Prognostic value of deoxyribonucleic acid aneuploidy in primary non-small-cell lung carcinomas and their metastases

The ploidy status of the deoxyribonucleic acid of a malignant lung tumor provides additional information besides histologic grading and tumor staging according to lymph node infiltration and tumor metastasis. Ninety-nine surgical specimens from patients with non-small-cell lung carcinoma were investigated by flow cytometry. Deoxyribonucleic acid aneuploidy was found in 48 % of the primary tumors. Patients with deoxyribonucleic acid–euploid tumors showed better survival ($p < 0.01$) than those with deoxyribonucleic acid–aneuploid carcinomas independent of tumor stage. Deoxyribonucleic acid ploidy status of the primary tumor was compared with that of N2 lymph node metastases in 29 cases. Seven samples showed a change from deoxyribonucleic acid aneuploidy in the primary tumor to deoxyribonucleic acid euploidy in the lymph node metastases. Survival was significantly better for patients with euploid primary tumors and lymph node metastases, followed by patients with deoxyribonucleic acid–aneuploid primary tumors and euploid lymph node metastases. Survival was poorest in patients with deoxyribonucleic acid–aneuploid primary tumors and lymph node metastases. It was observed that only the simultaneous determination of deoxyribonucleic acid ploidy of primary tumors and lymph node metastases permits accurate prognostic evaluation in case of lymph node infiltration. (J THORAC CARDIOVASC SURG 1992;104:1476-82)

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The pathoanatomic classification of lung carcinomas has largely remained unchanged in recent decades. Evaluation is based on staging of the tumor according to the TNM classification and International Union Against Cancer (UICC) classification and on grading by histologic evaluation of the degree of tissue differentiation.[1] These classifications do not, however, consider the variable biologic behavior and biochemical characteristics of the tumor and, therefore, are only of limited value for prognosis. It is desirable to find further variables to characterize the biologic aggressiveness of the malignant cells to determine the individual prognosis of each patient.[2] Quantitative flow cytometric deoxyribonucleic acid (DNA) analysis as a measure of the total DNA content of tumor cells provides useful information on the degree of chromosomal instability in human cancers.[3]

The ploidy status from different solid tumors has been investigated in numerous studies,[3-5] but not much is known about DNA distribution in lung carcinoma. It was the purpose of this study to measure the DNA content and the ploidy status of non-small-cell lung carcinomas (NSCLC) and to establish its prognostic value in a long-term follow-up investigation. The DNA content was investigated in a significant number of N2 lymph node metastases and compared with that in the respective primary tumor.

Patients and methods

Patients. The study comprised 99 patients (38 to 84 years, mean age 61) with NSCLC determined from operative specimens. Seventy fresh tissue specimens were taken immediately after operation. An additional 29 specimens from different

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Table I. *Tumor stage (UICC 1987, TNM classification) and DNA ploidy status of patients with NSCLC*

TNM classification	T1	T2	T3	N0	N1	N2	M0	M1
DNA euploid	8	32	10	19	15	17	49	2
DNA aneuploid	4	32	6	12	14	22	44	4

UICC classification	I	II	III	IV
DNA euploid	17	10	22	2
DNA aneuploid	8	8	28	4

Table II. *Histopathologic diagnosis and DNA ploidy status of 99 patients with NSCLC*

	Squamous cell carcinoma	*Adeno-carcinoma*	*Large-cell carcinoma*	*Total*
DNA euploid	24	21	6	51
DNA aneuploid	24	20	4	48
Total	48	41	10	99

Table III. *Grading and DNA ploidy status in 99 patients with NSCLC*

	I	*II*	*III*
DNA euploid	5	27	19
DNA aneuploid	5	25	18
Total	10	52	37

patients were investigated from paraffin-fixed material to increase the number of cases with N2 lymph node infiltration. Histologic sections were used to determine whether the tumor cell population of the material was higher than 30%.

