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A M E R I C A N C O L L E G E O F
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Flow Cytometric Analysis and Cytokeratin Typing of Human Lung Tumors*

A Preliminary Study

Alexander Guber, M.D.; Razia Cohen, M.Sc.;
Ronni Ronah; Israel Zan-Bar, Ph.D.; Benjamin Geiger, Ph.D.; and
Israel Bruderman, M.D., F.C.C.P.

In the current study a comparative analysis of keratin typing and DNA content was carried out in human lung tumors from transthoracic fine needle aspiration biopsies (TFNAB) (18 patients) or from surgically resected tumor tissues (14 patients). According to the cytologic and histologic features, 2 of the 32 tumors were diagnosed as benign tumors, 11 as squamous cell carcinomas, 12 as adenocarcinomas, and 7 as undifferentiated large cell carcinomas. Two cases in the adenocarcinoma and one in the undifferentiated large cell carcinoma groups were pulmonary metastasis or second primary tumors. Malignant cells of tumors which reacted positively with KK8.60 anticytokeratin polypeptides No. 10 and 11 (and hence contain keratinizing cells) displayed

diploid DNA content in a flow cytometric assay regardless of their cytologic or histologic appearance. In contrast, all tumors which lacked such positive cells (most of which were defined as adenocarcinomas and undifferentiated tumors) were hyperdiploid. The close correlation between high DNA content and both malignancy and the absence of advanced squamous differentiation (keratinization) suggests that such combined analysis may provide new tools for the cytologic diagnosis and prognosis of lung cancers.

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TFNAB = transthoracic fine needle aspiration biopsy;
MoAb monoclonal antibody; DI = DNA index

The diagnosis and classification of human tumors comprise crucial steps for an accurate prognosis and selection of optimal therapeutic procedures.

For lung tumors, a common diagnostic approach is the cytologic examination of transthoracic fine needle aspiration biopsy (TFNAB) specimens.¹⁻⁵ While this examination often provides valuable information on the nature of the tumor, it is often handicapped by limited morphology of distinct cellular structures and the inherent absence of distinctive histotypic-morphologic features.^{6,7}

A more precise differential diagnosis of TFNAB specimens may thus be obtained by specific immunocytologic examination of such tumors using a variety of markers such as cytokeratin polypeptides as described in our previous study.⁸ The results of that study indicated that a significant proportion of lung tumors originally diagnosed by cytology as squamous cell carcinomas, are in fact, adenocarcinomas or mixed adeno-squamous tumors. Thus, cytokeratin typing, using various monoclonal antibodies (MoAb), provides more precise information on the origin of tumors than currently attainable by cytologic examination only.

In an attempt to obtain additional information on TFNAB samples of lung tumors, which may bear on their biological properties, we have carried out a comparative analysis of keratin immunocytologic typing and DNA content analysis in the same specimens. This approach was based on the rationale that the clinical and biological behavior of tumors (including degree of malignancy and aggressiveness) may be related to the state of proliferation of the tumor cells. Flow cytometric analysis of propidium-iodide stained nuclei provides quantitative information on cell ploidy and the percentage of cells in the different phases of the mitotic cycle.^{9,10} The reported results suggest that in contrast to benign tumors and normal tissues, malignant cells, especially adenocarcinomas and undifferentiated tumors, contain significantly higher levels of DNA.¹¹⁻¹⁴

MATERIALS AND METHODS

The diagnosis of lung cancer (total 32 patients) was made either from TFNAB material (18 patients), or from surgically resected tumor tissue (14 patients). According to the cytologic and histologic features, 2 of the tumors were diagnosed as benign tumors, 11 as squamous cell carcinomas, 12 as adenocarcinomas, and 7 as undifferentiated large cell carcinomas. Two cases from the adenocarcinoma group and one from the undifferentiated large cell carcinoma group were pulmonary metastasis or second primary tumors. Of the 32 patients, 29 were men and 3 women with an average age of 62.6 years (Table 1).

Sample Collection and Preparation

Surgical specimens of the tumor tissue were snap-frozen in liquid nitrogen-cooled isopentane and kept at -70°C. Single cell suspensions of tumor tissues were obtained by mincing the specimen with scissors and forcing it through wire mesh.¹⁵ Cells were maintained in RPMI 1640 medium (Biolab, Jerusalem, Israel). To

*From the Chest Department, Sapir Medical Center, Meir General Hospital, Kfar Saba (Drs. Guber and Bruderman, Messrs Cohen and Ronah); the Department of Human Microbiology, Sackler School of Medicine, Tel-Aviv University, Tel-Aviv (Dr. Zan-Bar); and the Department of Chemical Immunology, the Weizmann Institute of Science, Rehovot, Israel (Dr. Geiger). Dr. Geiger is the recipient of the Erwin Neter Chair in Tumor and Cell Biology.

