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Flow Cytometric Analysis of DNA Ploidy Level in Paraffin-embedded Tissue of Non-small-cell Lung Cancer

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Abstract—*Investigations regarding the prognostic value of DNA content (ploidy) and proliferative characteristics [percentage of cells in S-phase or S-phase fraction (SPF)] have been greatly facilitated by the application of flow cytometry (FCM) using nuclei isolated from paraffin-embedded tissue. We have applied this technique to tumor sections from patients presenting with non-small-cell lung cancer (NSCLC) in* 1980 *and* 1981. *From* 67 *out of* 115 *patients material of sufficient quantity and quality was obtained to perform DNA–FCM. A multivariate analysis including stage of disease (UICC), age, tumor histology and treatment modality was performed to examine the prognostic significance of DNA–FCM in NSCLC. Aneuploidy was found in* 65% *of cases. In our study, the DNA content was not related to histology, stage of disease or treatment modality, nor to the length of survival (log rank test* P = 0.62). *Calculation of SPF was possible in* 49/67 *cases. The SPF was not related to histology, stage of disease or treatment modality, but a significant prognostic value was found for survival; patients with a high SPF died earlier* (P = 0.04) *and this was especially true for squamous cell carcinoma* (P = 0.02). *This study demonstrates the prognostic importance of DNA–FCM-derived information in NSCLC using a multivariate analysis; however further prospective studies in larger patient populations are needed.*

INTRODUCTION

NON-SMALL-CELL lung cancer (NSCLC) is a heterogeneous disease comprising a wide spectrum of tumors with different biological, morphological and therapeutic hallmarks. Clinical criteria such as stage, age, loss of weight and performance status have prognostic implications, but only indirectly reflect the biological properties of the tumor itself. Flow cytometric (FCM) studies of nuclear DNA content have shown a high incidence of ploidy abnormalities in NSCLC [1–6], but the clinical significance of these data remains in doubt [7, 8].

FCM determination of DNA ploidy levels in nuclei isolated from paraffin-embedded tissue greatly facilitates investigations regarding the DNA content and proliferative characteristics of NSCLC [9]. It allows the retrospective analysis of patients with sufficient follow-up to assess the prognostic significance of DNA content and proliferative activity.

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The aim of the present study was to examine the prognostic significance of DNA–FCM in NSCLC. For this a multivariate analysis including other prognostic factors such as stage of disease, age, histology and treatment modality was performed.

MATERIALS AND METHODS

Paraffin-embedded tumor tissue from all NSCLC patients treated in our institution between July 1980 and December 1981 was used for FCM. The material included surgically removed tissue and tissue removed from endobronchial tumor by rigid or flexible bronchoscope. No metastatic tissue has been used.

FCM was performed according to the method previously detailed [9]. In short, a thin slice of paraffin-embedded tissue or the whole material (in case of small tissue fragments) was scraped, cleared of paraffin with two changes of xylene for 1 h at room temperature and rehydrated in a sequence of xylene/ethanol, 100%, 96%, 70% and 50% ethanol for 30 min, followed by overnight incubation at 37°C in 0.25% trypsin (DIFCO) in citrate buffer

Table 1. Patients' characteristics and results of flow cytometry

Tumor histology	No. of cases	Survival (wks)	DNA Index	% cells S-phase
Tumor type				
Squamous cell carcinoma	59	0–230 + (30)	1–2.51 (1.45)	3–28 (13)
Adenocarcinoma	6	34–230 + (119)	1–1.85 (1.57)	9–22 (14.5)
Other	2			
Stage (UICC)				
I	5	65–230 + (118)	1–2.51 (1.28)	9–22 (12)
II	18	0–230 + (57.5)	1–2.24 (1.76)	3–21 (15)
III	29	3–220 (38)	1–1.90 (1.00)	4–28 (15.5)
IV	15	4–93 (4)	1–2.41 (1.22)	3–21 (9)
Treatment				
Surgery	17	0–230 + (101)	1–2.11 (1.57)	6–22 (14.5)
Radiotherapy	30	3–220 (54)	1–2.51 (1.23)	3–25 (15)
Supportive care	20	2–93 (4)	1–2.41 (1.29)	3–28 (12.5)

Figures in parentheses are medians.