Clinical staging of the tumors was based on the criteria of the UICC (1987). The patients' disease was staged at the time of operation (Table I). The histopathologic diagnoses comprised squamous cell carcinoma, adenocarcinoma, and large-cell carcinoma (Table II). The histologic classification of the tumors was assessed according to the grading system in Table III. Histopathologic grading of the tumors was performed according to the World Health Organization grading system.[6]

In all patients with tumor stage I, II, and III disease, curative resection was undertaken with the intent of leaving no macroscopic residual tumor behind. All patients with stage III disease whose tumor could not be totally removed were excluded from the study. A total of 23 pneumonectomies and 76 lobectomies were performed together with mediastinal lymphadenectomy.

Cell-fresh tissue. Approximately 0.25 gm of fresh tissue was removed from the lung cancer tissue. The specimens were taken from three different locations (peripheral, middle, and central) in the tumor to receive a representative cross section of the tissue. The samples were immersed in a 0.15 mol/L NaCl solution buffered with HEPES 5 mmol/L (HBS buffer) to pH 7.5 and cooled to 0° to 4° C during the following procedures until cell staining. The tissue samples were minced separately with a McIlwain electric tissue chopper (The Mickle Co., Gomschall, England), which was equipped with five parallel razor blades. The chopped tissue was immersed in 5 ml HBS buffer and filtered through a V2A steel sieve with 60 μm wire mesh. The cell suspension was washed twice by centrifugation in 50 ml HBS buffer at a specific gravity of 200 for 10 minutes.

Cell-fixed tissue. The preparation of a cell nuclei suspension of the material from 29 primary tumors and from their corresponding N2 lymph node metastases embedded in paraffin was done according to a modified method as described by Hedley and colleagues.[7] Both the cell nuclei suspension and the fresh total cell suspension were stained with 5 μl propidium iodide (2 mg/ml in HBS-buffer, Sigma Chemie, Deisenhofen, Germany) per 250 μl cell sample.

Flow cytometry. A Fluvo-Metricell flow cytometer[8] (Heka-Electronic Lambrecht, Pfalz, Germany) was used for simultaneous determination of the red propidium iodide fluorescence and cell nuclear volume. Monodispersed latex particles of 5 μm diameter (Paesel & Lorei GmbH & Co., Frankfurt, Germany) to which fluorescein isothiocyanate was covalently bound served as the internal volume and fluorescence standard in the measurement. The coefficient of variation (standard deviation/mean particle volume, 100%) for particle fluorescence and volume was between 1.8% and 2.2%.

An HBO-100 mercury high-pressure mercury arc lamp was used to excite the fluorescence between 400 and 500 nm by epi-illumination. The cellular fluorescence was measured between 550 nm and 770 nm with a photomultiplier tube. The maximum height of the logarithmically amplified volume and linearly amplified fluorescent signal of each cell nucleus was digitized with a resolution of 4096 channels and transferred to magnetic tape. The evaluation of the measurement was done with computer programs that had been developed previously. They allowed the measurements to be represented as monoparametric histograms.[9-11]

Computer-based analysis with the DIAGNOS1 program[12] (Valet, Max Planck Institute, Martinsried, Germany) permitted automatic scanning to the left and right of the G_0/G_1 peak within the DNA distribution curve (Fig. 1). A sample was considered to be DNA-aneuploid if a second DNA peak was detected whose height was at least 25% of the maximum amplitude of the G_0/G_1 peak and which showed a DNA index greater than 1.10. The DNA index was defined as the quotient between the G_0/G_1 peak of normal epithelial and inflammatory cells and the G_0/G_1 phase of the tumor sample.

