

Prognostic impact of DNA-image-cytometry in neuroendocrine (carcinoid) tumours

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Abstract. Establishing prognosis proves particularly difficult with neuroendocrine tumours (NETs) as a benign looking histology can be associated with a malignant behaviour. In order to identify prognostic factors we examined 44 gastrointestinal and pulmonary, paraffin-embedded NETs histologically and immunohistochemically. DNA-image-cytometry was used to examine 40 of these. We found that poor differentiation (corresponding to a Soga and Tazawa type D) and infiltrative growth correlated with a poorer prognosis. Moreover, parameters determined by diagnostic DNA cytometry like the 5c-exceeding rate, the 2c-deviation index, DNA-grade of malignancy, DNA-entropy and the type of DNA histogram were found to be of prognostic relevance. Morphometric parameters like the form factor and the mean nuclear area were relevant for survival, tumour recurrence and metastasis. However, in the multivariate analysis the only independent risk factor was the histological differentiation. The 5c-exceeding rate is a good objective risk factor, which can be used particularly in cases in which only a fine needle biopsy is available. Direct comparison of the histology and the 5c-exceeding rate in the multivariate analysis suggests that the 5c-exceeding rate taken as sole prognostic factor might be of higher prognostic relevance than the histology but larger studies are needed to confirm this.

1. Introduction

Neuroendocrine tumours (NETs) are rare lesions and occur with an incidence of 2.0/100,000 for men and 2.4/100,000 for women according to a recent Swedish study [16]. The most important problem concerning these tumours is that lesions with the same histological appearance can behave completely different. Therefore, despite new classification concepts and some progress concerning the knowledge of the molecular base of NETs it is impossible to predict their behaviour with certainty [20]. This is particularly true when only biopsy material is available which does not allow an evaluation of the invasive edge of the lesion. In the literature there are many studies evaluating prognostic factors of NETs. Localization, local invasiveness, the size of the lesion as well as the mitotic count have been regarded as the most decisive factors [20]. However, there are reports on cases with large mesen-

terial lymph node deposits originating from ileal NETs of a few millimetres in size [27].

In the present study we asked whether diagnostic DNA image cytometry is better at predicting the biological behaviour of NETs than morphological parameters.

2. Material and methods

2.1. Material

In this retrospective study we examined the paraffin-embedded NETs of 44 patients which had been removed endoscopically or by open surgery. To obtain follow-up data we reviewed the hospital notes, sent questionnaires concerning clinical presentation and progress to the referring doctors, and we contacted the registrars' office concerning survival data. The follow-up varied between 0 to 108 months. One patient was lost to follow-up as he had moved away. The questionnaires were answered in 28 cases. Two patients had been treated with chemotherapy and two with chemotherapy and interferon for secondary tumours.

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2.2. Morphology and immunohistochemistry

We reviewed all the routinely performed HE stained sections to confirm the diagnosis and classify the tumours according to Soga and Tazawa. They classified NETs (carcinoids) depending on their growth pattern into five different types. Tumours of type A have solid, nodular growth pattern, type B a trabecular, and type C a tubular or acinar growth pattern. Tumours of type D are “atypical” with many mitoses, a solid growth pattern, and may have necroses. They represent the highly differentiated neuroendocrine carcinoma of the current classification concept. Type E stands for any tumour which shows two or more of the above histologies [26]. Goblet cell carcinoids formed another group. We also assessed whether the tumour grew invasively or had set metastases. For the immunohistochemistry we used cuts of 5–8 μm from each tumour. The slides were deparaffinated and rehydrated. Then the endogenous peroxidase was inhibited with a mixture of 1% H_2O_2 and methanol and rinsed in PBS. The slides were incubated for 60 minutes with the primary antibody at room temperature. All the primary antibodies apart from chromogranin (Histoprime) were from DAKO. The cuts were then rinsed three times in PBS and incubated with the secondary antibody anti-rabbit or anti-mouse IgG (both from Dianova) in a dilution of 1 : 40 for 45 minutes. Once more the slides were rinsed three times with PBS and then incubated for 5 minutes with DAB. The reaction was stopped under running tap water. A counter stain with haemalum was performed and the slides were mounted.

