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Prognostic value of flow cytometrically determined DNA-ploidy, intracellular pH and esterase activity of non-small cell lung carcinomas

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Abstract

30 surgical specimens of patients with non-small cell lung carcinomas (NSCLC) were investigated. Significant increases of intracellular pH values in epithelial and inflammatory cells, in the percentage of dead epithelial and inflammatory cells and in the cell volume of vital inflammatory cells in cancerous lung tissue were encountered. Furthermore, decreases of the esterase activity of vital epithelial cells and of the percentage of free cell nuclei were observed. The DNA aneuploidy in 36.6% of the tumours was frequently associated with non-squamous cell carcinomas and stage II, III, IV tumours. Patients with DNA aneuploid tumours had a significantly shorter survival rate than those with DNA euploid tumours. Within the different tumour stages a similar tendency was observed which was, however, only significant in stage III tumour patients. Stage III tumours constitute therefore a heterogeneous entity with a worse prognosis for DNA aneuploid tumour patients. The intracellular pH values and esterase activity as well as the cell volume, the percentage of free cell nuclei and dead inflammatory or epithelial cells contained no significant prognostic information.

Key words: Flow cytometry; DNA-ploidy status; Prognosis; Non-small cell lung carcinoma

Introduction

The prognosis of patients suffering from non-small cell lung carcinoma has been based on clinical staging and histological cell type. These classifications do not, however, consider the variable biological behaviour and biochemical characteristics of the tumour and are therefore only of limited value for prognosis.

From a clinical standpoint, surgery is the accepted mode of treatment in stage I and II lung cancer. Surgical carcinoma curability is limited, however, in stage III because of tumour extension and mediastinal lymph node metastases [13]. A more precise prognostic estimate is desirable to facilitate the decisions about potential preoperative chemotherapy and the extension of mediastinal lymphadenectomy in stage III carcinoma.

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The determination of biochemically well-defined parameters, such as cellular DNA, which represents a measure of the total chromosomal DNA content of tumour cells by flow cytometry, has been valuable for the establishment of patient prognosis in a variety of tumours [7]. Depending on tumour type and author [1, 6, 16, 19, 23] between 40 and 85% of lung tumours are DNA aneuploid. The determination of additional biochemical parameters such as intracellular esterase activity and pH values as well as the volume of single cancer and inflammatory cells have been of practical interest for tumour diagnosis [12].

Flow cytometric methods for the simultaneous measurement of intracellular pH values and esterase activity of vital cells in conjunction with the determination of the DNA distribution of dead cells and the multifactorial analysis of the data with the DIAGNOS1 program system were recently developed [26].

The purpose of this study was to investigate to what degree patient prognosis was predictable by DNA ploidy or biochemical parameters.

Materials and Methods

Patients

Fresh tissue specimens from 9 female and 21 male previously untreated lung cancer patients with a mean age of 58 years (range: 44–72 years) were taken immediately after operation. The clinical staging of the tumours was based on the criteria of the International Union against Cancer (UICC 1987) and patients were staged at the time of surgery (Table 1). Histo-pathological grading of the tumours was performed according to the WHO grading system. All tumours examined were non-small cell lung carcinomas: 16 squamous cell carcinomas, 11 adeno-, 3 large cell carcinomas. The histologic examination and classification of the tumours was carried out according to the grading system in Table 2. 8 patients were treated by pneumonectomy, 21 by lobectomy, and in 1 patient by a lower bilobectomy. All patients in stage I, II and III were operated with a curative intention with the removal of all accessible tumour tissue.

