Ability of bivariate cytokeratin and deoxyribonucleic acid flow cytometry to determine the biologic aggressiveness of resectable non–small cell lung cancer

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Objective: The purpose of this study was to clarify the significance of bivariate cytokeratin and DNA flow cytometry for analysis of the biologic aggressiveness of resectable non–small cell lung cancer.

Methods: In 92 patients who underwent curative operations, the DNA ploidy status and S-phase fractions of the cancer cell populations inside the tumors were analyzed by a cytokeratin gating technique with paraffin-embedded specimens and were correlated with the surgical results.

Results: Ninety tumors yielded assessable DNA histograms. DNA diploidy was detected in 25 tumors with a mean S-phase fraction of 14.3% ± 4.7%, and DNA aneuploidy was detected in 65 tumors with a mean S-phase fraction of 15.1% ± 7.1%. The 5-year overall and recurrence-free survivals were 73.3% and 70.3%, respectively. Multivariate analysis showed that only TNM staging was a prognostic factor after surgery. There was a negative correlation between the logarithms of S-phase fraction and the disease-free interval for 22 patients with proven recurrence ($P = .006$). The tumors with high S-phase fractions recurred more rapidly than did those with low S-phase fractions.

Conclusion: In a bivariate analysis of cytokeratin and DNA flow cytometry in resectable non-small cell lung cancer, the S-phase fraction appeared to be correlated with the disease-free interval. However, DNA ploidy and S-phase fraction were not predictive of either recurrence or survival after operation. Thus DNA flow cytometry may be of limited use for the analysis of the biologic aggressiveness of lung cancer.

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Primary lung cancer has recently become the most common cause of cancer-related death in Japan, and the incidence of this malignancy continues to rise.[1] Moreover, the marked biologic aggressiveness of lung cancer causes patients to have a poor prognosis despite vigorous treatment. The surgical removal of a lung tumor offers a chance for cure, but the overall survival after resection for patients with non–small cell lung cancer (NSCLC) is far from satisfactory. To improve the prognosis of lung cancer, good biomarkers that can be used to guide treatment strategy must be identified, and an innovative multimodal approach that includes effective adjuvant therapy needs to be developed.

DNA ploidy and proliferative activity analyzed by flow cytometry have been established as useful indicators for the biologic aggressiveness of various neoplasms.[2,3] Because flow cytometry is fast and quantitative, it has the potential to be

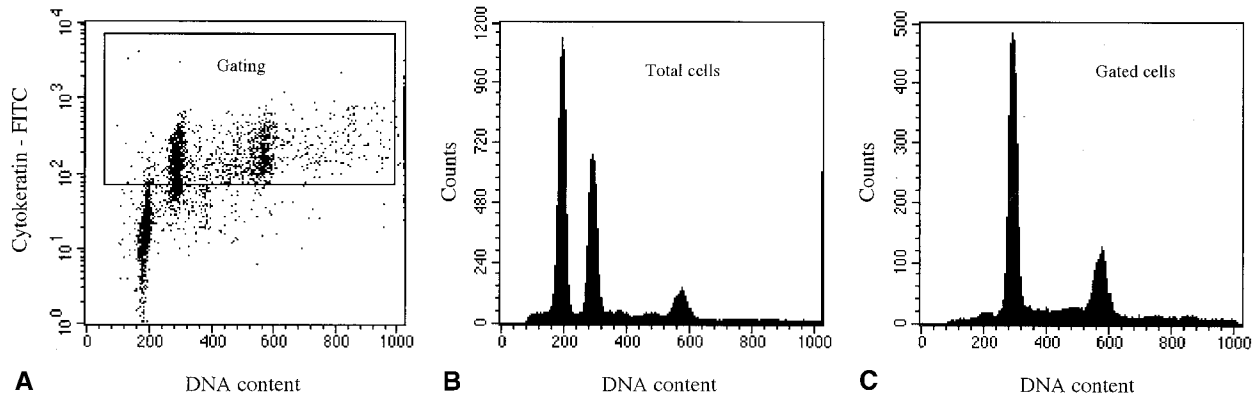

Figure 1. Bivariate cytokeratin and DNA flow cytometry. A, Dot plot of DNA content *(x-axis)* versus cytokeratin immunofluorescence *(y-axis)*. *Box* indicates gate for cytokeratin immunoreactivity. B, DNA histogram of total cells with SPF of 12.8%. C, DNA histogram of cytokeratin-positive cells with SPF of 14.1%.

