Virchows Archiv A Pathol Anat (1992) 421:121–125

Virchows Archiv A
Pathological Anatomy and Histopathology

Flow and image cytometric study of pancreatic neuroendocrine tumours: frequent DNA aneuploidy and an association with the clinical outcome

Kalle A. Alanen[1], Ursula G. Falkmer[2], Pekka J. Klemi[1], Heikki Joensuu[3], and Sture Falkmer[2]

[1] Department of Pathology, University of Turku and University Central Hospital of Turku, Turku, Finland
[2] Department of Tumour Pathology, Karolinska Institute and Hospital, Stockholm, Sweden
[3] Department of Oncology and Radiotherapy, University of Turku and University Central Hospital of Turku, Turku, Finland

Received February 17, 1992 / Received after revision April 9, 1992 / Accepted April 10, 1992

Summary. Eighteen pancreatic neuroendocrine (NE) tumours were analysed for nuclear DNA content by image cytometry (ICM) and flow cytometry (FCM). The DNA indices (DIs) obtained by ICM were somewhat higher than those obtained by FCM, but a major disagreement was present only in 1 case. Thirteen patients had been followed up at least for 6 years after the diagnosis or until death. At 6 years of follow-up all 4 patients with a tumour with a DI ≥ 1.8 by ICM had died from their NE tumour or had metastatic disease, whereas all 9 patients with a smaller DI had no evidence of the disease ($P=0.001$). The DIs calculated from the FCM data also correlated well with the final outcome ($P=0.01$). A high incidence of DNA aneuploidy was found by both methods in histologically and clinically benign NE tumours; 12 (67%) were DNA aneuploid by FCM and 16 (89%) by ICM. It is concluded that pancreatic NE tumours are frequently DNA aneuploid, and both cytometric DNA methods give prognostic information in these tumours. The presence of DNA aneuploidy should not be considered as a sign of malignant behaviour in pancreatic NE tumours, whereas a large DI is associated with poor prognosis.

Key words: Islet-cell neoplasms – Insulinoma – Gastrinoma – Glucagonoma – DNA index

Introduction

Pancreatic neuroendocrine (NE) tumours may be cured by surgical excision, but are sometimes inoperable due to local infiltration, and may give rise to distant metastases. Clinical, histopathological, and immunohistochemical data often fail to predict the clinical outcome of the disease (Heitz 1984; Klöppel and Heitz 1988).

Correspondence to: K. Alanen, Department of Pathology, University of Turku, Kiinamyllynkatu 10, SF-20520 Turku, Finland

Cytometric assessment of the nuclear DNA content has become a commonly used technique in clinical pathology (Auer et al. 1989). It is mainly used in "malignancy grading" of different types of cancer, where it is supplementary to more conventional clinical and histopathological examinations. Carcinomas that consist of neoplastic parenchymal cells with a DNA euploid (diploid or tetraploid) DNA distribution pattern usually have a favourable clinical course, whereas tumours with a DNA aneuploid pattern often behave aggressively (Fallenius et al. 1988; Auer et al. 1989; Forsslund and Zetterberg 1990; Haugen and Mjölneröd 1990). Histologically benign adenomas of the endocrine organs such as the thyroid, the parathyroids, the pituitary and the adrenals may be DNA aneuploid with a small DNA index (DI), and the presence of DNA aneuploidy appears to have little prognostic influence in these tumours (Joensuu and Klemi 1988).

Pancreatic NE tumours are also frequently DNA aneuploid (Stipa et al. 1987; Alanen et al. 1990; Graeme-Cook et al. 1990; Donow et al. 1991), but it is currently not known whether DNA aneuploidy is an unfavourable prognostic factor in pancreatic NE tumours or not. Stipa et al. (1987) measured the nuclear DNA content of 11 insulinomas and 14 gastrinomas by image cytometry (ICM), and found DNA ploidy values of 2.5 N or greater and/or the 5 N exceeding rate of 1% or greater to occur more often in NE tumours with metastases than among those without. In line with their findings, Donow et al. (1991) found survival to be shorter in patients with malignant and DNA aneuploid pancreatic NE tumours than in those with DNA diploidy, whereas Graeme-Cook et al. (1990) failed to find any relationship between the nuclear DNA content measured with DNA flow cytometry (FCM) and outcome in their series of 14 NE tumours.