Cells

0.5 g tissue was removed from lung cancer tissue and from normal lung tissue of the same patient. The specimens were taken from 3 different locations (peripheral, middle and central) of the tumour to attain a representative cross-section of the tissue. The samples were immersed in 0.15 M NaCl solution buffered with 5 mM HEPES to pH 7.35 (HBS-buffer) and cooled to 0–4°C during the following procedures until cell staining. The tissue samples were separately minced with a McIlwain electric tissue chopper (The Mickle Company, Gomshall, UK), which was equipped with five parallel razor blades. The chopped tissue was taken up in 5 ml HBS-buffer, sucked 50 times back and forth without bubbles using a 1 ml Eppendorf type pipette with a plastic tip whose opening was cut open to 1.5–2 mm diameter and filtered through a V2A-steel sieve with 60 µm wire distance. The cell suspension was washed twice by centrifugation in 50 ml HBS at 200 × g for 10 min. 5 µl of a dye cocktail containing 2 mg/ml propidium iodide (PI, Sigma, Deisenhofen), in DMF (dimethylformamide) to stain the DNA of dead cells and of 1 mg/ml 1.4 diacetoxy-2,3 dicyanobenzene (ADB, Paesel, Frankfurt, Germany) as pH and esterase indicators for vital

cells were added to the remaining 250 µl of fresh cell sample. ADB diffuses into cells and is cleaved by intracellular esterases into the fluorescent pH-indicator 2,3 dicyano-hydroquinone (DCH) and non fluorescent acetate. Due to its negative electric charge DCH accumulates in vital cells.

Flow cytometry

The cell volume and two fluorescences were simultaneously measured with a Fluvo II flow cytometer (HEKA- Elektronik, Lambrecht, Germany). The cell volume was determined from the change in electrical resistance as the cell passed hydrodynamically focused through the center of a cylindrical orifice (85 µm diameter, 100 µm length) at an electrical current of 0.15 mA. HBS buffer was used as sheath fluid. Fluorescence was excited by a HBO 100 W high-pressure mercury arc lamp (Osram, Munich, Germany) between 300 and 400 nm. The emitted cellular fluorescence was collected between 418–440 nm as blue fluorescence of DCH and between 500–750 nm as green fluorescence of DCH and red fluorescence of propidium iodide. The maximum amplitude of the two fluorescent signals and of the volume of each cell was digitized by 4096 step analog digital converters and the digitized values were collected in list mode on magnetic tape. The data on the tapes were evaluated by the DIAGNOS1 program system [26]. DNA aneuploidy was automatically determined by the CALC program: the DNA distribution was scanned to the left and right side of the G_0/G_1 peak and called aneuploid when an additional discrete DNA peak higher than 30% of the maximum amplitude of the G_0/G_1 peak was encountered. Shoulders of nearly DNA-euploid cancer cells were not detected by the aneuploidy subroutine. They cause, however, an abnormally high coefficient of variety (CV) of the G_0/G_1 peak and an abnormally high number of cells in the $S+G_2/M$ proliferation compartment.

Statistical analysis

The method for survival analysis was the statistical failure time model with censored data according to Kaplan and Meier [11]. The data were analyzed using the BMDP Statistical Software (Los Angeles, CA, 1983). For comparison of the functions of different populations the log-rank test was used. After a median observation interval of 48 months, follow up data were obtained from hospital or office charts and telephone calls to referring physicians or patients' families when necessary.

Results

Cells of 30 patients suffering from non-small cell lung carcinoma were examined by a multiparameter flow cytometric list mode measurement. The list mode data were analyzed by the DIAGNOS1 program system, where the data display as three dimensional cube display gave a quick overview for each cell sample and permitted a qualitative evaluation of the number of dead and vital cells and of the amount of cell debris (Figure 1A). Morphologically intact cells exhibited both a substantial cell volume signal and either an esterase activity, for vital cells, or a distinct DNA value for dead cells. Bare cell nuclei showed a DNA signal associated with a small volume signal. Enucleated cells appeared only with a volume signal in the cell debris region. The cube graph in addition indicated the distribution of the cells into small volume inflammatory lymphocytes and granulocytes and large volume epithelial and tumour cells.