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Reprint requests: Dr. Guber, Sapir Medical Center, Meir General Hospital, Kfar Saba, Israel 44281

Table 1—Demographic Characteristics of Lung Cancer Patients*

Diagnosis	Tissue/TFNAB	No.	Age, yr (r)	M/F
SQ	7/4	11	64.0 ± 16.7 (44-83)	9/2
AD	2/10	12	65.7 ± 7.2 (57-76)	12/0
UND	3/4	7	64.0 ± 14.6 (40-80)	6/1
Benign	2/0	2	61.0 ± 19.8 (47-75)	2/0
Total	14/18	32	62.6 ± 13.1 (40-89)	29/3

*AD, adenocarcinoma; SQ, squamous cell carcinoma; UND, undifferentiated carcinoma; (r), range.

obtain a true representation of the tumor, relatively large segments of tissue were processed.

The TFNAB was done using a 5-inch, 22-gauge needle. Again to obtain a true representation of the tumor cells, the needle was moved inside the tumor through the depth several times before taking it out, enabling multiple sampling. The majority of cells recovered from the needle were used for Papanicolaou staining¹⁶ and light microscopic evaluation while the rest were suspended in RPMI 1640 medium and used for DNA analysis or keratin typing.

DNA Analysis

Propidium-iodide staining of nuclei was carried out according to the procedure developed by Vindelov et al,¹⁷ and the fluorescence of about 10,000 nuclei was measured per specimen by flow cytometer (FACS 440, Becton Dickinson). The data were analyzed according to the method described by Dean.¹⁸ As normal diploid control specimens, human lymphocytes obtained from normal donors were used. The DNA index (DI) for each specimen was calculated from the ratio of the mean value of G_0/G_1 peak in the patient sample and in the control diploid cells. Diploid tumor populations were defined as having a single G_0/G_1 peak at the same range as the G_0/G_1 peak for the control cells, $DI = 1.0 \pm 0.1$ (0.1 is twice the standard deviation of the mean G_0/G_1 peak of the control diploid lymphocytes). Tumors were considered hyperdiploid if there was a G_0/G_1 peak with DI value higher than 1.1.

Cell Cycle Analysis

Cell cycle analysis was performed for both the diploid and hyperdiploid tumors. The percentage of normal stromal and infiltrating cells mixed in the diploid peaks was estimated by Papanicolaou

staining of the same cell suspensions. Assuming that these normal cells were all diploid, the percentage of tumor cells in each phase of the cell cycle was recalculated.

Cytokeratin Typing

Cytokeratin typing was performed using immunofluorescence microscopy according to the method previously described.⁸ The anticytokeratin MoAb used in this study was KK8.60 (Sigma USA), which specifically reacts with human cytokeratins No. 10 and 11, and is a specific marker for keratinizing epithelia such as well differentiated squamous cell carcinomas.^{19,20}

Statistical Analysis

The differences in DI values between the KK8.60 positive and negative groups were analyzed by unpaired Student's *t* test.

RESULTS

Flow-Cytometric Analysis of DNA Content in Tumor Cells

The basis for the results presented here was flow cytometric analysis of DNA content in cells derived from TFNAB samples or frozen solid tumors. To obtain an accurate estimate of the diploid and hyperdiploid cell populations, we have examined the DNA content in normal human lymphocytes and cytologically determined the percentage of normal (presumably diploid) stromal and infiltrating cells within each tumor sample. As shown in Figure 1A, normal lymphocytes had a major peak (91 percent of the cells) with DI value of 1.0 and a minor peak of cells with roughly double the amount of DNA. We propose that the former peak represents diploid cells at the G_0/G_1 phase of their cycle while the second corresponds to diploid cells fixed at the G_2/M stage in the cycle.^{9,10} Benign tumors (for example fibroma, Fig 1B) and some of the malignant tumors (well differentiated squamous carcinoma, Fig 1C) had only diploid cells with DI values similar to those of the normal lymphocytes (1.01 and 0.93, respectively), though the percentage of cells at