Euploid tumors had a DNA index of 1.0, whereas DNA-

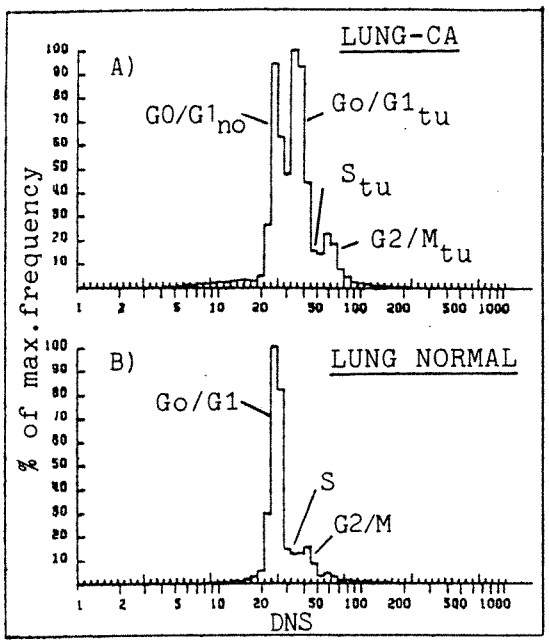

Fig. 1. Representative single histograms of DNA distribution of NSCLC (**A**) and of normal control tissue (**B**). *CA*, cancerous; *no*, normal; *tu*, tumor; *DNS*, deoxyribonucleic acid.

aneuploid tumors had an index less than 0.8 or greater than 1.10. DNA peaks that could not be defined according to the stated criteria and were similar to DNA-euploid cells were not rated as being DNA aneuploid. These particular samples, however, showed an abnormally high coefficient of variation in the G_0/G_1 peak or a high number of cells in the S and G_2/M proliferation window. Normal lung and lymph node tissues were used as reference samples.

Statistical analysis. After a median observation interval of 52 months, follow-up data were derived from hospital or office charts and telephone calls to outside physicians. Survival analysis was obtained according to Kaplan and Meier.[13]

A stepwise regression analysis was used to test the influence of certain independent clinical and pathologic variables (sex, operation, histologic type, tumor stage, grading) and the ploidy status of the tumor. The effect of these variables on survival was studied with the proportional hazard regression model of Cox.[14]. Differences of variable distribution between euploid and aneuploid tumors were examined with regard to statistical significance with Pearson's χ^2 test.

Results

A total of 99 patients with NSCLC were examined by flow cytometry and the data were analyzed by the DIAGNOS1 program system. Of the 99 specimens forty-eight showed an additional DNA peak representing an expression of the G_0/G_1 peak of the carcinoma and were classified as DNA aneuploid.

Mainly euploid tumors (17/25) were present in tumor stage I disease (Table I). The DNA ploidy status was not

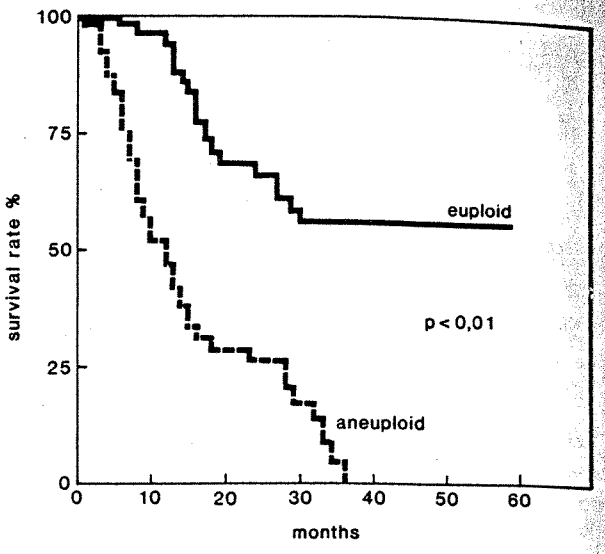

Fig. 2. Actuarial survival of all patients with NSCLC (by Kaplan-Meier method) regardless of clinical stage according to DNA ploidy level.

associated with a particular histologic type. DNA euploidy was found in 24 of 48 cases of squamous-cell carcinoma, 21 of 41 cases of adenocarcinoma, and 6 of 10 cases of large-cell carcinoma (Table II). No correlation could be found between tumor grading and DNA ploidy status ($p < 0.99$, Table III).

Sixty-two of the 99 patients died within the observation interval of 52 months (Table IV). The patients with DNA-aneuploid tumors showed significantly shorter survival times than those with DNA-euploid tumors. The median survival time for patients with DNA-aneuploid tumors was 12.1 months, whereas the survival time was 33.8 months for patients with euploid tumors ($p < 0.01$). Of the patients with DNA-aneuploid tumors, 87.5% died within the observation interval, but only 39.2% of patients with DNA-euploid tumors died during this time ($p < 0.01$, Fig. 2, Table IV).