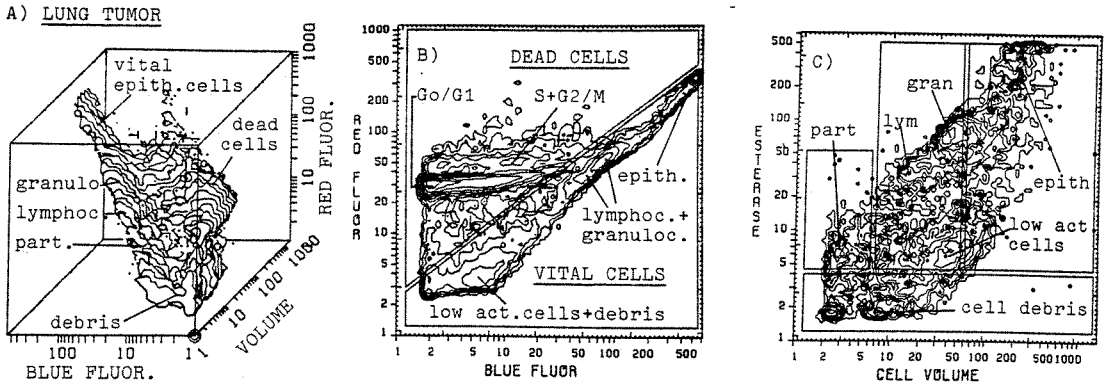

Fig. 1. A) Cloud display of ADB/PJ stained cells of a human lung carcinoma. A total of 31 279 cells and particles are contained in the graph; **B)** Projection of the cube content of Fig. 1A onto the front wall of the cube. Vital and dead cells can be separated for further quantitative evaluation in the blue versus red fluorescence histogram. **C)** Cell volume versus esterase activity of vital cells obtained from Fig. 1B by gating the list mode data on the compartment of vital cells.

During the subsequent analysis the list mode data were displayed in the blue/red fluorescence projection of the cube which allowed the distinction between dead (=propidium iodide-positive) and vital (DCH-positive) cells (Figure 1B).

The vital cells were gated out by the program and displayed in the volume versus blue fluorescence projection (Fig. 1C). The green fluorescence intensity was taken as a measure of the esterase activity and the ratio of green to red fluorescence of the vital cells as a measure of the intracellular pH, as described earlier [27, 28]. The dead cells were gated and reanalysed in the volume versus red fluorescence projection (Figure 2). The two dimensional contour plot allowed the automated discrimination between the G_0/G_1, the S, and the G_2M phases in normal lung tissue (smaller cells) and in the larger tumour cells. The DNA of the dead tumour cells was displayed as single histogram (Figure 3A,B) to detect the cell cycle distribution, DNA aneuploidy and proliferative status of the tumour cells.

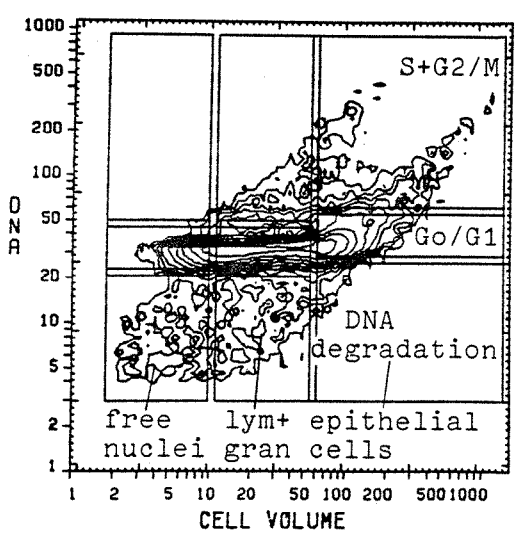

Fig. 2. Cell volume vs DNA of the dead cells. The graph is obtained by gating on the compartment of dead cells in Fig. 1B. The DNA distribution of the epithelial cells, of the inflammatory cells and of the free nuclei were evaluated in the G_0/G_1 resting phase, the $S+G_2/M$ proliferation phase and the DNA autolysis phase.