In an earlier FCM study on pancreatic NE tumours from one of our laboratories it was not possible to show an association between the nuclear DNA ploidy pattern of the neoplastic cells and the clinical course, although tumours with a "multiploid" DNA pattern tended to

behave more aggressively (Alanen et al. 1990). We have now re-investigated these tumours by ICM and added 2 cases to the series. A longer follow-up time is also now available. In contrast to our previous study, a clear association could now be found between a large DI and unfavourable clinical course of the neoplastic disease.

Materials and methods

Formalin-fixed and paraffin-embedded specimens from 18 patients with pancreatic NE tumours were analysed by means of both FCM and ICM. The FCM results have been reported in part elsewhere (Alanen et al. 1990); the 2 new cases (F/58, Table 1) were 1 insulinoma and 1 hormonally inactive NE tumour (M/60, Table 3). One case from our previous series could not be analysed with ICM, because of the lack of tissue. The clinical and histopathological data are summarized in Tables 1–3. None of the patients was given any treatment before surgery. The original histopathological classification of the tumours is given in the tables. The cases where the pathologist had not been able to decide whether or not the tumour was malignant in nature are listed to be of uncertain malignancy in the tables. Synaptophysin immunostaining was used to confirm the NE origin of the tumours (Chejfec et al. 1987), and antisera against insulin, gastrin, glucagon, pancreatic polypeptide, somatostatin and vasoactive intestinal polypeptide were used to

Table 1. Clinical, histopathological and DNA cytometric data of insulin-producing pancreatic neuroendocrine (NE) tumours

Sex/age at operation	Size (cm)[a]	DNA cytometric data				Histo-patho-logical classi-fication	Course of disease	Follow-up time (years)
		FCM		ICM				
		Ploidy	DI	Ploidy	DI			
F/38	1	An	1.39[b] 1.43 1.45	Tetr	1.8	Benign	NED	7
M/22	2	An	1.35[b] 1.41	An	1.5	Benign	NED	23
M/38	1	An	1.33[b] 1.43	An	1.5	Benign	DID	7
F/58	1	An	1.25	An	1.4	Benign	NED	1
F/62	1	An	1.25[b] 1.31 1.32	An	1.3	Benign	DID	7
F/68	2	An	1.30	An	1.5	Benign	NED	6
F/56	3	An	1.16	An	1.4	Benign	NED	18
M/60	2	Di Di	1.00[b] 1.00	An	1.3	Benign	DID	2 months

An, Aneuploid; Di, diploid; Tetr, tetraploid; NED, no evidence of disease; DID, died from intercurrent disease (cor pulmonale, post-operative subphrenic abscess, and subarachnoid haemorrhage)

[a] The largest diameter of the tumour nodules is given
[b] Two or three blocks of the same tumour were analysed

Table 2. Clinical, histopathological, and DNA cytometric data of the gastrin-producing pancreatic NE tumours (or immunohistochemically gastrin/glucagon-expressing NE tumours)

Sex/age at operation	Size (cm)[a]	DNA cytometric data				Histo-patho-logical classi-fication	Course of disease	Follow-up time (years)
		FCM		ICM				
		Ploidy	DI	Ploidy	DI			
F/41	2	Mu	1.22 1.93	Mu	1.4 2.0	Malign	Liver metastases	9
F/59	8	An Di	1.24[b] 1.00	An	1.3	Benign	DID	16
M/20	4	Di	1.00	Di	1.1	Uncertain	NED	9
M/58	0.5	Di	1.00	An	1.3	Benign	DID	16
M/44	3	Di	1.00	Di	1.0	Benign	NED	4

Mu, Multiploid; for the other abbreviations, see Table 1. The deaths from intercurrent diseases were caused by pulmonary embolism and by cerebral thrombosis.

[b] Two blocks of the same tumour were analysed. This case was initially interpreted as DNA diploid, although there was a small extra peak with a DI 1.24 present in the FCM DNA histogram. However, after finding a similar extra peak by ICM, the interpretation of the FCM histogram was revised

Table 3. Clinical, histopathological, and DNA cytometric data of the immunohistochemically non-defined pancreatic NE tumours

Sex/age at operation	Size (cm)	DNA cytometric data				Histo-patho-logical classi-fication	Course of disease	Follow-up time (years)
		FCM		ICM				
		Ploidy	DI	Ploidy	DI			
F/34	8	Mu	1.33 1.93	Mu	1.3 1.9	Malignant	Progressive abdominal tumour	4
M/60	5	An	1.70	Tetr	2.2	Malignant	Died from NE tumour	6
F/51	20	Di	1.00	Tetr	2.0	Malignant	Died from NE tumour	6
M/70	7	An	1.56	Tetr	2.1	Uncertain	Died from NE tumour	5,5
F/33	7	Di	1.00[a]	An	1.3	Uncertain	NED	5

[a] Eleven blocks of the same tumour were analysed. For abbreviations, see Tables 1 and 2

characterize the tumour cells. The immunohistochemical data are given in more detail in the previous report (Alanen et al. 1990).