The prognostic value of the DNA ploidy status was independent from tumor stage. Patients with DNA-euploid tumors survived longer in all stages than patients with DNA-aneuploid tumors (Fig. 3, Table V).

To define a uniform subgroup, we investigated 29 primary tumors and their corresponding tumor-infiltrated N2 lymph nodes in patients with stage III disease from paraffin-fixed material. There were 18 primary tumors with DNA euploidy and 11 with DNA aneuploidy. Twenty-five metastatic lymph nodes showed euploidy as opposed to four with aneuploidy. In seven patients the DNA ploidy status of the primary tumor and of the lymph node metastasis differed in that the primary tumor

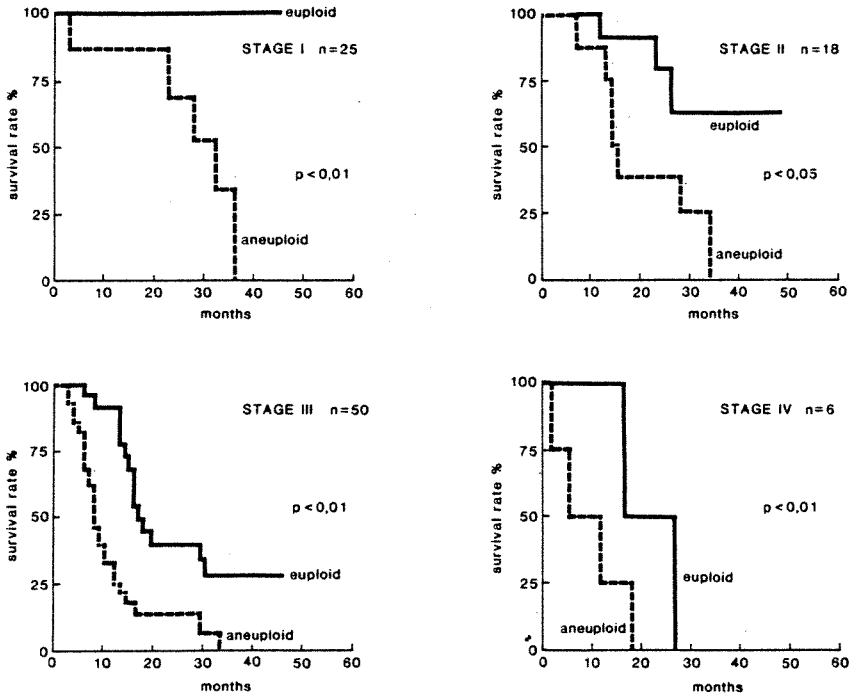

Fig. 3. Actuarial survival (Kaplan-Meier) of patients with NSCLC according to stages I through IV and DNA ploidy level.

Table IV. *Survival and DNA ploidy status of patients with NSCLC*

Stage	*DNA ploidy status*	*No. of patients*		
		Total	*Dead*	*Alive*
I	DNA euploid	17	—	17
	DNA aneuploid	8	5	3
II	DNA euploid	10	3	7
	DNA aneuploid	8	8	—
III	DNA euploid	22	15	7
	DNA aneuploid	28	26	2
IV	DNA euploid	2	2	—
	DNA aneuploid	4	4	—
Total patients	DNA euploid	51	20	31
	DNA aneuploid	48	43	5

Table V. *Median survival times (in months) of patients with NSCLC according to DNA ploidy status*

Stage	*DNA euploid*	*DNA aneuploid*	*Log rank test*
I	All alive	25.5	$p < 0.01$
II	>36	14.5	$p < 0.05$
III	16.5	8.0	$p < 0.01$
IV	22.0	8.5	$p < 0.01$

displayed DNA aneuploidy while the metastasis was DNA euploid. The best survival prognosis was found in those patients whose primary tumor and lymph node metastasis were both DNA euploid (median survival rate, 17.0 months), followed by patients whose primary tumor was aneuploid and the lymph node metastasis euploid (median survival rate, 9.0 months). Patients with DNA-aneuploid primary tumors and metastases had the worst