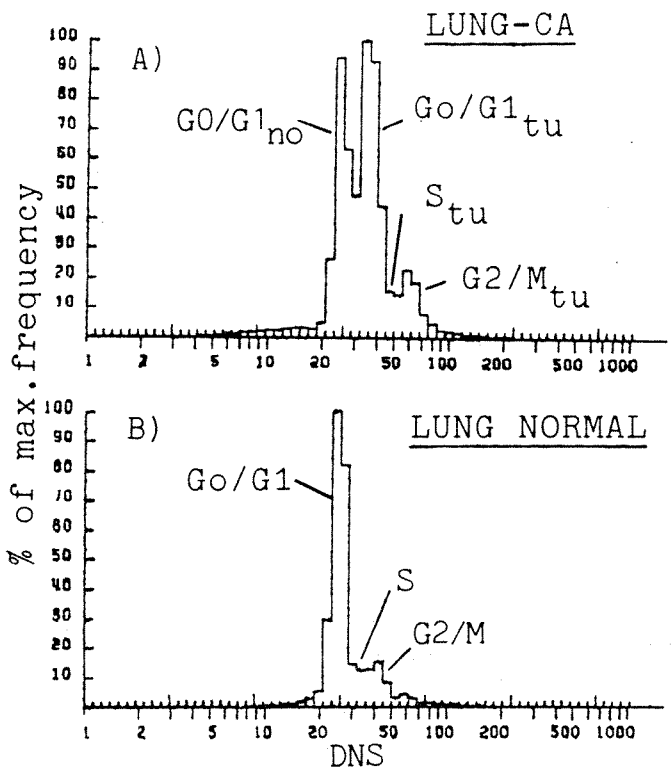

Fig. 3. Representative single histograms of the DNA distribution of non-small cell carcinoma of the lung (**A**) and of a normal control tissue (**B**). The number of cells (ordinate) is plotted against the fluorescence intensity (abscissa). Aneuploidy was quantitated with the DIAGNOSI program system [26].

A total of 50 parameters were calculated for each measurement from the two parameter histogram of Figures 1B,C, and 2. All results were subjected to a diagnostic evaluation by a multifactorial analysis with the DIAGNOSI program system.

In tumour stage I, mainly DNA euploid tumour (7 out of 8), were encountered (Table 1). DNA euploidy was frequently associated with squamous cell carcinoma of the lung (Table 2). No correlation was observed between tumour grading and the DNA ploidy status (Table 2).

17 of the 30 patients died within the observation interval of 48 months (Table 3). 11 of the 30 malignant tumours (i.e. 36.6%) showed measurable DNA aneuploidy

Table 1 Tumour stage (UICC 1987, TNM-Classification) and ploidy status of patients with non-small cell lung carcinoma

	TNM-classification					
	T1	T2	T3	N0	N1	N2
DNA euploid	4	14	3	10	6	4
DNA aneuploid	0	6	3	3	5	2
	UICC-classification					
	I	II	III	IV		
DNA euploid	7	4	6	2		
DNA aneuploid	1	3	4	3		

Table 2 Histopathological diagnosis, tissue grading and DNA ploidy of 30 patients with non-small cell lung carcinoma

	Histology			
	Squamous cell	Adeno	Large cell	Total
DNA-euploid	11	5	3	19
DNA-aneuploid	5	6	–	11
Total	16	11	3	30
	Grading			
	I	II	III	Total
DNA-euploid	4	6	9	19
DNA-aneuploid	2	8	1	11
Total	6	14	10	30

Table 3 DNA-ploidy status of patients with non-small cell carcinoma of the lung according to flow cytometric analysis after a median observation interval of 48 months

Stage	DNA-ploidy status	No. of pts. (Total)	No. of pts. (Dead)	No. of pts. (Living)
I	DNA-euploid	7	0	7
	DNA-aneuploid	1	0	1
II	DNA-euploid	4	1	3
	DNA-aneuploid	3	3	0
III	DNA-euploid	6	4	2
	DNA-aneuploid	4	4	0
IV	DNA-euploid	2	2	0
	DNA-aneuploid	3	3	0
Total	DNA-euploid	19	7	12
	DNA-aneuploid	11	10	1

(Table 3). The patients with DNA aneuploid tumours had significantly shorter survival times than those with DNA-euploid tumours (Table 4). 90% of the patients with DNA-aneuploid tumours died within the observation period, but only 37% of patients with DNA-euploid tumours (Figure 4; Table 4).