Sections 50 μm thick were cut from the paraffin blocks for DNA cytometry, and were deparaffinized as described earlier (Alanen et al. 1990). FCM was done using a FacStar flow cytometer (Becton-Dickinson Immunocytometry Systems, Mountain View, Calif., USA), and DNA was stained with propidium iodide. For each DNA histogram 20×10^3 particles were analysed. The DI was defined as the relative DNA content of an aneuploid stem line as compared with the diploid peak, and was calculated by dividing the modal channel number of the aneuploid stem line by that of the diploid stem line. The peak with the lowest DNA content was taken as the diploid peak. In 7 cases, between 2 and 11 blocks of the same tumour were analysed revealing intratumoral variation in the DNA content (Askensten et al. 1989).

ICM was performed as described in detail elsewhere (Askensten et al. 1990; Ullen et al. 1991). Briefly, 30-μm-thick sections were cut from the paraffin blocks. They were deparaffinized twice in 3 ml Histoclear, and the specimens were rehydrated in decreasing concentrations of ethanol (100% – 90% – 70%), and washed twice in phosphate buffered saline (PBS). The enzymatic disintegration was performed in 3 ml freshly prepared 0.05% pronase solution (Sigma, 9.7 units/mg) for 30 min at 37° C in a shaking water bath with intermittent vortex mixing. The disintegrated nuclei were then filtered through a nylon mesh with a 30 μm pore size. The nuclei were washed twice in PBS and resuspended in Carbovax (2% polyethylene glycol in 50% ethanol). They were spun down on poly-L-lysin-coated glass slides at 1,250 *g* for 15 min, and air-dried at room temperature.

The subsequent Feulgen staining technique, including acid hydrolysis, was performed in 5 M hydrochloric acid at room temperature for 1 h. The specimens were assessed on a CCD-camera based image analysis system (Ahrens System, Institut für Meß-Technik, Bargteheide, Hamburg, FRG). The measurement was made at a wavelength of 546 nm. Granulocytes and lymphocytes found within the same sample were used as an internal control, and no correction coefficient for different absorption between the nuclei of control and neoplastic cells was used (Ullen et al. 1991). At lest 150 intact, structurally identified tumour cell nuclei were measured in every specimen. The modal value from the DNA histograms was used for the calculation of the DI in a manner analogous to that for the FCM DIs (Ullen et al. 1991). DNA diploid tumours were considered to have a DI between 0.8 and 1.2, and DNA tetraploid between 1.8 and 2.2. We realize that these values may be debated, but they are used in the literature.

Statistical analysis of survival between the groups with a low and a high DI was done by comparing the number of patients with no evidence of the disease at 6 years from the date of the diagnosis with Fisher's exact test. Patients with a shorter survival time available ($n=4$, Tables 1–3), and the patient who died from an intercurrent disease 2 months after the diagnosis were not included in the analysis. The *P* values are two-sided.

Results

As a rule, the DIs obtained by ICM were somewhat higher than those by obtained FCM, except in 3 cases, where they were similar (Tables 1–3). In addition, a discrepancy was seen in 1 case, where the nuclear DNA distribution pattern of an immunohistochemically non-defined NE tumour was found to be of the diploid type by FCM but of the tetraploid type by ICM (Table 3).

The nuclear DNA ploidy of 7 of the 8 insulin-producing NE tumours was found to be DNA aneuploid by FCM. The corresponding results with the ICM technique were essentially the same. One insulinoma with a DI about 1.4 by FCM had a somewhat larger DI of 1.8 by ICM, and another tumour that was DNA diploid by FCM was aneuploid with a DI 1.3 by ICM (Table 1). The DIs were lower than 1.5 by FCM, but a DI as high as 1.8 was obtained from one tumour by ICM. All tumours could be radically removed at surgery, no lymph node metastases were present, and none of the tumours has given rise to distant metastases during the follow-up of 1–23 years. No sign of malignant type of growth pattern was present at histopathological examination in any of the cases.