2.3. DNA-image-cytometry

DNA-cytometry was performed on cytopspins prepared from nuclear suspensions after enzymatic cell separation using pepsine as described earlier [4]. Due to lack of material in four cases it was only performed on 40 samples. For preparation we cut around the tumour outlines in the paraffin block and prepared the nuclear suspension from two to three 70 μm sections. Small tumours were cut out from the blocks completely. The suspension was applied on slides covered with poly-L-lysine and these were air-dried. Afterwards the slides were stained in a modified Varistain 24-3 staining machine. Initially they were fixed according to Böhm [7], then hydrolysed in 4 N HCl for 55 minutes at 27.5°C. This was followed by staining according to Feulgen during 60 minutes using pararosaniline as dye [4]. Measurement was performed

using the DNA-cytometer CM-1 (Hund, Wetzlar). This was developed at RWTH Aachen in cooperation with the company ABOS (Munich, Germany). It consists of a microscope Hund 500 LL, equipped with an interference filter with a 570 ± 10 nm half-value width and a device that keeps the voltage at the halogen lamp constant. An objective with 40 \times magnification was used. The microscope is connected via a calibrated camera and a monitor with a personal computer for image analysis.

Nuclei to be measured were marked with a cursor on the monitor. Integrated optical densities (IOD's) were measured for each nucleus after automatic segmentation of its contour using a watershed algorithm. Nuclei were thus detected using their local background for IOD measurements. A software-based glare error correction was performed. In each slide 20 granulocytes were measured for internal calibration. The mean IOD of the granulocytes was defined as the 2c DNA content of a normal diploid nucleus. We did not use a correction factor. The CV-values of the IOD's of the reference cells were below 5% [15]. In each slide 200 tumour cells were measured. We calculated the position of the first and second DNA-stemline, the 5c exceeding events (5cEE) and 5c exceeding rate (5cER), the 2c deviation index (2cDI), the DNA-entropy, the DNA-malignancy grade, the mean DNA-content of the tumour cells, the mean formfactor, the mean nuclear area, the standard deviation of DNA-ploidy, the coefficient of variation of the DNA-ploidy and the DNA-stemline interpretation according to Böcking [4–6,28].

2.4. Statistical analysis

The statistical analysis was performed using Systat Student version 1.0 except for the Cox regression which was performed using SPSS software version 10. *P* values below 0.05 were considered as statistically significant.

All patients were included in the statistical analysis. When only a local, endoscopic resection of the tumour was performed we considered that we did not know whether the subjects had metastasis to the lymph nodes or not. Correlation between two parameters was measured with the Pearson's Chi-Square while significance of the Kaplan–Meyer curves was measured by Tarone–Ware.

The thresholds for the different DNA variables were derived from Kaplan–Meyer statistics with balanced groups.

3. Results

3.1. Study population

Of the 44 patients 20 (45.5%) were men and 24 (54.5%) were women, 65.9% of the patients survived. One patient was lost to follow-up via the registry as he moved to a different country. Of the questionnaires that had been sent to the general practitioners 28 were answered. In 13.8% of the cases the patients suffered from a secondary tumour. In eight cases death was due to metastatic spread of the NET or of secondary tumours. In five cases the cause of death was not known and one patient died of a post-operative cardio-vascular shock.

The mean age was 54.9 years with a range from 19 to 79 years. An age ≥ 55 years was associated with a shorter survival time ($p < 0.002$) and a higher incidence of local ($p = 0.012$) and distant metastases ($p < 0.03$) and tumour recurrences ($p < 0.006$). Eight of our patients with NETs of the appendix were in the younger age group. To ensure that the difference in prognosis was not due to the higher incidence of the appendiceal NETs in the younger group we checked whether these differences persisted once these patients were excluded. Even then there remained a difference in survival and metastases and recurrences depending on the age though only the difference in survival remained statistically relevant ($p < 0.02$).

3.2. Localization and therapy

The distribution of the tumours throughout the pulmonary and gastro-intestinal tract is shown in Table 1. NETs of the appendix have a good prognosis as long as the tumour does not extend to the caecum. In the latter case the frequency of local recurrences ($p = 0.077$) and metastases (lymph node, $p = 0.013$; distant, $p < 0.04$) increases compared to localized appendiceal NETs as NETs of other localizations. There was a tendency of longer survival of the patients with NETs solely affecting the appendix compared to patients with tumours in other localizations, but the difference was not statistically significant ($p < 0.066$). If the tumours were located in the jejunum or ileum the chances of survival were worse than in other localizations ($p < 0.045$). The patients who had a palliative operation had a poorer prognosis than patients with a curative or endoscopic operation ($p < 0.00065$). The frequency of secondary metastases in the lymph nodes ($p < 0.003$) and in other organs ($p < 0.002$) was higher with palliative operations than with radical operations and endoscopic resection.