(3 mM spermine tetrachloride, 0.5 mM Tris, pH 7.6). After vortexing and filtration 2–3 × 10^6 cells were stained according to the method of Vindelov *et al.* [10]. Analysis of cellular DNA content and percentage S-phase cells [or the S-phase fraction (SPF) in the histogram] was performed on a FACS IV cell sorter (Becton and Dickinson, Sunnyvale, CA, U.S.A.); histograms with a coefficient of variation exceeding 8% were not used.

The proliferative activity was calculated by counting the number of cells with a DNA content between G_1 and G_2/M values. In cases with less than 30% admixture of diploid cells, the percentage of aneuploid S-phase cells was calculated, without corrections for the presence of diploid S- and G_2/M-phase cells. Patients were grouped according to DNA index (DI) into three categories: DI = 1.0, 1.1 ≤ DI ≤ 1.74 and DI ≥ 1.75. The percentage of S-phase cells was taken as a measure of proliferation. Patients were divided into two categories: SPF ≤ 17% and SPF > 17%. The different groups were compared with the log rank test and survival curves were estimated by the Kaplan–Meier method. Furthermore, the data were analysed with the Cox regression model.

RESULTS

From 67 out of 115 patients material of sufficient quantity and quality was available to perform FCM for DNA analysis; calculation of SPF was possible in 49 of these 67 cases. The distribution of the patients with respect to tumor histology, stage (according UICC) and treatment is shown in Table 1. Distribution of histology and stage is fairly typical for the Dutch situation. Most patients with stage III were irradiated including some patients with stage I or II not suitable for surgery. Twenty patients got only supportive care for various reasons, including poor performance status. DNA aneuploidy was present in 44/67 cases (65%). As seen in Table 1, there are no significant associations between DNA index and tumor histology, tumor stage or treatment modality. This is also the case with SPF, although the rather small number do not exclude a rather weak association with for instance histology. Neither the DNA content of all NSCLC patients (log rank test $P = 0.62$) (Fig. 1) nor the DNA content of squamous cell patients (SCC) (log rank test $P = 0.59$) (Fig. 2) was related to the length of survival. However, SPF was of significant prognostic value: patients with a high SPF died earlier ($P = 0.04$) (Fig. 3): this was especially true for SCC ($P = 0.02$) (Fig. 4).

Variables considered to be of possible prognostic significance were sex, age, tumor histology, treatment modality, stage of disease, DNA index and percentage of cells in S-phase. Regression analysis with the Cox regression model determined two factors as having significant prognostic value, namely SPF and treatment modality. The results of this analysis are shown in Fig. 5 for NSCLC and Fig. 6 for SCC. These results are summarized in Table 2 (NSCLC) and Table 3 (SCC), which show that the relative risk of dying of NSCLC or SCC with SPF > 17% is 2.52, respectively 3.64 times greater than with SPF ≤ 17%.

DISCUSSION

The results presented in this report fit into an accumulating amount of data regarding the value of DNA–FCM-derived parameters as indicators of prognosis [4, 5, 8]. Studies in this area have been greatly facilitated by the development of methods to use archival material. Since then, an aneuploid