The DNA ploidy of 3 of the 5 gastrin- or glucagon-producing tumours was found to be diploid by FCM, and 2 were diploid by ICM (Table 2). One tumour was considered to be DNA multiploid by both techniques. This patient was found to have liver metastases 6 years after surgery, whereas all the other patients with a gastrin/glucagon-producing NE tumour and with a DI ≤ 1.3 had no evidence of disease 4–16 years after surgery. All glucagon-producing tumours were completely excised in all cases, and no metastases were present at the time

of operation. Three of the tumours were classified histopathologically as benign, 1 was considered to have a questionable malignant potential, and 1 was assessed as malignant.

The DNA ploidy of the 5 immunohistochemically non-functioning NE tumours was aneuploid (or tetraploid) by ICM, and only 2 were diploid by FCM (Table 3). One tumour was DNA multiploid by both methods. The DIs were generally higher than in insulin- or gastrin/glucagon-producing NE tumours. The size of the non-functioning NE tumours was also usually larger, and in 3 cases radical removal of the entire tumour mass by surgery was not possible. These 3 tumours were also histopathologically classified as malignant, whereas the presence of histopathological criteria for malignancy remained uncertain in the remaining 2 cases. Three of the patients have died from their NE tumours, 1 is still alive with a progressively growing abdominal tumour mass, and only 1 patient has no evidence of the disease 5 years after surgery.

No significant intratumoral heterogeneity was found in cases where two or more blocks were analysed from the same tumours (Table 1–3).

Thirteen patients had been followed up at least for 6 years after the diagnosis or until death. After 6 years of follow-up all 4 patients with a tumour with a DI ≥ 1.8 by ICM had died from their NE tumour ($n=3$) or had metastatic disease ($n=1$, patient F/41, Table 2), whereas all 9 patients with a smaller DI had no evidence of the disease ($P=0.001$). The DIs calculated from the FCM data also correlated well with the final outcome; 9 of the 10 patients with a tumour with a DI < 1.5 were alive at 6 years from the diagnosis, whereas all 3 patients with a DI > 1.5 had died from NE tumour ($P=0.01$).

Discussion

In our previous FCM DNA study on pancreatic NE tumours (Alanen et al. 1990) as many as 7 (88%) out of the 8 clinically and histopathologically benign insulinomas were found to consist of neoplastic cells with a DNA aneuploid DNA content. This incidence is higher than that usually found in most human carcinomas or malignant lymphomas (Hedley 1989). A high incidence of DNA aneuploidy was now also found by ICM, thus confirming our earlier observation with FCM alone. Consequently, the presence of DNA aneuploidy in pancreatic NE tumours is not a reliable sign of malignant clinical behaviour (Graeme-Cook et al. 1990; Donow et al. 1991).

The DIs calculated from the ICM DNA histograms were almost constantly higher than those obtained by FCM. Feulgen absorption measured from the internal diploid standard cells (granulocytes and lymphocyte nuclei) may be 5–15% lower than the expected diploid value (Böhm and Sandritter 1975), which could explain the difference. This DI shift was originally reported for fresh cells, but has now also been found by one of us when deparaffinized formalin-fixed material has been analysed (U.G. Falkmer, unpublished data).

Only one major discrepancy between the two methods was encountered. One pancreatic NE tumour with a DI 1.0 in FCM analysis was found to have a DI 2.0 by ICM. The discrepancy could be explained by intratumoral heterogeneity and the presence of only a small amount of tetraploid cells in the FCM sample (Fallenius et al. 1988; Askensten et al. 1989). However, no solid evidence of intratumoral heterogeneity was obtained in multiple analyses of 7 of the NE tumours in the present series.

As we have demonstrated earlier, tissue autolysis may produce artefact peaks in the DNA histogram (Alanen et al. 1989). In the present study such artefacts are an unlikely explanation for the high incidence of DNA aneuploidy found. According to our experience such false peaks are rare (Joensuu et al. 1990), and most of the starting material in the present study was rapidly frozen before fixation.

