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DNA ploidy and cell-cycle analysis in pancreatic and ampullary carcinoma: flow cytometric study of formalin-fixed paraffin-embedded tissue

H. Baisch[1], G. Klöppel[2], and Brigitta Reinke[1]

[1] Institute of Biophysics and Radiobiology, University of Hamburg, Hamburg, Federal Republic of Germany
[2] Department of Pathology, Free University of Brussels, Brussels, Belgium

Summary. The cellular DNA content of formalin-fixed, paraffin-embedded specimens from 47 ductal adenocarcinomas of the pancreas and 5 adenocarcinomas of the ampulla of Vater was analysed using flow cytometry. Ploidy and the fraction of cells in the S and G2M phases were determined and correlated with tumour stage and grade as well as patients' survival. Cell populations with aneuploid DNA content were observed in 15% of the tumours. The S+G2M fractions ranged between 1% and 10%. Compared to non-neoplastic tissue of the pancreas the S+G2M fraction was significantly higher in the carcinomas. Cox regression analysis revealed the S+G_2M fraction as an independent prognostic factor ($p<0.05$). Ploidy was of no prognostic value for survival, but correlated weakly with tumour stage and tumour grade. All patients without lymph node metastases at time of surgery had diploid tumours. Aneuploidy was restricted to tumours in advanced stages and tended to be more frequent in high-grade tumours.

Key words: Pancreatic adenocarcinoma – Prognosis – Aneuploidy – DNA index

Introduction

Cellular DNA content abnormality is a well-recognised feature of human tumours. The percentage of tumours with aneuploid cell populations varies for different types of neoplasm (Frankfurt et al. 1984). In addition to the ploidy level reflecting the existence of an abnormal DNA stem line, an estimation of proliferation activity can be obtained by measuring the fraction of cells in the various phases of cell cycle. Flow cytometry provides a fast and precise method for determination of DNA aneuploidy. The proportions of cells in the phases of the cell cycle can be determined from the same measurement; however, these data are less precise because of the presence of debris in the sample. Flow cytometric measurements have proved useful in supplementing clinical and histopathological findings in the classification of malignant disease (Friedlander et al. 1984; Baisch et al. 1986; Koss et al. 1989). Aneuploid cellular DNA content and high proliferative activity have been associated with a poorer prognosis for different tumour types (Hedley et al. 1985; Otto et al. 1984; Tribukait 1987).

The aim of the present study was to elucidate the prognostic and biological value of cellular DNA content and the fraction of cells in the various phases of the cell cycle as determined by flow cytometry in pancreatic and ampullary carcinomas. These were determined in material obtained from paraffin blocks of 52 tumours and were evaluated in a retrospective analysis in combination with certain aspects of pathology and survival. Because of the possible interference of the various prognostic factors, multivariate analysis was used to assess the value of cellular DNA content for survival.

Offprint requests to: H. Baisch, Institute of Biophysics and Radiobiology, UKE, Martinistrasse 52, D-2000 Hamburg 20, Federal Republic of Germany

Materials and methods

Formalin-fixed and paraffin-embedded resection specimens of pancreatic or ampullary carcinomas were investigated ($n=52$). Tumours located in the head ($n=40$) or the ampulla of Vater ($n=5$) were removed by Whipple's resection, tumours in the body or tail of the pancreas ($n=7$) by partial left-sided pancreatectomy. Normal pancreatic tissue from organ donors and patients with focal chronic pancreatitis was used as controls. Tumour stage at time of surgery was determined according to the 1987 updated TNM classification and is compiled in Table 1. Survival time was defined as the period between surgery and death due to pancreatic cancer. The patients ranged in age from 37 to 75 years (mean age 60 years); 34 were male and 18 female.