Table 1

Localization of the NETs		
	Localization	Frequency
Foregut	Bronchus	3 (6.8%)
	Stomach	5 (11.4%)
	Papilla Vateri	3 (6.8%)
	Pankreas	3 (6.8%)
	<i>Total</i>	<i>14 (31.8%)</i>
Middlegut	Small intestine	6 (13.6%)
	Meckel's diverticulum	1 (2.3%)
	Valvula Bauhini	1 (2.3%)
	Appendix	10 (23%)
	Appendix and caecum	4 (9.1%)
	Colon ascendens	2 (4.5%)
	<i>Total</i>	<i>24 (54%)</i>
Hindgut	Colon transv./desc.	1 (2.3%)
	Rectum	5 (11.4%)
	<i>Total</i>	<i>6 (13.6%)</i>

3.3. Size and histology

The size of the tumours had been reported in only 24 cases. The smallest tumour was 0.3 cm in diameter, the largest 9 cm and the average was 3.6 cm. Unlike other authors we could not find a correlation between tumour size and survival, metastasis or recurrences but this is probably due to our small numbers.

Most of the tumours, i.e. 26, were of the mixed type E (65.9%), seven of which had parts that were poorly differentiated (type D). Nine were of the type AC and 6 of the type AB. Twelve tumours (27.3%) were of type A, two of type B, one of type C. We also had three goblet cell carcinoids, one of which consisted purely out of goblet cells and the others had parts, which corresponded to type B or type AB, respectively. We found that NETs which were poorly differentiated or of the goblet cell type were more frequently associated with lymph node metastases ($p = 0.001$), distant metastases ($p = 0.0002$) and a higher lethality ($p < 0.00001$) than other types (Fig. 1). Infiltrative growth of the tumours was also associated with more frequent distant metastases ($p < 0.04$) and higher lethality ($p = 0.006$).

3.4. Immunohistochemistry

NSE immunoreactivity was found in 41 of 44 cases (93.2%) and chromogranin immunoreactivity in 31 of 44 cases (70%). There was no evidence that the expression of these antigens was associated with a different prognosis. In case of the peptide hormones a

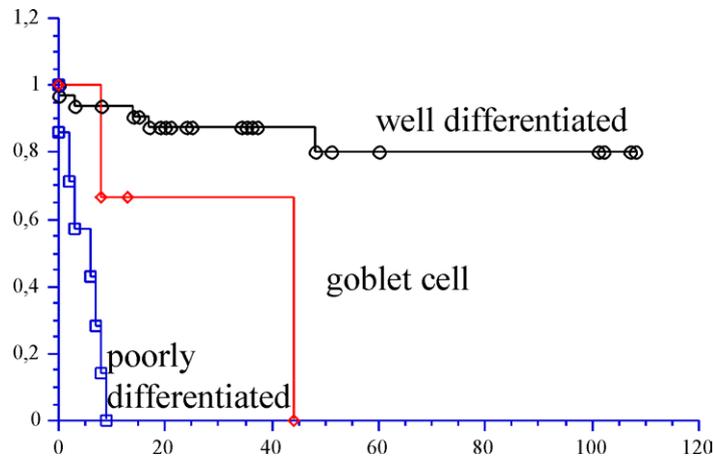


Fig. 1. Univariate survival analysis (Kaplan–Meier) of 44 patients with carcinoid tumours. Patients with well-differentiated carcinoid tumours have a significant better prognosis compared to goblet-cell carcinoids or poorly differentiated tumours.

positive reaction was found for pancreatic polypeptide and somatostatin in 25%, serotonin in 40.9%, glucagon in 13.6% and for gastrin in 6.8% of the carcinoids. Tumours of the pancreas, papilla vateri, appendix and rectum frequently produce several hormones. The survival of patients with tumours which reacted to pancreatic polypeptide seemed to be somewhat better ($p < 0.05$) but was not reflected in a significantly lower frequency of local tumour recurrence or lower incidence of metastases.

In half of our patients a positive reaction to S-100 was found and in 55% we found sustentacular cells in the tumour tissue. Metastasis into other organs occurred less frequently in patients with S-100 positive tumours ($p = 0.002$) or tumours that contained sustentacular cells ($p < 0.02$).