As expected, the median survival time decreased with increasing tumour stage (Table 4). Patients with DNA-euploid tumours survived, however, in all stages longer than patients with DNA-aneuploid tumours. The discrepancy in survival time between patients with DNA-aneuploid and DNA-euploid tumours was statistically significant in stage III while only a tendency was observed in stage II and IV as well (Figure 5). The only patient in stage I who developed a tumour recurrence with liver metastases had a DNA-aneuploid tumour. Median survival time for patients with

Table 4 Median survival times (MST in month) of patients with non-small cell carcinoma of the lung according to DNA ploidy after a median observation time of 48 months

Stage	DNA Euploid	SE	DNA Aneuploid	SE	Log-rank test
I	>48		>48		
II	36.00	9.8	23.00	5.0	0.3636
III	24.33	4.1	10.50	1.4	0.0071
IV	22.00	5.0	9.67	4.2	0.2769
Total	37.21	3.6	15.3	3.2	0.0007

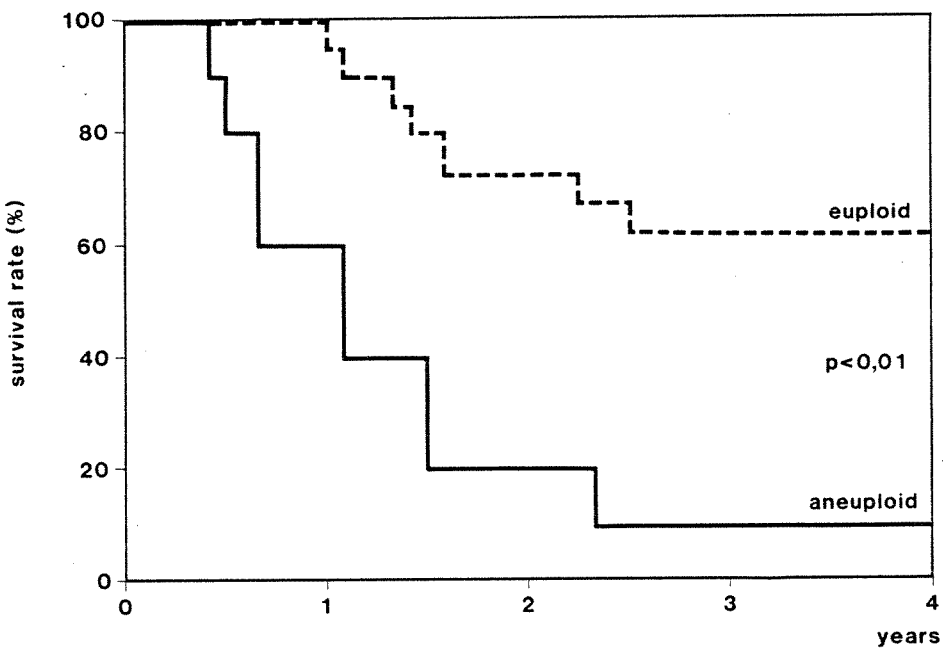

Fig. 4. Actuarial survival of all patients with non-small cell carcinoma of the lung regardless of their clinical stage according to the DNA ploidy level.

DNA-aneuploid tumours was 15 months, while it was 37 months for patients with DNA-euploid tumours.

Concerning the functional cell parameters (Table 5), tumour samples contained fewer vital cells but more vital epithelial cells than normal lung tissue. The cell volume, esterase activity and esterase activity concentration of tumour cells was lower than in normal tissue. The intracellular pH was slightly elevated in malignant cells. The cell volume, esterase activity concentration and intracellular pH were elevated in inflammatory cells.

Differences existed also between DNA-euploid and DNA-aneuploid tumour cells. Living DNA aneuploid tumour cells were significantly smaller (by about 20%) than DNA euploid tumour cells (Table 6). The intracellular pH in the vital DNA aneuploid tumour cells was by about 0.1 pH units more acidic than in the DNA euploid tumour cells. Furthermore a lower esterase activity was detectable in the DNA aneuploid cells.