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a reliable method for investigators. The reliability of the data measured by flow cytometry may depend strongly on the quality of the sample, rather than on the capability of the cytometer. In practice, the data from cancer tissue include errors caused by stromal cells and infiltrating lymphocytes inside the tumors, and this inaccuracy may have affected the results in previous studies of proliferative activities.[4] Recently, an immunofluorescent staining step for the cytokeratins of cancer cells has been adopted in the preparation for flow cytometry to detect only cancer cells and not nonepithelial cells, and this has provided more accurate cancer-specific analyses of clinical specimens.[5,6]

The aim of this study was to clarify the ability of bivariate cytokeratin and DNA flow cytometry with paraffin-embedded specimens to predict the biologic aggressiveness of resectable NSCLC. Accordingly, the associations of tumor DNA ploidy and S-phase fraction (SPF) with the surgical prognoses were assessed. Furthermore, to verify whether tumors with a high proliferative activity grow faster and recur earlier, the disease-free intervals (DFIs) of patients with recurrence after surgery were correlated with their SPF values.

Patients and Methods

Patients

This study was performed on 92 patients with primary NSCLC who underwent curative operations and received no induction therapy between January 1, 1993, and December 31, 1996, at the University Hospital, Kinki University School of Medicine, Osaka, Japan. Written, informed consent was obtained from all patients. The patients were reexamined every 3 months for the first 2 years and at an interval of 6 months thereafter, according to the routine follow-up scheme. The follow-up records updated to December 31, 2000, were used for this study.

Flow Cytometry

From each formalin-fixed and paraffin-embedded specimen obtained from surgically resected lung carcinoma, 4-μm thick slices for hematoxylin and eosin staining and 70-μm thick slices for flow cytometry were prepared. After the 70-μm sections were removed from paraffin and rehydrated, the tumor-enriched areas were cut off from the sections during viewing of the corresponding hematoxylin and eosin stained area. The samples were incubated with 0.1% collagenase (Worthington Biochemical Corporation, Lakewood, NJ) in phosphate-buffered saline solution, pH 7.4, with 1% bovine serum albumin at 37°C overnight. After washing in phosphate-buffered saline solution, samples were digested with 0.2% pepsin in 1N hydrochloric acid and 0.9% saline solution for 20 minutes at 37°C. The digested samples were filtered through a nylon mesh and washed in saline solution buffered with tris(hydroxymethyl)aminomethane, pH 7.4, supplemented with 1% bovine serum albumin (BSA-TBS). Finally, the single cells were suspended in 100 μL of BSA-TBS. The suspensions were incubated with 20 μL of the monoclonal anticytokeratin antibody reagent (1:500) clone C-11 (Sigma, St Louis, Mo) or MOPC-21 (Sigma), an isotype control reagent of mouse immunoglobulin G1, for 90 minutes at 4°C. The samples then were incubated sequentially with 20 μL of a fluorescein isothiocyanate-conjugated anti-mouse immunoglobulin G antibody reagent and 5 μg of 7-amino-actinomycin D (Sigma) for 30 minutes at room temperature. After washing, 5×10^6 cells were resuspended in 1 mL of BSA-TBS. The samples were analyzed with a FACS Calibur (Becton Dickinson, San Jose, Calif). Fluorochromes were excited with a single 488-nm argon laser. Fluorescein isothiocyanate fluorescence was detected through a 530 ± 15 nm band-pass filter, and 7-amino-actinomycin D fluorescence was detected through a 650-nm long-pass filter. For each sample at least 30,000 events were counted. Samples with a coefficient of variation of more than 8% were excluded from the flow cytometric analyses, according to the recommendation for useful S-phase determinations by Shankey and associates.[4] The acquired data were analyzed with ModFit software (version 2.0; Verity Software House, Topsham, Maine).

TABLE 1. Pathologic factors and DNA flow cytometric measurements

Factors	DNA ploidy pattern: All patients (n = 92)	Diploid (n = 25)	Aneuploid (n = 65)	*P* value*	SPF (n = 90)	*P* value†
Histologic type				.33		.32
Adenocarcinoma	44	10	32		13.6% ± 6.6%	
Squamous cell carcinoma	35	12	23		15.5% ± 5.5%	
Adenosquamous carcinoma	8	3	5		16.8% ± 8.6%	
Large cell carcinoma	5	0	5		18.2% ± 8.6%	
Primary tumor (T)				.25		.71
pT1	33	8	24		14.9% ± 7.6%	
pT2	41	10	30		15.4% ± 5.9%	
pT3	15	7	8		13.9% ± 6.5%	
pT4	3	0	3		12.7% ± 2.4%	
Regional lymph nodes (N)				.64		.14
pN0	68	17	49		14.4% ± 6.4%	
pN1	11	3	8		13.6% ± 7.0%	
pN2	13	5	8		18.2% ± 6.1%	
Pathologic stage				.27		.69
IA	27	6	20		13.8% ± 7.2%	
IB	28	6	21		15.2% ± 5.8%	
IIA	2	0	2		13.8% ± 8.4%	
IIB	18	6	12		14.7% ± 6.8%	
IIIA	14	7	7		16.9% ± 7.1%	
IIIB	3	0	3		12.7% ± 2.4%	

