreactive for HCG-alpha, 3 of which were obviously benign. With respect to the DNA content of these PET there was no firm association between ploidy and HCG-alpha positivity in PET. Although HCG-alpha was most commonly expressed in the subgroup of malignant aneuploid PET, its general expression rate was similar in diploid and aneuploid PET (31% versus 36%). A close relationship between HCG-alpha immunoreactivity and increasing DNA content is therefore unlikely.

In summary, we feel that DNA cytometry, either by flow or image techniques, may be helpful in predicting the aggressiveness and thus the prognosis of malignant PET. No help can be expected for the assessment of malignancy in nonfunctioning PET. Whether this is true for functioning tumours remains to be seen.

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