Paraffin sections (3 μm) from blocks mainly containing tumour tissue were stained with haematoxylin and eosin (H&E). These sections were used to determine the histological grade. All pancreatic and ampullary carcinomas belonged to the category of ductal adenocarcinoma. Histological grading was performed as previously described (Klöppel et al. 1984); three grades of malig-

Table 1. Stage and site of pancreatic carcinoma

Stage	Ampullary carcinoma	Pancreatic carcinoma	
		head	body + tail
T_1 N_0	–	3	–
T_2 N_0	2	6	2
T_2 N_1	3	25	5
T_2 N_2	–	5	–
T_2 N_1 M_1	–	1	–
Total	5	40	7

nancy were distinguished: grade 1 corresponding to well-differentiated, grade 2 to moderately differentiated and grade 3 to poorly differentiated carcinomas. The most relevant variables used in grading were glandular differentiation, nuclear polymorphism and frequency of mitoses.

For flow cytometric measurement 30-μm sections were cut from the paraffin blocks used for histological assessment. These sections were processed into single cell suspensions according to the slightly modified method described by Hedley et al. (1983). Separate single cell suspensions were performed from at least two sections of each paraffin block. Dewaxing was carried out using two changes of 3 ml C68 (Technicon, Bad Vilbel, FRG) for 10 min at room temperature and followed by rehydration in a sequence of 100%, 96%, 70% and 50% ethanol and distilled water for 10 min at room temperature. Single cell suspensions were obtained mechanically by syringing (diameter of cannula 2 mm) 20 times and enzymatically by incubating in 5% trypsin at 37° C for 45 min. The cell suspension was stained with Ho33258, a DNA-specific fluorescence dye (Herzog and Schütze 1968) and then filtered through a nylon gauze (53 μm). The DNA distribution was measured on a flow cytometer ICP 22 (Phywe, Göttingen, FRG).

A histogram was considered to be diploid if its DNA distribution showed a single G_1 peak. Any additional G_1 peak was taken to indicate aneuploidy. The DNA index (DI) was calculated as the ratio of aneuploid to diploid G_1 peak channel. Since tumour material always contains normal diploid cells, these cells were used as an internal standard. A tumour was only considered as aneuploid if the abnormal DNA stem line was reproducible in all samples measured. The histogram in Fig. 2 upper chart represents a diploid and Fig. 2 lower chart an aneuploid DNA distribution. The coefficient of variation (CV) of the G1 peak of diploid and aneuploid cells had a mean of 8.5%. After background subtraction and correction for clumped cells (Beck 1980) the fractions of cells in the various phases of the cell cycle were analysed using the rectangular method described previously (Baisch et al. 1975).

The survival data were analysed using Cox's proportional hazard regression model to evaluate the effect of flow cytometric parameters on survival, other possible prognostic factors such as the age of patient and the site of tumour being taken into account (Cox 1972). Survival curves were calculated using the method according to Kaplan and Meier (1958). Differences between actuarially calculated survival curves were assessed by Breslow and Mantel-Cox. In the survival analysis the patients still alive were considered as censored observations. Differences between the fractions of cells in the S+G2M phases were assessed according to Kolmogoroff (1933) and Smirnoff (1939).