3.5. DNA-cytometry

The histograms showed three different patterns of DNA-distribution (Fig. 2). The first showed a single DNA-stemline near 2c and only a few values at 4c. The second revealed a smaller stemline around 4c besides that at 2c, the latter containing most of the cells and the third pattern showed a second stemline at 4c comprising more cells than the first one (Fig. 2). Whereas pattern 1 corresponds to the euploid–diploid-type, pattern 2 is identical to the euploid–polyploid-type, and pattern 3 is the aneuploid–peritetraploid DNA-histogram-type as defined by the 4th ESCAP-consensus-report [15]. 43.2% of our cases revealed just one stemline around 2c. The DNA-stemlines were found at 1.97c on average. Two stemlines, the first of which was larger than the second, we found in 43.2%. The least num-

ber of cases (11.4%) showed the third pattern of DNA-distribution with two stemlines, the second of which was larger than the first. The NETs in the three different groups showed a significantly different behaviour concerning the frequency of recurrences, metastases and survival (Table 2, Fig. 3). Mean DNA-ploidy, the position of the DNA-stemline and the coefficient of variation of the DNA-stemline did not correlate with prognosis. On the other hand, a 2cDI greater 0.4 ($p < 0.003$), a 5cER of ≥ 0.5 ($p < 0.005$), a DNA malignancy grade of 0.26 or greater ($p < 0.003$) and a DNA-entropy greater 3.55 ($p < 0.004$) was associated with a higher mortality (Fig. 3). The higher mortality associated with these different DNA-parameters was also reflected in a higher frequency of metastasis and local recurrence (Table 2). A 5cER > 1.5 corresponding to greater than three 5cEE was related to a more marked difference in mortality and all of the tumours in this group metastasised to the lymph nodes ($p = 0.002$) and into other organs ($p = 0.001$). Compared to the 5cER > 0.5 this test was therefore more specific but less sensitive (Table 2).

3.6. Morphometry

A higher mortality was found for the cases in which the mean area of the nucleus exceeded $38.6 \mu\text{m}^2$ ($p < 0.03$) and those in which the formfactor was ≤ 1.27 ($p < 0.04$) (data not shown). There was no correlation between the morphometric parameters and frequency of tumour recurrence and of metastases.

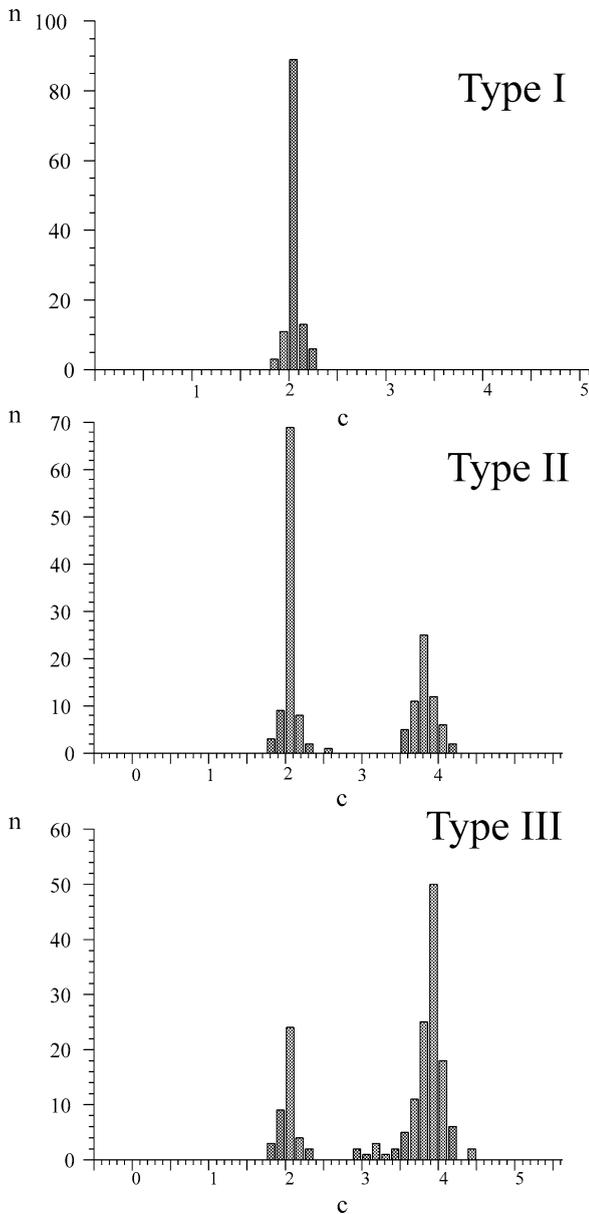


Fig. 2. Examples of different histograms to exemplify prognostical relevant DNA distribution in NETs.