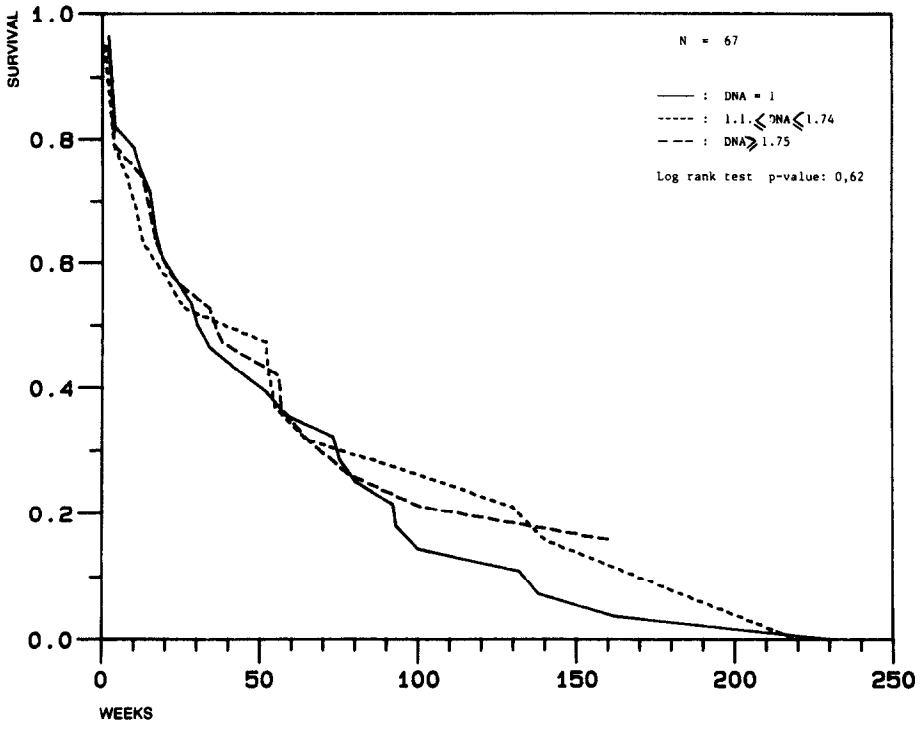

Fig. 1. Kaplan–Meier survival curves of all NSCLC with diploid and aneuploid DNA content.

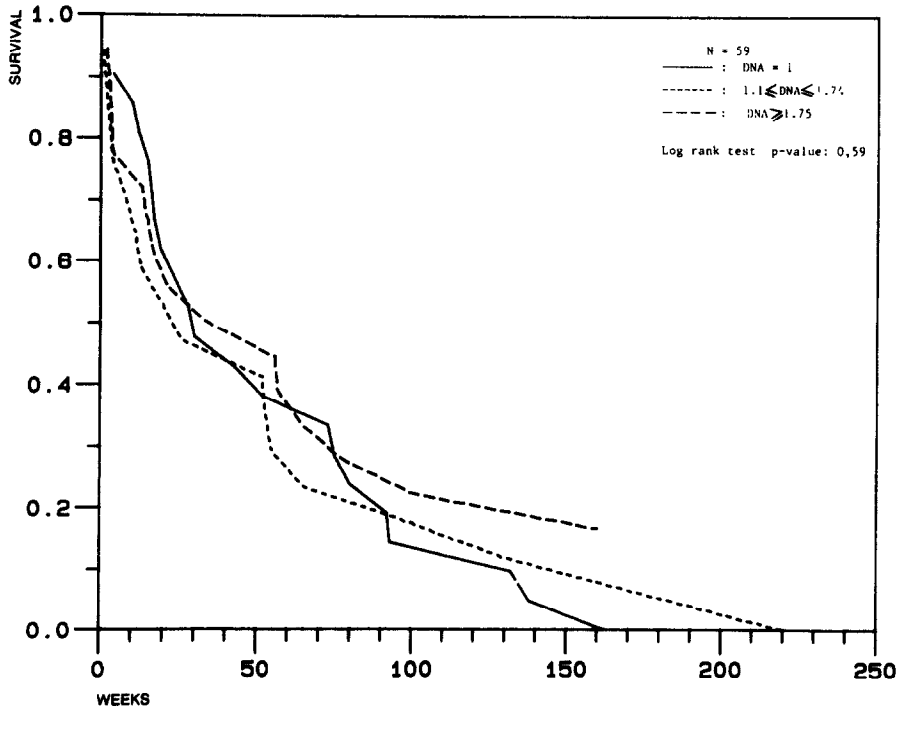

Fig. 2. Kaplan–Meier survival curves of squamous cell carcinoma with diploid and aneuploid DNA content.