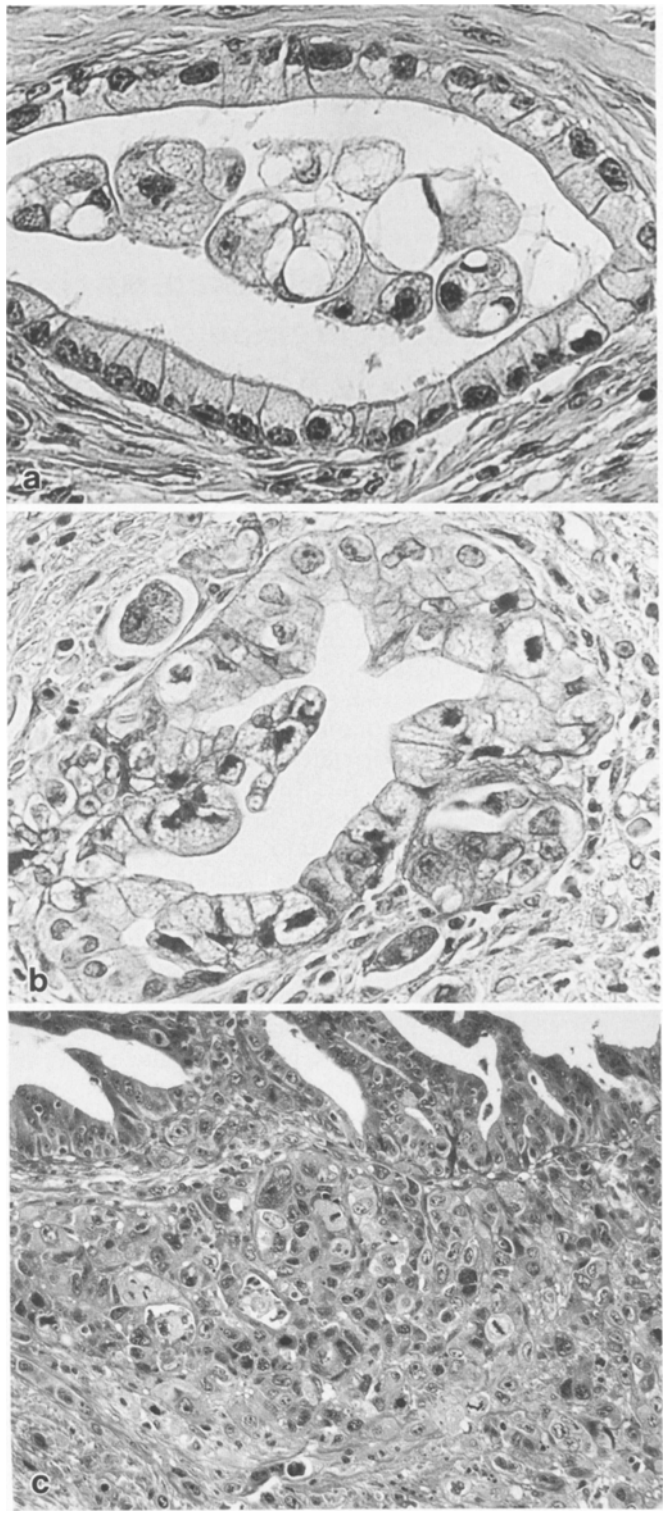

Fig. 1. Histological sections of pancreatic carcinomas, H&E stained. **a** Pancreatic ductal adenocarcinoma, well-differentiated glandular pattern, grade 1, ×600. **b** Moderately differentiated pattern, grade 2, ×600. **c** Poorly differentiated pattern, grade 3, ×240

Results

Histological sections of pancreatic tumours investigated in this study are shown in Fig. 1. Grading reflects differentiation stages. Typical flow cytometry histograms with a DI of 1 (diploid) and DI greater than 1 (aneuploid) are shown in Fig. 2. We obtained 40 (85%) diploid and 7 (15%) aneuploid tumours; 5 histograms could not be analysed because insufficient intact cells could be iso-

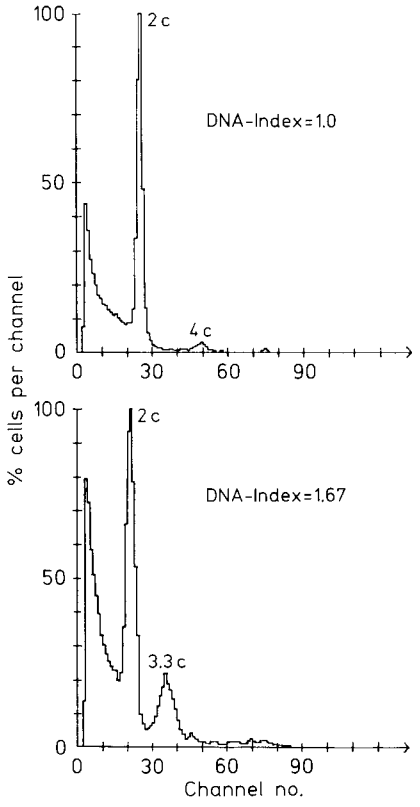

Fig. 2. DNA distribution of paraffin-embedded pancreatic carcinoma measured by flow cytometry. The *upper chart* shows a diploid tumour (G1 at *2c*, G2M at *4c*), the *lower chart* represents a tumour with an aneuploid tumour cell line