SPF is shown as mean ± SD.
*P value for difference in DNA ploidy.
†P value for difference in SPF distribution.

The SPF was calculated from a DNA histogram of the cells gated for cytokeratin positivity (Figure 1). The SPF values were divided into two groups: a high-SPF group, defined as having SPF greater than the median of all assessable tumors, and a low-SPF group, defined as having SPF less than the median.

Statistical Analysis

Where appropriate, the data were expressed as mean ± SD. Differences between two sets of data were tested with the *t* test, the Mann-Whitney test, or the Kruskal-Wallis test for continuous variables and with the χ^2 test for categorical variables. Correlations between two variables were evaluated with the Spearman rank correlation coefficient. The probability of cumulative cancer-related survival was calculated by the Kaplan-Meier method. Patients who died of unrelated causes were treated as censored cases. Differences in survival were tested with the log-rank test for univariate analysis and with the Cox's proportional hazard model for multivariate analysis.

Results

The patient population consisted of 70 male and 22 female patients, with a mean age of 63.4 years (range 27-82 years). The lung tumors were histologically classified according to the World Health Organization criteria as follows: adenocarcinoma (n = 44), squamous cell carcinoma (n = 35), adenosquamous carcinoma (n = 8), and large cell carcinoma (n = 5).[7] The disease status was determined according to the TNM staging system: stage IA (n = 27), stage IB (n = 28), stage IIA (n = 2), stage IIb (n = 18), stage IIIA (n = 14), and stage IIIB (n = 3) (Table 1).[8] All patients underwent a curative operation combined with mediastinal lymphadenectomy. A lobectomy was performed in 83 patients, bilobectomy was performed in 2 patients, and pneumonectomy was performed in 7 patients. Among the lobectomy procedures, 8 patients underwent extended resections with removal of the involved chest wall and 1 patient had the involved pericardium removed. The median follow-up period was 44.7 months (range 1-83 months). Twenty-two patients had disease recurrence, and 27 patients died. There were 5 operative deaths, 17 cancer-related deaths, and 5 unrelated deaths.

Of all 92 tumors, 2 tumors had DNA histograms with a coefficient of variation greater than 8.0% and were therefore excluded. The remaining 90 tumors yielded adequate DNA histograms, with a mean coefficient of variation of 6.3 ± 1.2. DNA diploidy was detected in 25 tumors and DNA aneuploidy was detected in 65 tumors. The mean SPF of the 90 analyzable tumors was 14.94% ± 6.5%, and the median

TABLE 2. Multivariate analysis of independent prognostic factors after surgical therapy according to the Cox proportional hazards model

	Overall survival			Recurrence-free survival		
Variable	Hazard ratio	95% Confidence interval	*P* value	Hazard ratio	95% Confidence interval	*P* value
Histologic type						
Adenocarcinoma (n = 42)	3.2	0.7-13.5	.11	1.2	0.4-3.2	.77
Squamous cell carcinoma (n = 33)	1			1		
Pathologic stage						
I (n = 52)	1	1				
II (n = 20)	1.6	0.3-8.5	.57	1.8	0.5-6.0	.30
III (n = 15)	21.2	5.1-86.7	<.0001	5.8	1.9-17.2	.001
DNA ploidy pattern						
Diploid (n = 24)	1	1				
Aneuploid (n = 61)	4.2	0.8-20.0	.07	2.5	0.8-7.8	.10
SPF						
Low-SPF group (n = 44)	1	1				
High-SPF group (n = 41)	1.5	0.3-6.2	.58	1.5	0.5-4.3	.43

was 14.6%. The mean SPFs of the DNA diploid and aneuploid tumors were 14.3% ± 4.7% and 15.1% ± 7.1%, respectively, which were not significantly different (P = .64). Thus DNA diploid and aneuploid tumors had equal proliferative activities. Moreover, the DNA ploidy and SPF were also independent of the conventional pathologic factors (histologic type, TNM classification, and pathologic stage; Table 1).