3.7. Cox model

All the parameters which were relevant for the prognosis in the univariate analysis were entered into the Cox model and tested for their independence. Unfortunately, it had to be broken off after the sixth step due to the limited number of cases. At that stage all six parameters entered were still independent and were associated with quite high relative risks (Table 3).

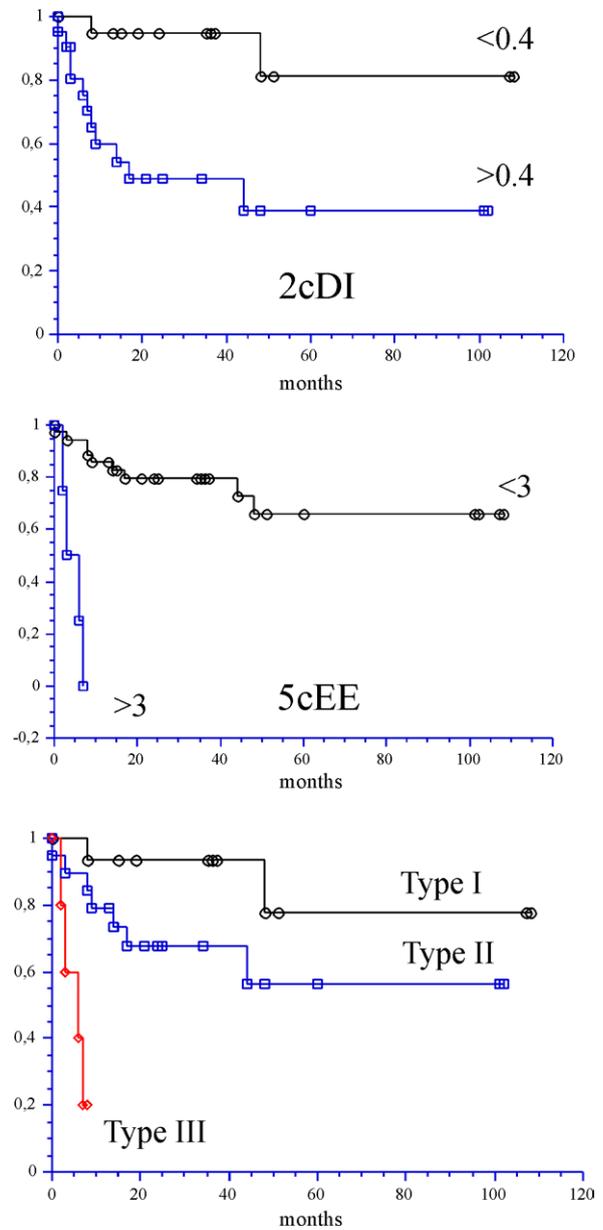


Fig. 3. Univariate survival analysis (Kaplan–Meyer) of 44 patients with carcinoid tumours. Patients with carcinoid tumours with a 2cDI <math><0.4</math>, a 5cEE of <math><3</math> and type I histogram have a significant better prognosis.

4. Discussion

Prediction of biological behaviour of neuroendocrine tumours (NETs) is still a difficult matter. The aim of the present study was to evaluate DNA cytometry as a prognostic tool in NETs of different localizations, and to compare its value with other prognostic fac-

Table 2
Relationship between DNA parameters and malignant behaviour

	2cDI		DNA-MG		DNA-entropy		5cEE		5cER		Histogram type		
	≤0.4	>0.4	<0.26	≥0.26	<3.55	≥3.55	0	>0	<1.5	≥1.5	I	II	III
Local recurrence	0	41.2%	0	41.2%	5.9%	35.5%	0	41%	16.7%	50%	0	31.1%	50%
LN metastases	11.8%	55%	11.8%	55%	11.8%	55%	11.8%	55%	27%	100%	14.2%	41.2%	66.7%
Distant metastases	5.6%	45%	5.6%	45%	11.1%	40%	11.1%	55%	17.6%	100%	5.6%	27.8%	83.3%

Table 3
Results of the Cox multivariate analysis

Sequence of parameters entered in the Cox-model	Significance	Relative risk exp (B)	95% confidence interval for exp (B)	
			lower	upper
(1) 5cEE > 3	0.045	13.76	1.1	178.5
(2) Operation	0.001	41.48	4.7	368.8
(3) Histological type	0.009	35.24	2.5	501.8
(4) Local recurrence	0.003	22.85	2.8	186.0
(5) Localization (small bowel)	0.014	56.26	2.2	1411
(6) Formfactor	0.032	8.37	1.2	58.4

tors known from the literature. We identified patient's age, localization of the tumour, structure of the invasive edge, histological type, invasion of the coecal base in appendiceal NETs, S-100 reactivity, the presence of sustentacular cells, and DNA cytometry as prognostic important parameters.