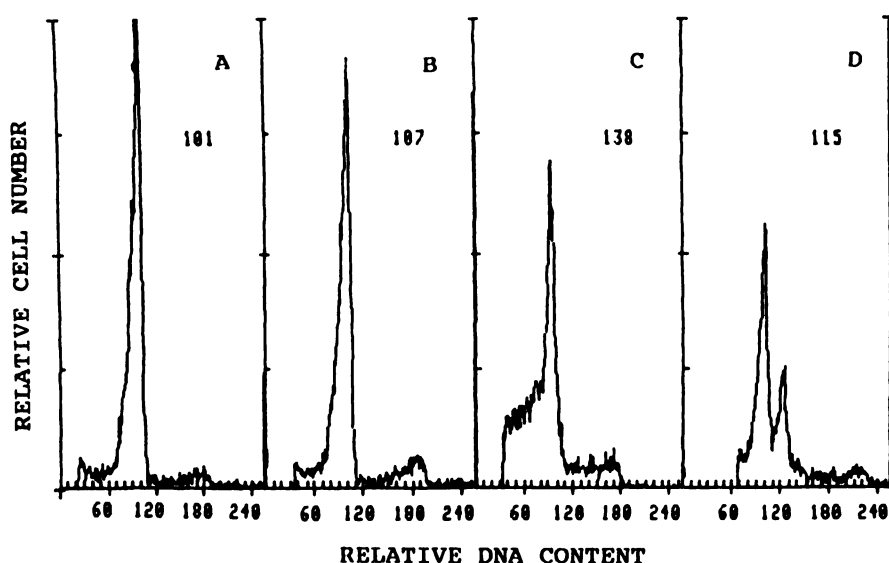


FIGURE 1. DNA content analysis from lung cancer patients. A, Normal lymphocytes ($DI = 1.0$, $G_0/G_1 = 91$ percent, $S + G_2/M = 9$ percent). B, Benign tumor-fibroma ($DI = 1.01$, $G_0/G_1 = 86$ percent, $S + G_2/M = 14$ percent). C, Well differentiated squamous cell carcinoma. The single diploid G_0/G_1 peak consists of both malignant cells and normal cells. The recalculated percentage of tumor cells in G_0/G_1 is 67 percent and in $S + G_2/M$ is 33 percent. D, Poorly differentiated squamous cell carcinoma. The initial G_0/G_1 peak ($DI = 1.06$) consists of diploid normal cells. The second smaller G_0/G_1 peak contains hyperdiploid tumor cells ($DI = 1.33$, $G_0/G_1 = 52$ percent).

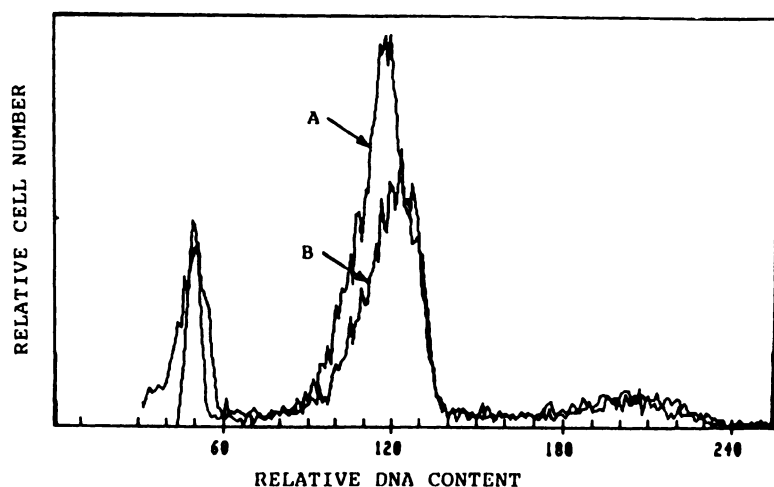


FIGURE 2. Comparisons of DNA profiles of TFNAB material (B) and tissue sample (A) of one patient with adenocarcinoma. The diploid and hyperdiploid peaks of A and B have essentially the same DI values (1.0 and about 2.5, respectively), and the percentages of cells under each peak are rather similar. Notice the diploid peak is at channel 45.

G_0/G_1 was somewhat lower (86 percent and 67 percent, respectively).