Differences of functional cell parameters were also observed between surviving ($n=13$) and deceased patients ($n=17$) for epithelial cells such as a lower pH value

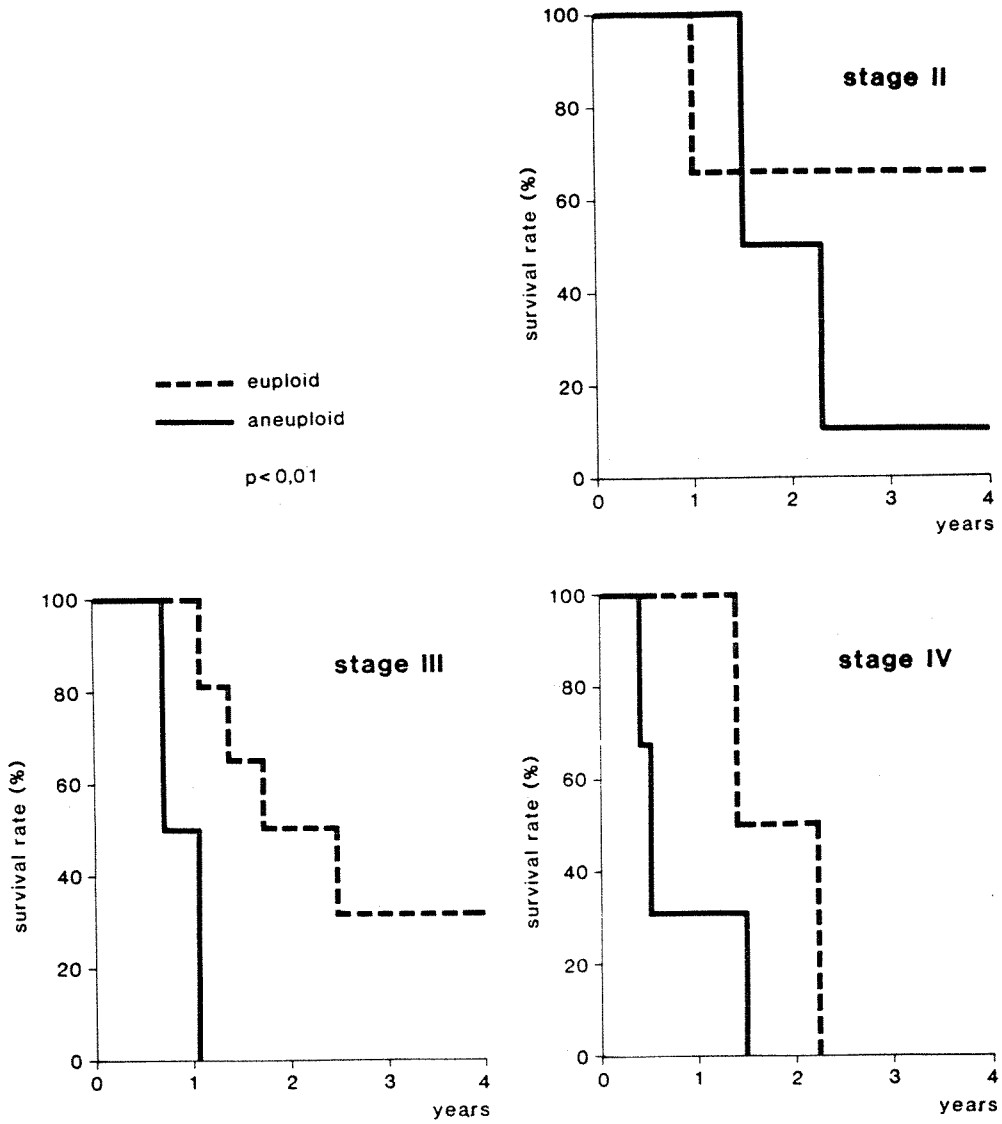

Fig. 5. Survival of stage II, III and IV patients with non-small cell carcinoma of the lung according to the DNA ploidy level.

of 7.28 ± 0.08 (SEM) versus 7.53 ± 0.06 and a smaller cell volume of 2.91 ± 0.28 as compared to 3.40 ± 0.32, but these parameters did not contain significant prognostic information. Other parameters such as esterase activity of epithelial cells and percentage of dead cells of all inflammatory and epithelial cells were not significantly different between surviving and deceased patients.