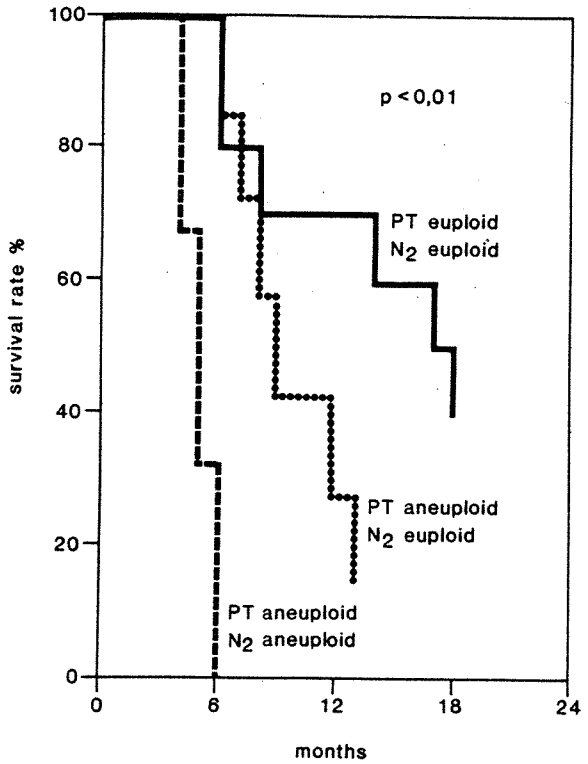

Fig. 4. Survival of 29 patients with NSCLC and N2 lymph node metastases according to DNA ploidy status. *PT*, Primary tumor.

prognosis with a median survival rate of 5.0 months (Fig. 4). The multivariate regression analysis showed that survival depended mostly on tumor stage and ploidy status and less on histologic type, grade of differentiation, or type of operation (Table VI).

Table VI. *Survival and clinical/histopathologic parameters (proportional hazard model according to Cox)*

Variable	*Coefficient of regression*	*SE*	p *Value*
DNA ploidy status	1.69	0.30	<0.001, S
Tumor stage	1.22	0.24	<0.001, S
Sex	0.91	0.40	<0.01, S
Histologic type	0.19	0.23	<0.29, NS
Grading	0.20	0.22	<0.35, NS
Operation	0.28	0.34	<0.68, NS

SE, Standard error; *S*, significant; *NS*, not significant.

Discussion

The preoperative and postoperative therapeutic management of patients with lung cancer depends greatly on the accurate assessment of prognosis. Variables such as tumor infiltration, lymph node involvement, histologic type, grading, and host performance status influence the outcome after surgical resection, but do not account for all the variations in survival observed in this patient group.[15]

An additional parameter for prognosis is the DNA ploidy status of a tumor. A DNA histogram of normal tissue contains a prominent cell peak of diploid DNA content representing the G_0/G_1 phase, that is, the resting phase of the cell cycle. A second smaller peak of tetraploid DNA in the same normal histogram depicts the proliferative phase of the cell cycle (Fig. 1).

Chromosomal aberrations caused by disturbances during the process of cell division are assumed to initiate the development of DNA-aneuploid tumors.[16-20] Loss of chromosomes and endomitotic polyploidization are depicted by additional aneuploid peaks within the histogram (Fig. 1).

The different DNA values apparently reflect the varying biologic behavior and aggressiveness of a tumor.[21] Several authors have measured the DNA content in colorectal, ovarian, bladder, and breast cancer.[22-25] In most of the tissues studied so far, DNA aneuploidy is correlated with poor prognosis.