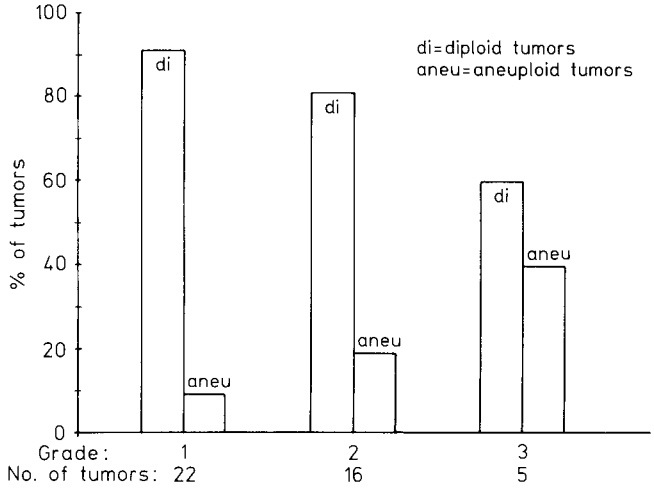

Fig. 3. Proportion of diploid and aneuploid tumours in grades 1–3

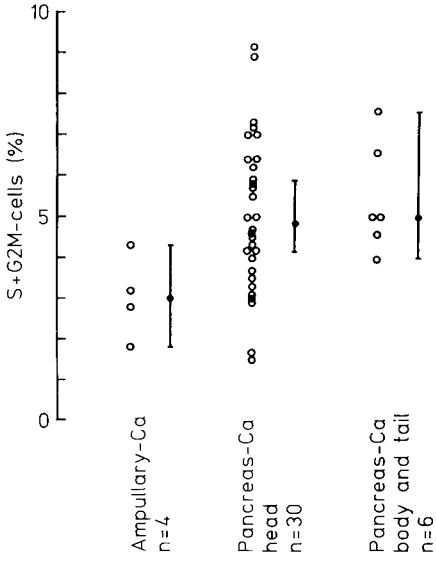

Fig. 4. Percentage of S+G2M phase cells measured by flow cytometry. Besides the values of single tumours median and 95% confidence limits of tumours at different location in pancreas are depicted

lated. Five aneuploid carcinomas had DI between 1.5 and 1.8 and 2 close to 2.0. All normal and pancreatitis samples were diploid. The proportion of aneuploid tumours among pancreatic head and pancreatic body and tail carcinomas was about the same (17% and 14%, respectively). All 5 ampullary carcinomas were diploid. Among the lymph-node-positive (N1) tumours of the pancreatic head 21% (6/28) were aneuploid while the 8 tumours without lymph node metastasis (N0) were diploid. Correlation of aneuploidy and grade revealed an increase in the percentage of aneuploid tumours with grade (Fig. 3).

The fraction of cells in S+G2M phase is an estimate of the proliferative capacity of tumours. We found 1–10% S+G2M cells (mean 4.7%) in our carcinoma samples, whereas for non-malignant pancreatic tissue 0.5–5% (mean 3.1%) were obtained. The correlation of this variable with location of tumour is shown in Fig. 4. Ampullary carcinomas showed lower proliferative activity than pancreatic carcinomas, but all values displayed considerable variations. No correlation between S+G2M proportion and stage or grade could be detected. The median S+G2M percentages were 4.6 and 4.9 for T1 and T2N0 tumours, respectively. The tumours of grade 1, 2 and 3 yielded median S+G2M values of 4.7, 6.2 and 4.6, respectively.