Only 1 of the 3 tumours with questionable malignant histopathological potential caused death within the follow-up of 5–9 years. This tumour had a large DI by both FCM and ICM, whereas the 2 remaining cases had a low DI (Tables 2 and 3). The good correlation between the DI and the clinical outcome suggests that the cytometric nuclear DNA ploidy pattern is of clinical value as a supplementary tool in establishing the prognosis of pancreatic NE tumours. We conclude that pancreatic NE tumours are frequently DNA aneuploid, and that NE tumours with a large DI are associated with an unfavourable clinical outcome.

Acknowledgements. This study was supported by grants from the Cancer Society of Finland, The Swedish Medical Research Council (Project No 718), the Swedish Diabetes Association, and the Cancer Society of Stockholm, Sweden. Dr. Alanen also acknowledges a Visiting Scientist Fellowship from Swedish Diabetes Association, allowing him to work at the Laboratory of Cell Analysis at the Department of Tumour Pathology, Karolinska Hospital, Stockholm.

References

Alanen KA, Joensuu H, Klemi PJ (1989) Autolysis is a potential source of false aneuploid peaks in flow cytometric DNA histograms. Cytometry 10:417–425

Alanen KA, Joensuu H, Klemi PJ, Martin S, Alavaikko M, Nevalainen TJ (1990) DNA ploidy in pancreatic neuroendocrine tumours. Am J Clin Pathol 93:784–788

Askensten UG, Rosen AK von, Nilsson RS, Auer GU (1989) Intratumoral variations in DNA distribution pattern in mammary adenocarcinomas. Cytometry 10:326–333

Askensten UG, Moberger B, Auer GU (1990) Methodological aspects of cytochemical DNA assessment of adenocarcinoma of the endometrium by means of image and flow cytometry using conventionally formalin-fixed and paraffin-embedded specimens. Arch Geschwulstforsch 60:209–216

Auer G, Askensten U, Ahrens O (1989) Cytophotometry. Hum Pathol 20:518–527

Böhm N, Sandritter W (1975) DNA in human tumors: a cytophotometrix study. Curr Top Pathol 60:151–219

Chejfec G, Falkmer S, Grimelius L, Jacobsson B, Rodensjö M, Wiedenmann B, Franke WW, Lee I, Gould VE (1987) Synaptophysin: a new marker for pancreatic neuroendocrine tumours. Am J Pathol 11:241–247

Donow C, Baisch H, Heitz PU, Klöppel G (1991) Nuclear DNA content in 27 pancreatic endocrine tumours: correlation with malignancy, survival and expression of glycoprotein hormone alpha chain. Virchows Arch [A] 419:463–468

Fallenius A, Auer G, Carstensen J (1988) Prognostic significance of DNA measurement in 409 consecutive breast cancer patients. Cancer 62:331–341

Forsslund G, Zetterberg A (1990) Ploidy level determinations in high-grade and low-grade malignant variants of prostatic carcinoma. Cancer Res 50:4281–4285

Graeme-Cook F, Bell DA, Flotte TJ, Preffer F, Pastel-Levy C, Nardi G, Compton C (1990) Aneuploidy in pancreatic insulinomas does not predict malignancy. Cancer 66:2365–2368

Haugen OA, Mjölneröd O (1990) DNA-ploidy as prognostic factor in prostatic carcinoma. Int J Cancer 45:224–228

Hedley DW (1989) Flow cytometry using paraffin-embedded tissue: five years on. Cytometry 10:229–241

Heitz PU (1984) Pancreatic endocrine tumours. In: Klöppel G, Heitz PU (eds) Pancreatic pathology. Churchill Livingstone, Edinburgh, pp 206–232

Joensuu H, Klemi PJ (1988) DNA aneuploidy in adenoma of endocrine organs. Am J Pathol 132:145–151

Joensuu H, Alanen KA, Klemi PJ (1990) Evidence for false aneuploid peaks in flow cytometric analysis of paraffin-embedded tissue. Cytometry 11:431–437

Klöppel G, Heitz PU (1988) Pancreatic endocrine tumours. Pathol Res Pract 183:155–168

Stipa F, Arganini M, Bibbo M, Straus F, Dytch H, Wied G, Horvath K, Kaplan E (1987) Nuclear DNA analysis of insulinomas and gastrinomas. Surgery 102:988–998

Ullen H, Falkmer UG, Collins VP, Auer GU (1991) Methodologic apsects on nuclear DNA assessment of gliomas with astrocytic and/or oligodendrocytic differentiation. Correlation of image and flow cytometric studies on paraffin-embedded specimen. Anal Quant Cytol Histol 13:168–176