Discussion

Non-small cell lung carcinoma (NSCLC) consists of a mixed group of tumours with distinct but overlapping histologic properties, clinical course and biologic behaviour. Surgery is the accepted mode of treatment in this combined group of tumours.

It is desirable to improve the treatment of NSCLC by the early determination of discriminatory prognostic factors [31]. Accurate preoperative information about the

Table 5 Mean ± SEM values of cell biochemical parameters in normal lung tissue and lung cancer (Au = arbitrary units)

	Tumor $n=30$	Normal $n=21$	$P<$ [a]
Vital cells			
% of all cells	31.0 ± 2.7	47.0 ± 3.7	0.01
% vital inflam. cells	80.4 ± 2.8	86.2 ± 2.1	0.1
% vital epith. cells	19.6 ± 2.8	13.8 ± 2.1	0.1
Dead cells			
% dead of all inflam. cells	67.4 ± 3.1	52.6 ± 4.1	0.05
% dead of all epith. cells	70.7 ± 3.8	56.1 ± 3.9	0.05
Epithelial cells			
Cell volume (Au)	3.01 ± 0.16	3.49 ± 0.18	0.1
Esterase activity (Au)	0.71 ± 0.09	1.13 ± 0.19	0.05
Esterase concentration (Au)	0.24 ± 0.03	0.31 ± 0.05	0.1
pH	7.48 ± 0.04	7.43 ± 0.04	0.05
% $S+G_2/M$	26.5 ± 2.8	31.9 ± 3.8	0.1
% debris	17.6 ± 3.2	16.8 ± 5.5	1.0
Inflammatory cells			
Cell volume (Au)	0.58 ± 0.02	0.47 ± 0.03	0.05
Esterase activity (Au)	0.23 ± 0.03	0.20 ± 0.02	0.5
Esterase concentration (Au)	0.51 ± 0.11	0.52 ± 0.06	1.0
pH	7.42 ± 0.05	7.34 ± 0.04	0.1
% $S+G_2/M$	23.2 ± 3.2	26.2 ± 2.8	0.1
% debris	10.9 ± 2.2	8.2 ± 2.8	0.1
Free nuclei			
% of all DNA contain. part.	32.9 ± 3.8	48.3 ± 3.4	0.05

[a] *P*: Student's *t*-test.

Table 6 Functional cell parameters of vital cells in DNA-euploid and DNA-aneuploid tumours (mean ± SEM)

	DNA-euploid ($n=30$)	DNA-aneuploid	$P<$
Cell volume epith. (AU)	3.29 ± 0.29	2.74 ± 0.22	0.05
Intracellul. pH epith.	7.45 ± 0.06	7.26 ± 0.11	0.01
Esterase activ. epith. (AU)	0.76 ± 0.13	0.63 ± 0.15	0.05

local and metastatic extension of the tumour and the histopathologic type of the tumour for staging and grading are essential for the decision whether surgery alone is the most appropriate treatment for the patients or whether they should have additional chemotherapy.

Chest x-ray, CT scan, mediastinal tomography, bronchoscopy and mediastinoscopy are generally accepted methods for preoperative staging. Sensitivity/specificity levels of diagnosis range between 50 and 80/86% for mediastinal tomography [5, 10, 17, 20] and for CT-scan [8, 9, 15, 21]. Sensitivity levels between 71 and 90% have been reported for mediastinoscopy [8, 18, 20, 24, 33] concerning detection of media-

stinal lymph node involvement. Tumour-staging is clinically particularly important for the application of chemotherapy protocols.