Figure 5 shows actuarial survival of the patients by site of the tumour. The difference in survival between

Table 2. Median survival time of patients after resection of pancreatic carcinoma in the head of the gland

	Grade of tumour			Node involvement		DNA ploidy	
	1	2	3	N_0	N_1	diploid	aneuploid
Number of patients	15	14	4	7	26	25	5
Median survival (months)	12	16	8	14	12	12	16

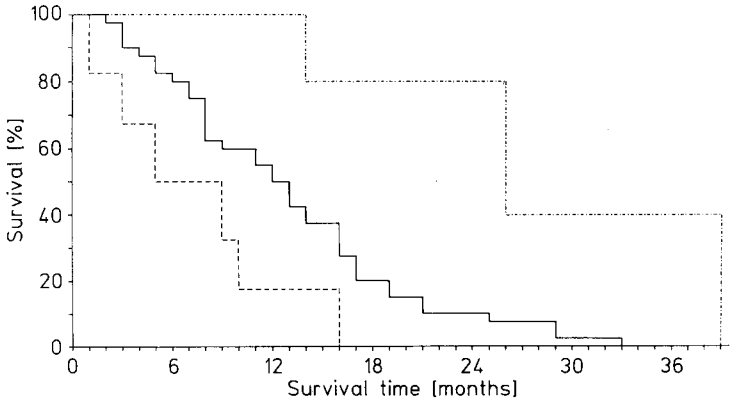

Fig. 5. Actuarial survival curves of tumours at different sites in pancreas: —.— ampullary carcinomas; —— pancreas head carcinomas; – – – pancreas body and tail carcinomas. *Abscissa:* Survival time post surgical removal of tumour

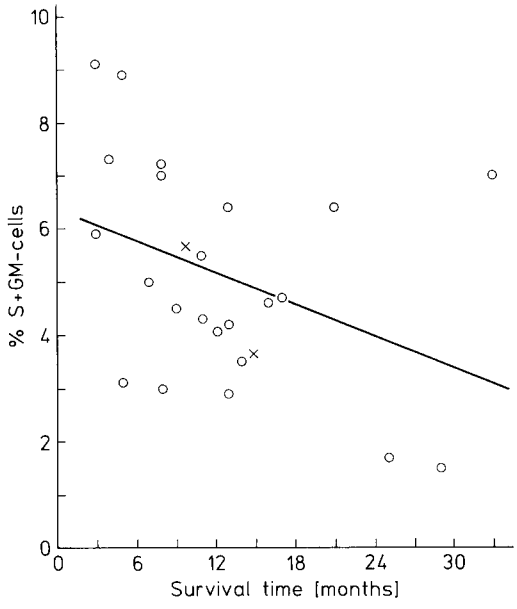

Fig. 6. Correlation between proportion of S+G2M phase cells and survival time of patients post surgical removal of tumour. *Straight line* represents regression analysis. o, Patients died of carcinoma; x, patients alive

ampullary, pancreas head, body and tail carcinomas was statistically significant ($p<0.01$). However, tumour grade, lymph node involvement and DNA ploidy was not correlated with survival time, as shown in Table 2. The grade 3 tumour patients may have the worst prognosis with a median survival time of only 8 months; however, this is not significant because of the small number of patients in this group.

Figure 6 shows the correlation of S+G2M percentage with survival time. Regression analysis yielded only a low correlation coefficient of -0.38; however, there seems to be a tendency for higher proportions of S+G2M to be associated with an unfavourable prognosis.

Discussion

The low percentage of aneuploid (DI > 1) ductal adenocarcinomas of the pancreas (15%) is striking in comparison with other malignancies. Frankfurt et al. (1984) reported aneuploidy rates in the range of 60–90% for various tumours (bladder, breast, colorectal, ovarian and lung carcinomas, and melanoma). In renal cell carcinoma we observed aneuploidy in approximately 50% (Baisch et al. 1984). This discrepancy between pancreatic carcinoma and other neoplasms raises the question whether flow cytometry measurements (FCM) of paraffin-embedded material are precise enough to reveal the exact incidence of aneuploidy in any tumour type. Hedley et al. (1984) observed a lower frequency of aneuploidy in paraffin-embedded material than in fresh tissue. In contrast, Macartney et al. (1986) showed that FCM results of formalin-fixed and paraffin-embedded material correlated well with information obtained from fresh tissue. Comparison of FCM paraffin method with Feulgen scanning absorption cytometry also yielded close correlation (Fossa et al. 1986). As we found a similar aneuploidy rate in a small series of freshly prepared pancreatic carcinoma tissue (1/7 tumours, unpublished results) as in the paraffin-embedded material, it appears that our present results compare with those of fresh material.