The most important finding of our study was the highly significant and multivariate independent prognostic importance of DNA cytometry. A 2cDI > 0.4, a DNA-malignancy grade ≥ 0.26, a DNA-entropy > 3.55, a 5cER ≥ 0.5 were of prognostic relevance. Similar to the study of Stipa et al. we found that a 5cER > 1.5% was the most specific but also the least sensitive parameter [29]. The 5cER ≥ 0.5 correlated also with the incidence of metastases and tumour recurrences. The 5cER has been found to be a good marker of malignancy in studies examining other tumours [13,24]. In the literature there are contradictory data on the importance of DNA cytometry in NETs. The largest study is by Padberg et al. who found a significant association between survival, tumour size and localization, TNM-stage and tumours belonging to type I/II or type III/IV according to Auer in DNA-cytometry [23]. Two investigations using flow cytometry revealed a correlation between DNA-aneuploidy and staging, tumour size, depth of invasion and also mortality in colorectal carcinoid tumours [8,31]. Blöndal et al. examined 15

NETs of the lung by flow cytometry and found that the benign tumours tend to be diploid [3]. Other studies found a relationship between the percentage of tumour cells lying above 2.5c and the survival time in patients with metastasized intestinal NETs [11,22]. Alanen et al. who examined NETs of the pancreas found that the DNA-index was >1.8 in the group of patients that died after 6 years of follow-up [2]. The group of Stipa et al. examined gastrinomas and insulinomas of the pancreas and found a correlation between metastatic spread, a 5cER > 1% and a DNA-stemline-ploidy > 2.5c. The ploidy was the more sensitive but also less specific prognostic factor [30]. However, there are other studies which did not show any correlation between DNA-cytometric parameters and prognosis [10,12,17]. In the study of Fitzgerald et al. 95 tumours were examined and no connection between DNA-cytometry and prognosis was found. They did not differentiate though between non-diploid and frankly aneuploid tumours which might blur the differences. These very different results are probably at least in part due to the fact that the definitions of DNA-aneuploidy varied from one study to another. The studies which established a correlation between DNA-parameters and prognosis tended to be image-cytometric studies. This might be an indication that the non-selective measurement of flow cytometric studies is inadequate to de-

tect nuclei with minor DNA aberrations. That nearly all NETs have chromosomal imbalances has recently been shown by CGH analysis. Gains as well as losses were found [30]. The difficulty in assessing prognostic factors in a relatively rare disease like NETs is that prospective studies are difficult. When past cases are reviewed the degree and thoroughness of the follow-up tend to vary greatly. The quality of the information regarding lymph node and distant metastases as well as local recurrences will therefore vary markedly from case to case and tend to obscure correlations of DNA-measurements and outcomes. The classification into three different histogram types correlated well with prognosis. Patients with NETs of DNA histogram type III developed distant metastases in 80% and 50% suffered from local recurrence. In contrast, patients with type I NETs developed metastases in less than 6.5% and had no local recurrences. $5cER > 1.5$ seemed the most useful tool for the identification of metastatic carcinoids. All the tumours in that group had developed metastases in the lymph nodes and in other organs. It was the first parameter which was taken into the Cox-model. This demonstrates that in our study it is the parameter which taken on its own predicts the survival best. The different relative risks attributed to the different risk factors in the Cox-model were quite significant though they also showed a wide variation. As the multivariate analysis had to be stopped after the sixth step a full evaluation was not possible.

Taken together, our data indicate that DNA cytometry is a useful objective tool to predict the prognosis of NETs. Other important factors were the histological differentiation, invasiveness and the localization of the tumour. DNA-cytometry can also be performed on smears of pre-operatively obtained (endo-) sonographically or CT-controlled fine-needle aspiration biopsies. Therefore not only a non-invasive specific diagnosis of NETs is possible (eventually by assistance of immunocytochemistry) but also the valid assessment of prognosis (occurrence of metastases, survival). Thus, in older patients or those who are not operable a tumour-resection can be avoided if the DNA-pattern gives evidence of a favourable prognosis.

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