DNA index has been shown in a relatively large series of patients with sufficient follow-up time to be associated with adverse prognosis in stage II breast cancer [11], colorectal cancer in Dukes' stage C [12] and stage III ovarian cancer [13]. Relationship between DNA characteristics and prognosis may, however, be modified by the development of effective systemic therapy; in neuroblastoma [14] and childhood ALL [15] patients with abnormal DNA appear to have a more favorable prognosis.

So far, studies on the prognostic importance of abnormal DNA content in NSCLC using fresh tumor material have been contradictory; at least some investigations have reported a poorer prognosis of patients with aneuploid tumors [8]. We have not been able to confirm these findings. One

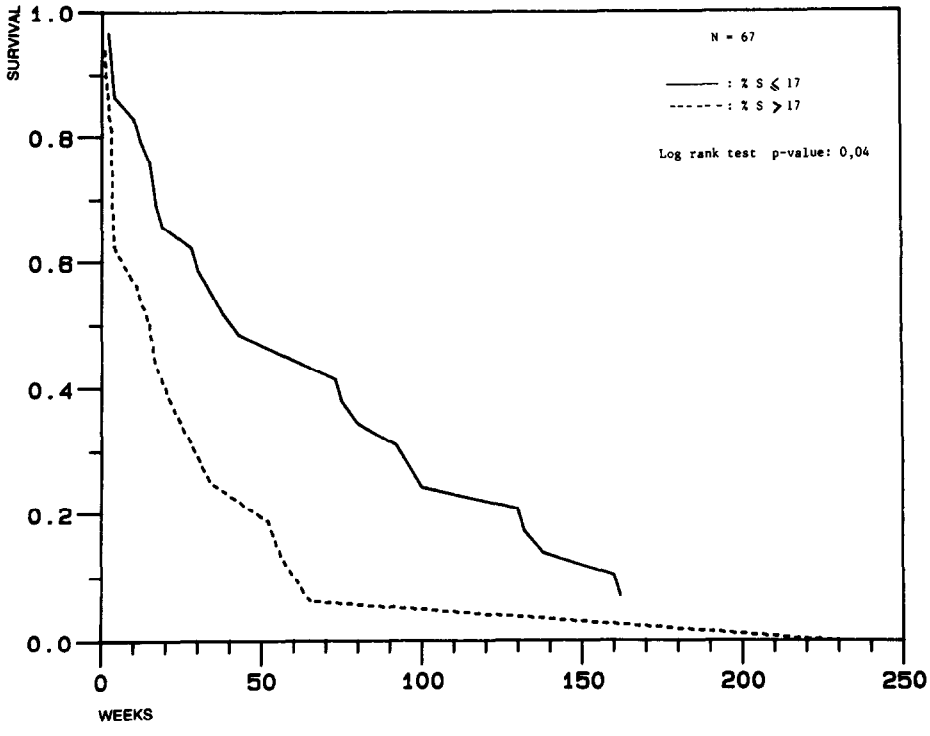

Fig. 3. Kaplan-Meier survival curves of all NSCLC with measurable proliferation phase (% S-phase).