The determination of proportion of cells in the phases of cell cycle is less precise. As shown in Fig. 2, the samples contained a considerable fraction of cell debris that possibly may account for the increase in the percentage of S-phase cells calculated from the histogram. It is noteworthy, however, that we obtained similar proportions of cells in S+G2M from fresh tissue (mean $6.4 \pm 4.7\%$) to those from paraffin material ($4.8 \pm 1.2\%$, unpublished results).

In accordance with the generally held view that aneuploidy is related to tumour progression, we found a correlation between aneuploidy and lymph node involvement. All aneuploid samples were found among N_1 tumours, whereas all N_0 tumours were diploid. Furthermore, the frequency of aneuploidy increased with grade (Fig. 3), a finding also observed in renal cell carcinoma (Otto et al. 1984). No correlation was found between the fraction of cells in S+G2M phase and stage or grade of the pancreatic tumours. This may be explained by the broad range of the S+G2M variable, indicating varying proliferative capacity of individual tumours.

The prognostic value of the different parameters obtained by flow cytometry and histology was tested with the Cox-model. This revealed that survival time after surgical removal did not correlate with aneuploidy, but was significantly ($p < 0.05$) related to proliferative activity (S+G2M), (Table 2, Fig. 6). A low proportion of cells in the S+G2M phase pointed to longer survival, a high S+G2M percentage to a shorter survival time. Prognostic relevance of S+G2M proportion was also reported for breast, bladder and head and neck carcinoma (Hedley et al. 1985; Tribukait 1987; Johnson 1985), but not for renal cell carcinoma (Baisch et al. 1986).

Retrospective studies on the prognostic value of cellular variables may be limited by the fact that the patients differ with respect to their treatment. Thus, the prognosis depends not only on the inherent biology of the tumour but also on the sensitivity with regard to the chemotherapy applied. In the present study, only one tumour (located in the head of the pancreas) responded to chemotherapy with a partial remission and hence prolonged survival of the patient. As the S+G2M value of this tumour was relatively high (7%) and hence suggestive of a short survival time, it may be speculated that tumours with a high proliferation rate are more sensitive to chemotherapy. In support of this suggestion are results from studies in bladder carcinoma showing that tumours with high proliferative capacity have a better responsiveness than those with a low proliferative capacity (Tribukait 1987).

Ploidy has been reported to be the main prognostic variable in a number of malignancies (Friedlander 1984; Baisch 1986; Rodenburg 1987; Cornelisse 1987). According to our results DI (ploidy) was not predictive with respect to prognosis of pancreatic carcinoma. This is at variance with the findings of Weger et al. (1987), who studied paraffin-embedded pancreas tumours by DNA image cytometry. Patients with near tetraploid (DI near 2) carcinomas (8/15) were still alive 70 months after surgery, while all 16 patients with near triploid tumours died within 18 months. Unfortunately no further details of this investigation were reported. Since our series included only a few aneuploid tumours, the issue of prognostic relevance of ploidy remains open for pancreatic carcinoma.

To identify patients with favourable versus a poor prognosis, all available parameters were used. In the multivariate analysis we found tumour location ampulla, head, body and tail to be the best predicting variable for survival time in accordance with previous reports (Klöppel 1979, 1984). Tumour grade and lymph node involvement were not correlated with survival time in the present study. This is in contrast to previous results, where survival time was at least related to grade (Klöppel 1984). The data in the literature concerning stage are less clear. While some authors reported longer survival of patients without lymph node metastasis (Cubilla 1978; Rückert and Kümmerle 1985; Funovics and Fritsch 1986), others found no correlation between lymph node involvement and survival (Edis et al. 1980, Herter 1982). Despite a slight correlation between tumour location and proportion of S+G2M (Fig. 5) in the present study, both variables proved to be of prognostic relevance in the multivariate analysis. For all other parameters determined no prognostic relevance was found. However, because of the wide range of all variables more data are needed to establish their significance for tumour progression and prognosis in patients with pancreatic carcinoma.

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