The cell and tissue grading information is estimated less important for clinical decisions since the majority of samples is classified as grade II tumours (Table 2).

Chemotherapy has been used in inoperable stage III tumours to reduce tumour mass prior to surgical intervention [13, 14]. Martini et al. [13] defined a group of patients with enlarged mediastinal lymph nodes evident on plain chest x-ray or with a marked widening of the carina at bronchoscopy. These patients benefited significantly from preoperative chemotherapy. The resectability rate was increased from 14 to 75% and 3 and 5 year survival rate was prolonged from 9% to 47 and 34% of these patients.

Beside the above mentioned preoperative staging techniques, the determination of additional subgrouping factors such as biochemical differences and the DNA ploidy status of a tumour could further improve the criteria for application of preoperative chemotherapy in stage III patients with N2 lymph node involvement. The results of this study show, interestingly, that DNA ploidy, in contrast to its limited value for tumour diagnosis [12] is the only significant prognostic parameter for survival amongst the flow cytometrically determined parameters (Table 4; Figure 4). DNA-euploid tumour patients survived significantly longer than patients with DNA-aneuploid tumours. This tendency was observed in all tumour stages; significantly, however, only in the total collective and in tumour stage III (Figure 5). Stage III patients with DNA-euploid tumours had an increased median survival time of 24.3 months versus patients with DNA-aneuploid tumours where the median survival time was only 10.5 months ($P < 0.01$) (Table 4).

The results demonstrate that stage III tumours cannot be regarded as a homogeneous group and that patients with stage III tumours can be classified according to DNA-ploidy into significantly different groups with respect to prognosis. This is in accordance with the results of other authors [31, 32, 34]. Volm et al. [31] observed that most of the patients with DNA-aneuploid tumours had stage III disease. It seems of interest in our study that DNA-euploidy was more frequently found in squamous cell carcinoma (69%) than in adenocarcinoma (45%). The incidence of DNA-aneuploidy in other studies was 40–85% [1, 6, 16, 19, 23] which is higher than the 36.6% of patients in our study. A reason for this difference may be the use of fresh cell preparations while other groups digest cellular proteins by pepsin prior to DNA staining. Enzymatic digestion is, however, incompatible with the determination of esterase activity and intracellular pH in vital cells and since the determination of these parameters was of a conceptual interest for this study, the disadvantage of higher coefficients of variation in the DNA distributions, which were in the order of 6–11% on a relinearised DNA scale was accepted.

An important problem in the determination of the DNA-ploidy status by flow cytometry is the heterogeneity of the solid tumours [25, 30]. The variability can be minimized by sampling the specimens from different locations of the tumour. Thus tumour heterogeneity can be detected more accurately by flow cytometry than by image analysis [4].

Several functional cell parameters are significantly different between lung cancer and normal lung tissue (Table 3). In the areas of cancerous lesions, for example, the frequency of inflammatory cells and also the cell volume of the inflammatory cells were significantly increased. The greater cell volume may represent a higher biological

activity in the course of the digestion of necrotic material. Samples from cancerous areas also contain a higher degree of dead cells and cell debris. These differences are helpful for diagnostic purposes [11] and could improve multiparameter analyses in the use of automated cytology [2].

There are also functional differences between vital cells of DNA-euploid and DNA-aneuploid tumour cells (Table 6). DNA-aneuploid tumour cells have a smaller cell volume than DNA-euploid cells. The larger DNA-euploid cells are more alkaline in pH-value than DNA-euploid cells, which may reflect a higher metabolic activity. The functional parameters do not, however, contain significant prognostic information concerning the survival of patients. It seems, therefore, of interest to check other cell biochemical parameters such as antigen expression [29] enzymatic activities [3] or oxido-reductive balance [22] for their potential prognostic values.

Clinically it seems particularly important to focus on biochemical tumour characteristics which predict the response of DNA-aneuploid stage III tumours with N2 lymph node involvement to preoperative chemotherapy more precisely than by staging and DNA parameters alone. This would further improve the results of tumour resection and survival of patients.

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