In contrast, many of the carcinomas contained a conspicuous peak with higher DI value. For example, in Figure 1D (poorly differentiated squamous cell carcinoma), the hyperdiploid peak (DI = 1.33) corresponding to the G_0/G_1 phase of hyperdiploid cells was accompanied by a complementary peak of the cells fixed at the $S + G_2/M$ phase of this cycle.

In view of the fact that TFNAB represent only a small sample of tumor cells, we determined whether the DI values obtained from TFNAB cells indeed represent the cell populations in the intact tumor by suspending cells from the same solid tumor from which TFNAB were taken, and subjecting them to flow cytometric analysis (three cases). Comparison of DI profiles of TFNAB and tissue samples (Fig 2) indicated that both the diploid and hyperdiploid peaks display essentially the same DI values (1.0 and about 2.5, respectively) and the percentages of cells under each peak are rather similar (differences in the diploid peak are mostly attributable to variations in the numbers of stromal cells included in the two samples). Based on these results it was assumed that TFNAB faithfully represent the cells in the solid tumor.

Table 2—Ploidy of Tumor Samples According to Histologic and Cytologic Diagnosis*

Diagnosis	Benign		AD		SQ		UND		No. of Samples
	T	TFN	T	TFN	T	TFN	T	TFN	
Diploid	2		1	5	4	3		2	7 10
Hyperdiploid			1	5†	3	1	3‡	2	7 8
				50%		36%		71%	47%

*AD, adenocarcinoma; SQ, squamous cell carcinoma; UND, undifferentiated carcinoma; T, tissue; TFN, transthoracic fine needle aspiration biopsy.

†Two samples with two distinct hyperdiploid peaks.

‡One sample with two distinct hyperdiploid peaks.

DNA Content in TFNAB of Different Lung Tumors

Flow cytometric analysis of different lung tumors is summarized in Table 2 and Figure 3. As shown, the benign tumors had DI values in the diploid range. Squamous cell carcinomas (as defined by cytologic and histologic criteria) were heterogeneous, with 7 out of 11 tumors containing diploid cells while the rest were in the hyperdiploid range. Tumors diagnosed as adenocarcinomas had highly diversified DI values. Of 12 tumors tested, the DI values of only 5 were in the diploid range, one exhibited a borderline value, and 6 were clearly hyperdiploid. The third group of tumors, namely undifferentiated carcinomas, displayed DI values similar to those of adenocarcinomas, ranging from 0.96 to 2.4. Interestingly, three of the tumors (two adenocarcinomas and one undifferentiated cell carcinoma) were heterogeneous and displayed two distinct peaks, both in the hyperdiploid range (Fig 3).

Cytokeratin Typing of Lung Tumors: Correlation With Flow Cytometry

Examination of cytokeratin expression in the various tumors was restricted here to staining with antibody KK8.60, specific for human cytokeratins No. 10 and 11. It had been previously shown that this antibody specifically reacts with keratinizing squamous epithelia.⁶

Immunofluorescent labeling with this antibody of 25 tumors out of 30 presented here revealed interesting features (Table 3 and Fig 3) which may be summarized as follows:

(a) All the tumors diagnosed as squamous cell carcinomas and positively stained with MoAb KK8.60 were diploid, suggesting close correlation between the degree of squamous differentiation and DNA content. Tumors displaying high DI values were all KK8.60 negative suggesting that they are either nonsquamous or squamous nonkeratinizing tumors.

(b) All tumors cytologically diagnosed as adeno-

Table 3—DNA Index of Tumor Samples According to Cytokeratin Typing*

MoAb KK8.60	DNA Index			
	Cytokeratin Typing	Cytologic Diagnosis		
		SQ	AD	UND
Positive	(12) 1.0† ± 0.05	(6) 1.00 ± 0.05	(5) 1.01 ± 0.05	(1) 1.03
Negative	(13) 1.53† ± 0.43	(4) 1.33 ± 0.02	(5) 1.51 ± 0.44	(4) 1.63 ± 0.41

*Numbers in parenthesis: samples stained with MoAb KK8.60; AD, adenocarcinoma; SQ, squamous cell carcinoma; UND, undifferentiated carcinoma.

†p = 0.007.

carcinomas or undifferentiated carcinomas, which were nevertheless, positively labeled with antibody KK8.60, contained DNA in the diploid range.

(c) All nonkeratinizing tumors (KK8.60 negative), regardless of their cytologic appearance, exhibited a hyperdiploid DNA content with the lowest DI value in the squamous cell carcinoma ($1.33 \pm .02$) and the highest in the undifferentiated carcinoma ($1.63 \pm .41$).