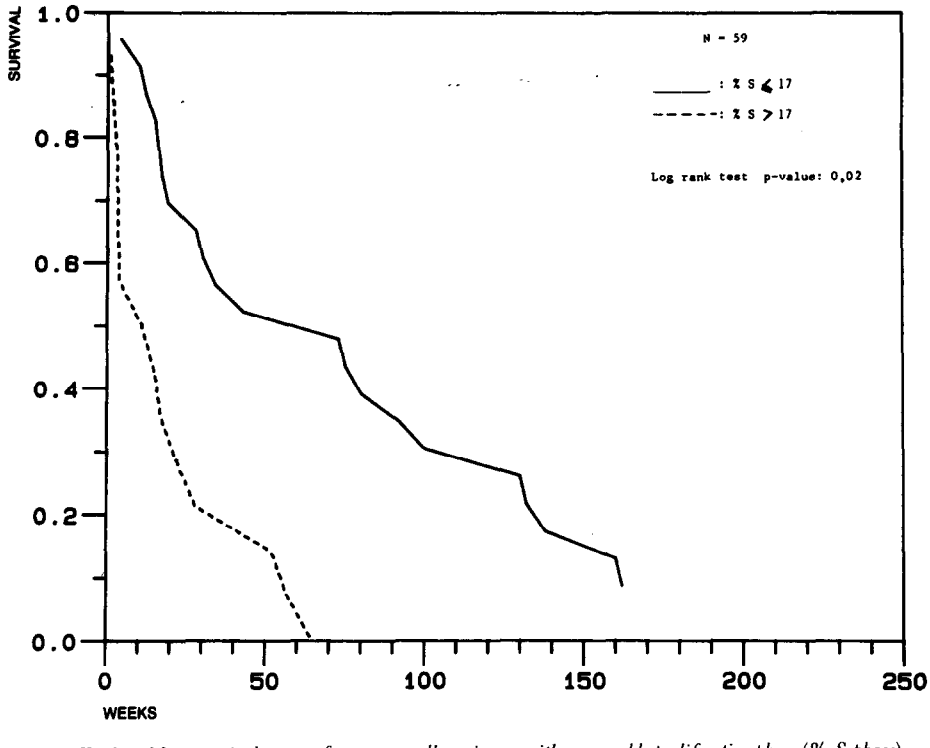

Fig. 4. Kaplan–Meier survival curves of squamous cell carcinoma with measurable proliferation phase (% S-phase).

possible explanation for this discrepancy with other common human malignancies may be the relatively poor prognosis and short survival time of NSCLC, as compared to, for instance, breast or colorectal carcinoma.

Apparently, in the majority of NSCLC patients (micro)metastases are present at the time of diagnosis and become clinically relevant after a short period of time. If, as we argued elsewhere, the DNA index is predictive of the presence rather than the behavior of metastases [12], this tumor characteristic will only be of limited importance in predicting the prognosis of patients with NSCLC.

In contrast to the DNA index, SPF in the histogram appeared to be a major prognostic indicator. In squamous cell carcinoma especially, SPF and

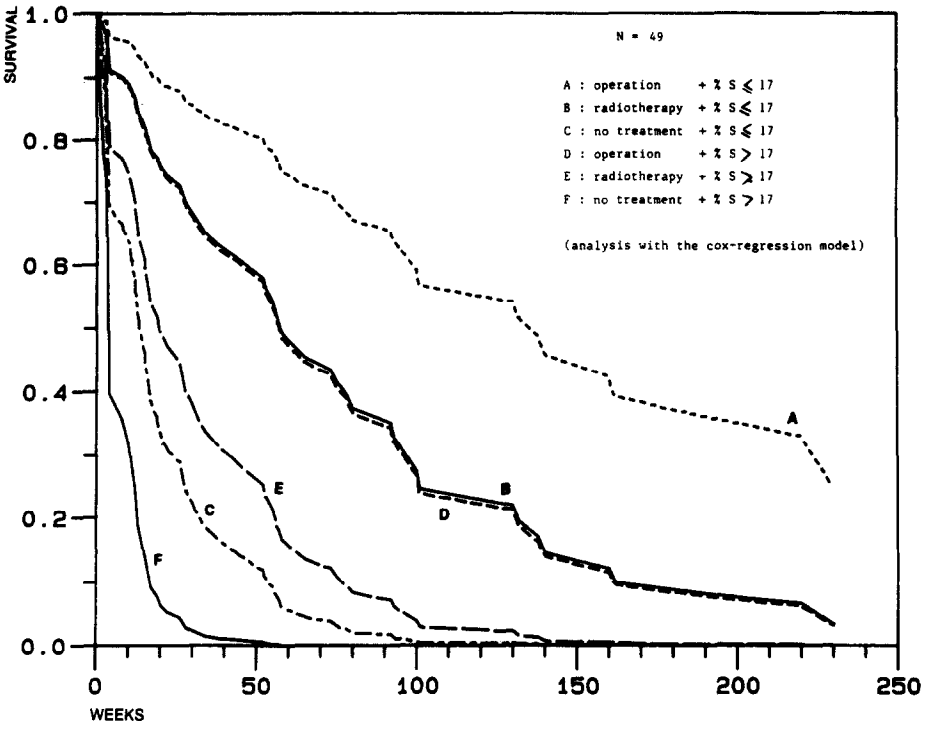

Fig. 5. Estimated survival curves of all NSCLC in relation to treatment and % S-phase.