In the present 4-year follow-up study we were able to show that DNA ploidy has a significant prognostic value in NSCLC. In all tumor stages, patients with DNA-euploid lung carcinomas had a significantly better survival than patients with DNA-aneuploid tumors (Table V, Fig. 3, $p < 0.01$). In the group with stage I disease no patient with a DNA-euploid tumor died. There were also significant differences in survival between patients with euploid and aneuploid tumors in stages II and III ($p < 0.05$ and $p < 0.01$, respectively).

In contrast to the results in a study by Ojala and co-workers,[26] patients with metastatic disease (stage IV) who had palliative resection showed different survival patterns when they were grouped according to the DNA euploidy and DNA aneuploidy of the tumors.

Hedley and associates[7] developed a method for the analysis of the cellular DNA content of paraffin-embedded pathologic material. This method permits the retrospective study of archived specimens. However, single-cell necrosis caused by fixation of the tissue may lead to some difficulty in interpreting the results. In such cases the DNA histogram shows wide peaks with a high coefficient of variation, thus the investigator may miss hidden aneuploid DNA peaks. However, when the results of DNA histograms of fixed specimens are compared with fresh samples of the same tumor they are identical in 94% of all cases.[27-29]

The DNA distribution of 29 primary tumors and their N2 lymph node metastases was investigated to obtain additional information that may aid in the surgeon's decision concerning whether surgical treatment alone or additional chemotherapy should be applied in such cases.

Martini and associates[30, 31] defined a group of patients with enlarged mediastinal lymph nodes on the plain chest x-ray film or with a marked widening of the carina at bronchoscopic study. With preoperative chemotherapy in this group of patients these investigators could increase the resectability rate from 14% to 75% and prolong 3- and 5-year survivals from 9% to 47% and 34%, respectively. We therefore investigated this subgroup of patients with N2 lymph node infiltration to find additional criteria for the selection of patients who could profit from adjuvant chemotherapy. We are currently beginning a therapeutic study with the aim to correlate the tumor response of chemotherapy in patients with stage III and IV disease who have DNA ploidy of the tumor cells.

In 7 of 29 cases we investigated, the ploidy status differed in the primary tumor and the lymph node metastasis. In all seven cases the primary tumor displayed DNA aneuploidy while the corresponding metastases were DNA euploid. The large variation between primary tumors and lymph node metastases with regard to ploidy status indicates that the flow cytometric analysis of lymph nodes alone gives only limited information about the primary tumors.[32] The simultaneous measurement of ploidy from both the primary tumor and lymph node metastasis permits an additional prognostic evaluation. In our study, patients with DNA-euploid primary tumors and metastasis lived significantly longer than patients with DNA-aneuploid primary tumors and DNA-euploid metastasis, followed by patients with DNA-aneuploid primary tumors and lymph node metastases (Fig. 4).

In the regression analysis, the DNA ploidy status was revealed as an independent prognostic parameter in view of survival (Table VI). The correlation of DNA ploidy with survival was even higher than for histologic tumor type and tumor grading. The DNA ploidy status of a bronchial carcinoma and the tumor stage had the same prognostic value. Surgical treatment, histologic classification, grading, and tumor stage were independent variables not associated with the DNA ploidy status (Tables I, II, and III).

An important problem in the determination of the DNA ploidy status by flow cytometry is the heterogeneity of the solid tumors. Olszewsky,[33] Frankfurt,[4] Aver,[34] Feichter,[35] and their associates demonstrated a high conformity of the DNA content of the primary tumor and lymph node metastasis. In our study the DNA ploidy status in the primary tumor and the metastases varied in nearly 25% of the cases. The fact that no DNA-aneuploid metastases were found from euploid primary tumors may be an indicator of a more pronounced heterogeneity in DNA-aneuploid primary tumors. For this reason the biologic variability within the individual samples was minimized by sampling the specimens from different locations of the tumor.

In conclusion, the determination of DNA ploidy status of lung cancer offers additional and independent prognostic information on survival. In cases of lymph node infiltration, only the simultaneous measurement of the DNA ploidy of the primary tumor and lymph node metastasis offers an accurate prognostic evaluation. A precondition, therefore, is the sampling of specimens from various locations of the tumor because of the heterogeneity of the cancerous lesion.

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