Cell-Cycle Analysis of Lung Tumors Using Flow Cytometry

Analysis of the flow cytometric profile enables a distinction between cells, either diploid or hyperdiploid, at G_0/G_1 phase of the cycle and those at the $S + G_2/M$ phase.^{9,10,15} In Table 4, the percentage of cells in each phase of the cell cycle is presented for each type of tumor.

As shown, normal cells or benign tumors contained about 90 percent of the cells at the G_0/G_1 phase and the rest in the $S + G_2/M$ phase. In contrast, the percentage of cells at the latter stage ("cycling cells")

was considerably higher in all malignant tumors. The overall percentage of cycling cells was very similar for squamous, adeno, and undifferentiated carcinomas (42 to 43 percent), though significant differences were found between the diploid and hyperdiploid subpopulations. Thus, the percentage of cycling cells in the diploid population was highest (about 55 percent) in adenocarcinomas and lowest (about 40 percent) in squamous cell carcinomas. Interestingly, in the hyperdiploid populations, the highest proportion of cycling cells was found in the squamous carcinomas and lowest in the adenocarcinomas.

Correlation Between DNA Content and Stage of Disease

According to TNM system, 4 out of 11 patients diagnosed as having squamous cell carcinoma were stage I with diploid DNA content in 2 of the tumors and hyperdiploid DNA content in the other 2. In the adeno and undifferentiated carcinomas, only one patient in each group was stage I with hyperdiploid DNA content. No difference was present in the distribution of diploid and hyperdiploid tumors in patients with stage II to IV disease.

DISCUSSION

The primary objective of this study was the development and preliminary analysis of novel diagnostic approaches which may complement the conventional cytologic examination of TFNAB of lung tumors. This approach was based on the rationale that the clinical and biological behavior of tumors may be related to the state of cell proliferation in the tumor.

In a previous study,⁸ we have established, using cytokeratin typing, that diagnosis based on cytologic criteria only may often be misleading. For example,