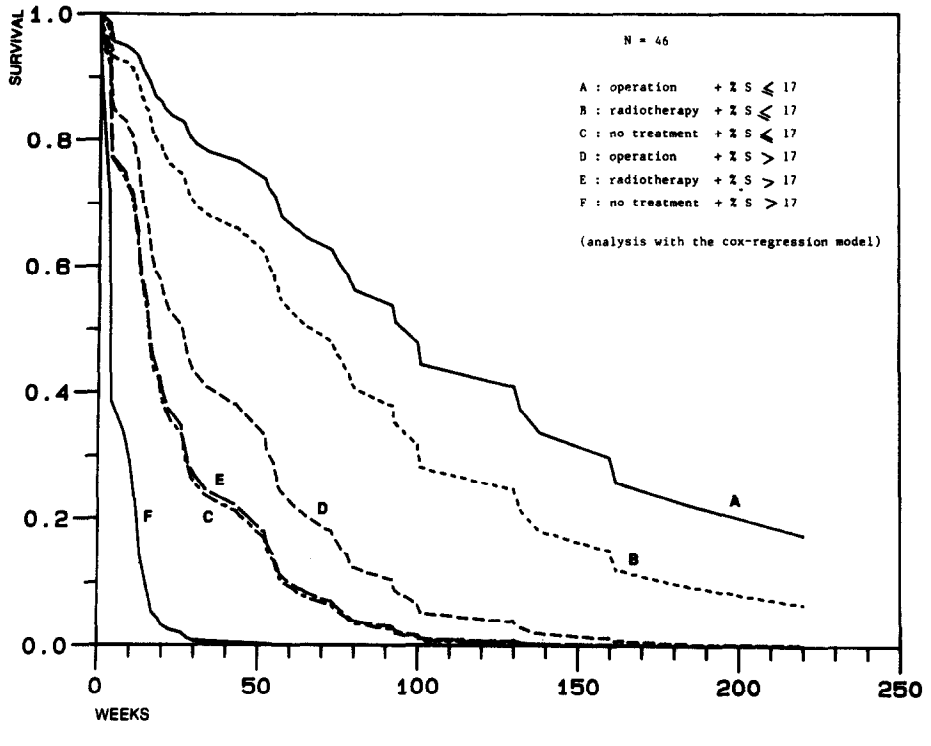

Fig. 6. Estimated survival curves of squamous cell carcinoma in relation to treatment and % S-phase.

Table 2. Variables of prognostic significance (NSCLC)

Variable	Coefficient	S.E.	Relative risk
Radiotherapy	0.91	0.37	2.48
Supportive care	2.27	0.44	9.70
% cells S-phase (>17)	0.93	0.31	2.52

Table 3. Variables of prognostic significance (SCC)

Variable	Coefficient	S.E.	Relative risk
Radiotherapy	0.44	0.33	1.56
Supportive care	1.77	0.43	5.85
% Cells S-phase (>17)	1.29	0.36	3.64

treatment modality (indirectly reflecting stage) were the most important factors. Here again prior reports [6, 8] have been contradictory and studies correlating results from other cytokinetic techniques such as tritiated thymidine labelling with prognosis are lacking. In interpreting these results, selection bias has to be taken into account. From 115 patients only 49, that is less than 50%, were available for S-phase counting, due to: (i) lack of enough tumor material and (ii) the presence of overlapping diploid and aneuploid populations in the DNA histograms. Therefore, there is a need to apply newer cytokinetic techniques which may increase the number of evaluable patients and provide more detailed cytokinetic information.

In fact, such studies, using incorporation of bromodeoxyurdine into the DNA of replicating cells [16], are in progress.

This is the first study to demonstrate the prognostic importance of DNA–FCM-derived information in NSCLC using a multivariate analysis. These data should be extended to larger patient populations. Moreover, more detailed and dynamic cytokinetic studies in NSCLC are indicated.

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