Survival after surgery was analyzed for 87 patients (excluding the 5 operative deaths). The 5-year overall survival and recurrence-free survivals for all 87 patients were 73.3% and 70.3%, respectively. According to histologic type, the 5-year overall and recurrence-free survivals were 68.3% and 71.9% for the 42 patients with adenocarcinoma, 80.9% and 70.5% for the 33 with squamous cell carcinomas, 80.0% and 62.5% for the 8 with adenosquamous cell carcinomas, and 75.0% and 75.0% for the 4 with large cell carcinomas, respectively. The histologic type did not significantly influence survival. According to the pathologic staging, the 5-year overall and recurrence-free survivals were 83.7% and 81.2% for the 52 patients with stage I disease, 71.4% and 70.2% for the 20 with stage II disease, and 39.7% and 34.3% for the 15 with stage III disease, respectively. Patients with stage I and III disease had a significant difference in overall survival (P = .0003), as did those with stage II and III disease (P = .03). The patients with stage I and III disease also had a significant difference in recurrence-free survival (P = .0006). According to DNA ploidy, the 24 patients with DNA diploid tumors had 5-year overall and recurrence-free survivals of 88.9% and 80.8%, respectively. The 61 patients with DNA aneuploid tumors had 5-year overall and recurrence-free survivals of 69.3% and 67.5%, respectively. Those with DNA diploid tumors tended to have a better prognosis than did those with DNA aneuploid tumors, but the differences in overall and recurrence-free survivals were not significant (P = .15 and P = .26, respectively). For the analysis of proliferative activity, the prognoses of the high- and low-SPF groups (above and below the median SPF, respectively) were compared. The high-SPF group (n = 41) had 5-year overall and recurrence-free survivals of 77.8% and 68.7%, respectively. The low-SPF group (n = 44) had 5-year overall and recurrence-free survivals of 73.8% and 74.0%, respectively. The high- and low-SPF groups had statistically equivalent overall and recurrence-free survivals (P = .82 and P = .27, respectively). Multivariate analysis showed that only TNM staging was a prognostic factor after surgery and that DNA ploidy and proliferative activity were not significant predictors (Table 2).

The DFIs from surgery to documented recurrence in 22 patients were correlated with the DNA flow cytometric parameters. The 4 patients with DNA diploid tumors had a mean DFI of 17.5 ± 7.0 months, whereas the 18 with DNA aneuploid tumors had a mean DFI of 17.0 ± 11.9 months. There was no significant difference in the DFI between those with DNA diploid and aneuploid tumors (P = .76). The high-SPF group (n = 12) had a mean DFI of 10.8 ± 3.6 months, whereas the low SPF group (n = 10) had a mean DFI of 24.5 ± 12.4 months. The DFIs of the high-SPF group were significantly shorter than those of the low-SPF

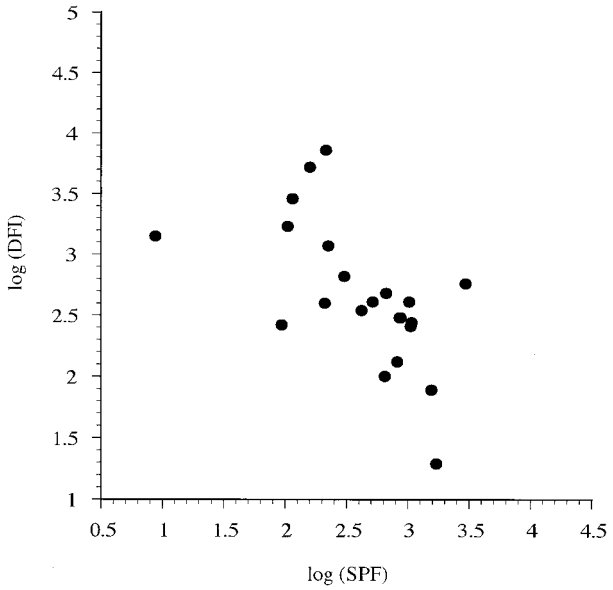

Figure 2. Correlation between natural logarithms of SPF (as percentage) and DFI (in months) among the patients with recurrence after surgery. These variables have significant negative correlation ($P = .006$).

group ($P = .003$). There was a significant negative correlation between the logarithms of SPF and DFI ($p = 0.006$), which indicates that the SPF was inversely proportional to the DFI (Figure 2).