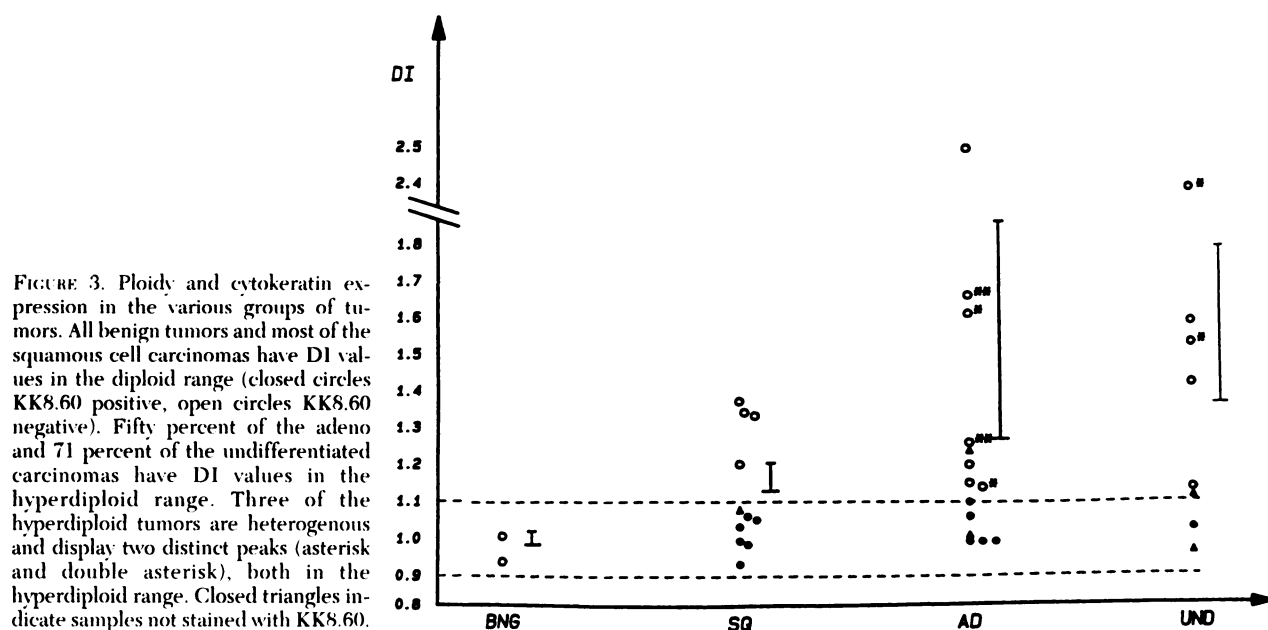


FIGURE 3. Ploidy and cytokeratin expression in the various groups of tumors. All benign tumors and most of the squamous cell carcinomas have DI values in the diploid range (closed circles KK8.60 positive, open circles KK8.60 negative). Fifty percent of the adeno and 71 percent of the undifferentiated carcinomas have DI values in the hyperdiploid range. Three of the hyperdiploid tumors are heterogenous and display two distinct peaks (asterisk and double asterisk), both in the hyperdiploid range. Closed triangles indicate samples not stained with KK8.60.

Table 4—Cell Cycle Characteristics of Lung Tumors*

Ploidy of Cells (N)	Phase of Cell Cycle	Normal Cells, %	Benign, %	AD, %	UND, %	SQ, %
Diploid	G ₀ /G ₁	91.0±0.01	87.0±2.0 (2)	46.0±17.0 (6)	52.0±1.4 (2)	60.0±25.0 (7)
	S + G ₂ /m	9±0.01	13.0±2.0	54.0±17.0	48.0±1.4	40.0±25.0
Hyperdiploid	G ₀ /G ₁	70.0±11.0 (4)	63.0±8.5 (4)	56.0±2.0 (3)
	S + G ₂ /m	30.0±11.0	37.0±8.5	44.0±2.0
Mean diploid & hyperdiploid	S + G ₂ /m	42.0±14.0	42.5±5.0	42.5±13.5

* (N), number of samples; AD, adenocarcinoma; SQ, squamous cell carcinoma; UND, undifferentiated carcinoma.

many tumors diagnosed as squamous cell carcinomas contain, in fact, simple epithelial cells typical of adenocarcinomas. This approach was extended here to flow cytometric analysis, combined with anticytokeratin 10/11 labeling. The data presented here provide interesting new information bearing on the biological properties of the various tumors. While the sample of tumors examined here is still limited, some preliminary conclusions may be drawn.

1. The DI values measured for each tumor were uniform and highly reproducible in TFNAB and in suspended cells from surgically resected tumors of the same patient.

2. All benign tumors examined displayed diploid DNA contents with slightly variable values of cycling and noncycling cells.

3. Malignant cells which positively react with KK8.60 anticytokeratin (and hence contain keratinizing cells) invariably displayed a diploid DNA content, while all tumors which lacked such cells were hyperdiploid.

4. Cells of the latter category (KK8.60 negative) were especially abundant in adenocarcinomas and undifferentiated tumors.

5. In two adeno and one undifferentiated carcinoma, two distinct hyperdiploid peaks were noted, suggesting the presence of at least two subpopulations of tumor cells in these tumors.

This close correlation between high DNA content and the absence of advanced squamous differentiation (keratinization) may provide new important tools for the diagnosis of lung cancers.^{21,22}

In some studies, ploidy was shown to be most indicative prognostic criterion, especially in squamous cell carcinomas.²³⁻²⁷ It is possible, therefore, that the clinical observation of longer survival time than expected in some patients with adeno or undifferentiated carcinomas is related to normal DNA content of the tumors. This possibility is corroborated by the present study in which the appearance of metastases or second primary tumors was related to high DNA content.

Additional information on the various tumors was derived from cell cycle analysis, based on the flow-cytometric data. Since we did not carry out cytophotometric-autoradiographic procedures to determine the

proportion of cycling cells, the data obtained here do not allow for the unequivocal distinction between cycling cells and noncycling, G₂ blocked cells.²⁸ Nevertheless, it is apparent that the percentage of S + G₂/M cells in all malignant tumors was relatively high up to approximately 55 percent compared with about 9 percent in normal cells and about 14 percent in benign tumors. This cell population was particularly abundant in the diploid population. It is still not clear what the biological basis is and clinical implications of the variability found between the various cell subpopulations, since the number of cases examined is small. However, the differences noted here appear to be significant and will provide the basis for extensive examination in the future.

In conclusion, it appears that the flow cytometric data, combined with immunocytochemical labeling for cytokeratins, may provide important diagnostic information on lung tumors, which might assist in the assessment of prognosis and the selection of optimal treatment. Additional studies are presently being conducted to further substantiate these claims and to demonstrate the prognostic significance of the findings.

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