Discussion

Analyses of the cell kinetics and various markers of cell proliferation may be reliable approaches to study the biologic aggressiveness of cancer.[2,3] To date, the development of programs that use the mathematic models to calculate the cell cycle fractions from a DNA histogram and a process for paraffin-embedded specimens reported by Hedley and coworkers[9] has led to the widespread clinical use of DNA flow cytometric analysis of cell proliferation. However, the downside to this rapid evolution is variability in sample preparation, which may cause discrepancies in reported results. It was therefore hoped that a standard, universally used procedure would be established.[4,10]

One of the technical problems in cell cycle analysis from a DNA histogram is the presence of nontumor cells inside tumors. When the population of nontumor cells is a significant percentage of the total, a large error in the flow cytometric data may result. To diminish these errors, the technique of cancer-specific flow cytometry with cytokeratin gating was recently established. Cytokeratins, a family of intermediate filament proteins, are components of epithelial cytoskeletons. This technique enables one to gate cancer cells differently from nonepithelial cells and can be conducted with paraffin-embedded tissue.[5,6] In the strict sense, however, this method cannot provide a cancer-specific analysis, because normal epithelial cells inside tumors have cytokeratins and can pass through the gate operation.

The establishment of a model to simulate tumor growth is helpful for predicting the clinical prognoses of patients with malignant tumors. Steel reported that the actual volume doubling time (T_{act}) is given by the following function: $T_{act} = \lambda \cdot T_s/[SPF \cdot (1 - \phi)]$, where T_s is the duration of DNA synthesis, λ is the correction factor, and ϕ is the cell loss factor.[11,12] Furthermore, taking logarithms of this equation leads to the following equivalent expression: $\log(T_{act}) = \log[\lambda \cdot T_s/(1 - \phi)] - \log(SPF)$. This study, which was performed under the assumption that the DFI is equivalent to T_{act} in this theoretical formula, demonstrated that the SPF is inversely correlated with the DFI in NSCLC. Therefore the significance of bivariate cytokeratin and DNA flow cytometry is just rough estimation of the DFI without determination of the SPF and T_s with a toxic method, such as the tritiated thymidine labeling method or the bromodeoxyuridine labeling method.[13-15]

It has been established that DNA ploidy is generally a good indicator of a tumor's biologic aggressiveness. Some studies of lung cancer that incorporated DNA flow cytometry found that the proliferative activity of DNA aneuploid tumors was higher than that of DNA diploid tumors,[16-18] whereas other studies found that there were no differences in proliferative activity between DNA ploidy patterns.[14,19,20] In this study the putative higher proliferative activity of DNA aneuploid tumors could not be proved for NSCLC, even with accurate SPF analysis with the cytokeratin gating method. Previous studies that found DNA diploid tumors to have low proliferative activity may have underestimated the SPF because of the presence of normal cells inside the tumors. To clarify whether there is any difference in the proliferative activity between DNA diploid and aneuploid tumors, a large-scale study will be necessary.

Whether DNA flow cytometry is useful in determining the surgical prognosis of patients with NSCLC remains a matter of debate.[2,3,21-27] Multivariate analysis in this study revealed that DNA ploidy and proliferative activity were not prognostic factors. The lack of significance of DNA ploidy with respect to both the surgical prognosis and the proliferative activity suggests that there may be little difference, if any, between DNA diploid and aneuploid NSCLC tumors in biologic aggressiveness. Similarly, patients with high-SPF tumors had the same prognoses as did those with low-SPF tumors, despite the previously mentioned correlation of SPF with the tumor growth rate. One possible explanation for this is that the SPF is not directly associated with the ability of a tumor to metastasize and therefore would not be a reliable predictor of recurrence. Because our statistical analysis included patients who did not have a recurrence and were possibly cured, there might be no

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significant difference in the survivals between the high- and low-SPF groups. The number of patients with stage II and III disease who had high risk of recurrence was small, and this sample-size issue could have affected other results in this study. Because of the small patient population with stage II disease, censored cases could largely bias the result of prognosis, which was indeed better than universal knowledge. Furthermore, with regard to future treatment strategies, it appears informative that tumors with DNA aneuploidy or high proliferative activity have good responses to chemotherapy.[28-30] For NSCLC, the prognoses of those with DNA aneuploid or high SPF tumors might be improved by the skillful use of adjuvant therapy.

In conclusion, more accurate DNA flow cytometric analysis of resectable NSCLC with the cytokeratin gating method revealed that the SPF value was correlated inversely with the DFI. However, DNA ploidy and SPF were not useful for predicting either recurrence or survival after surgery. The ability of DNA flow cytometric data to estimate the biologic aggressiveness of NSCLC is limited at best.

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