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A Study of Flow Cytometric Nuclear DNA Content in Lung Cancer: The Incidence of DNA Aneuploidy and Its Prognostic Implications

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Summary. **Flow cytometric nuclear DNA content (DNA ploidy) was measured in paraffin-embedded surgical specimens of 87 patients with primary lung cancer. Of the 87 tumor tissues, 17 (19.5%) showed a normal DNA stemline (DNA diploidy), and 70 (80.5%) an abnormal DNA stemline (DNA aneuploidy). Patients with DNA aneuploid tumors had significantly shorter survival times than those with DNA diploid tumors ($p < 0.001$). This study confirmed the high incidence of DNA aneuploidy as well as its prognostic implication in primary lung cancer patients. DNA aneuploidy seems to be a useful parameter for evaluating the degree of lung cancer malignancy.**

INTRODUCTION

Evaluation of the degree of malignancy of cancer cells serves to predict the prognosis as well as plan treatment for lung cancer patients. The degree of malignancy is usually estimated pathologically by evaluating the histological type and degree of differentiation of cancer cells. However, the pathological findings are not always correlated with the behavior and prognosis of the tumor itself. Recently, flow cytometry has enabled the measurement of the nuclear DNA content of tumor cells. The technique is considered stable, reproducible and objective, compared with the histological severity, which is judged by an experienced pathologist. Several studies have shown that an abnormal DNA stemline or DNA aneuploidy is a reliable factor predicting a poor prognosis in lung cancer.[1-5] However, there is still controversy over this point,[6] and the incidence of DNA aneuploidy also differs among previous reports. The purpose of this study is to determine the incidence of DNA aneuploidy and its prognostic implication in primary lung cancer.

MATERIALS AND METHODS

Patient Material

Flow cytometric nuclear DNA content was measured on paraffin-embedded surgical specimens of 87 patients. They were selected from 112 patients with primary lung cancer, who underwent surgery in our university hospital from November 1984 to February 1987. The selection was made according to the following basis: (1) the materials were adequate for histological and flow cytometric analysis; (2) the patients had survived for at least 4 weeks after surgery; (3) they could be followed up for more than 4 years. The tumor tissues of 87 patients were classified morphologically based on the criteria of the Japan Lung Cancer Society Study; there were 40 adenocarcinomas, 35 squamous cell carcinomas, 5 large cell carcinomas, and 7 small cell carcinomas. The postsurgical pathologic stages (pStage) were determined by the International Union Against Cancer classification. Thirty-seven of 87 patients had pStage I, 6 pStage II, 31 pStage IIIA, 9 pStage IIIB, and 4 pStage IV.

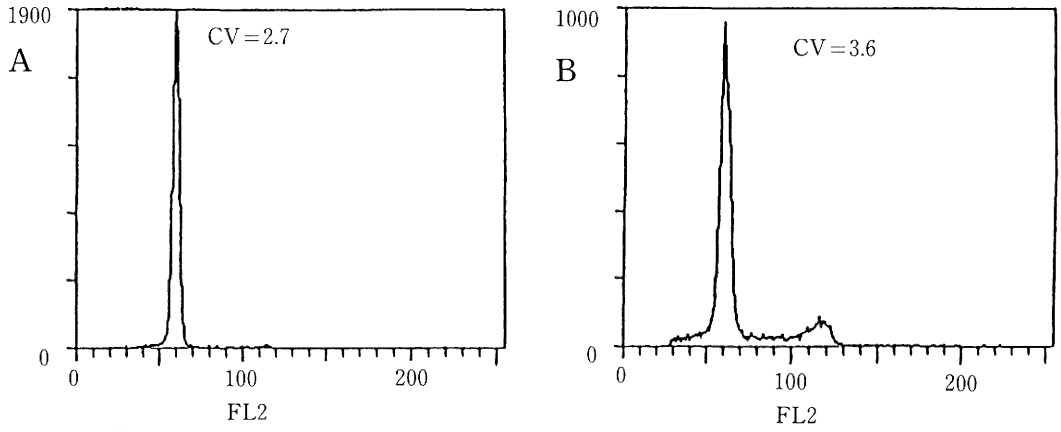
Flow Cytometric Analysis

One or two tissue blocks containing adequate cancer tissue from a primary tumor were selected from each case. One or two 50 μm thick sections were cut from each paraffin block. Single cell suspension was

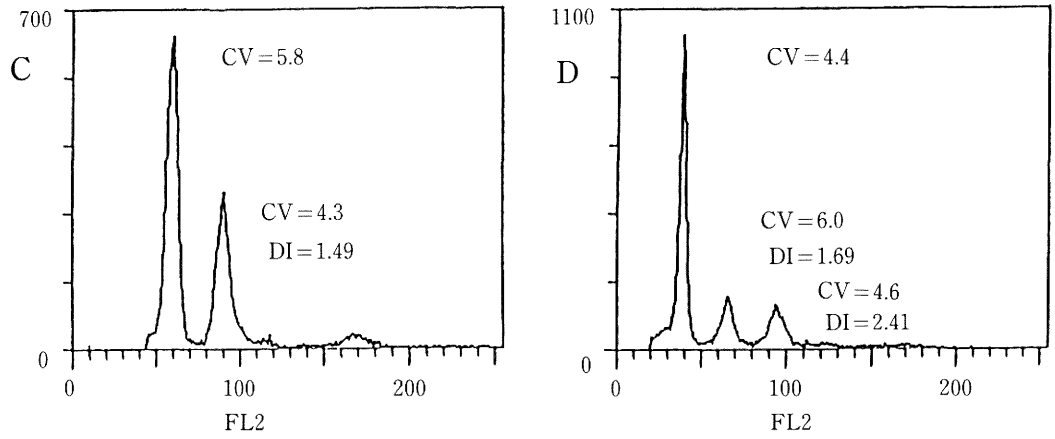
achieved by the modified method of Schutte et al.[7] Briefly, the sections were deparaffinized in xylene and rehydrated in a sequence of graded alcohols, and rinsed in distilled water. Then the sections were incubated in 0.25% trypsin in citrate buffer (3mM trisodium citrate, 0.1% v/v Nonidet P40, 1.5 mM spermine tetrahydrochloride, 0.5 mM Tris (hydroxymethyl)-aminomethane, pH 7.6) overnight at 37℃. After dispersion, the samples were filtered through a 60 μm nylon mesh. The DNA stain was carried out by the method of Vindeløv et al.[8] Flow cytometric analysis was carried out with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA). Ten thousand nuclei per sample were examined, and the nuclear DNA content (DNA ploidy) was displayed as a histogram. The normal DNA stemline (DNA diploidy) was defined as having a single G0/G1 peak (Fig. 1-A, B). The abnormal DNA stemline (DNA aneuploidy) was defined as having any distinct peak other than the G0/G1 peak (Fig. 1-C, D). In cases of DNA diploidy, one or two additional tumor blocks were reanalyzed in order to detect occult aneuploid peaks. When a DNA histogram pattern could not be classified with certainty, as DNA diploidy or DNA aneuploidy additional tumor blocks were reanalyzed until the histogram was clearly classifiable (Fig. 2).

Survival Analysis

Analysis of the results was carried out by Student's t test and the chi-square test. Survival times were calculated from the date of operation to the date of death or last follow-up date. Survival curves were estimated by the Kaplan-Meier method. Differences



A) DNA diploid without obvious S and G2/M phase.
B) DNA diploid with obvious S and G2/M phase.



C) DNA aneuploid containing one abnormal DNA stemline.
D) DNA aneuploid containing more than one abnormal DNA stemline.

Fig. 1. Representative examples of DNA histograms

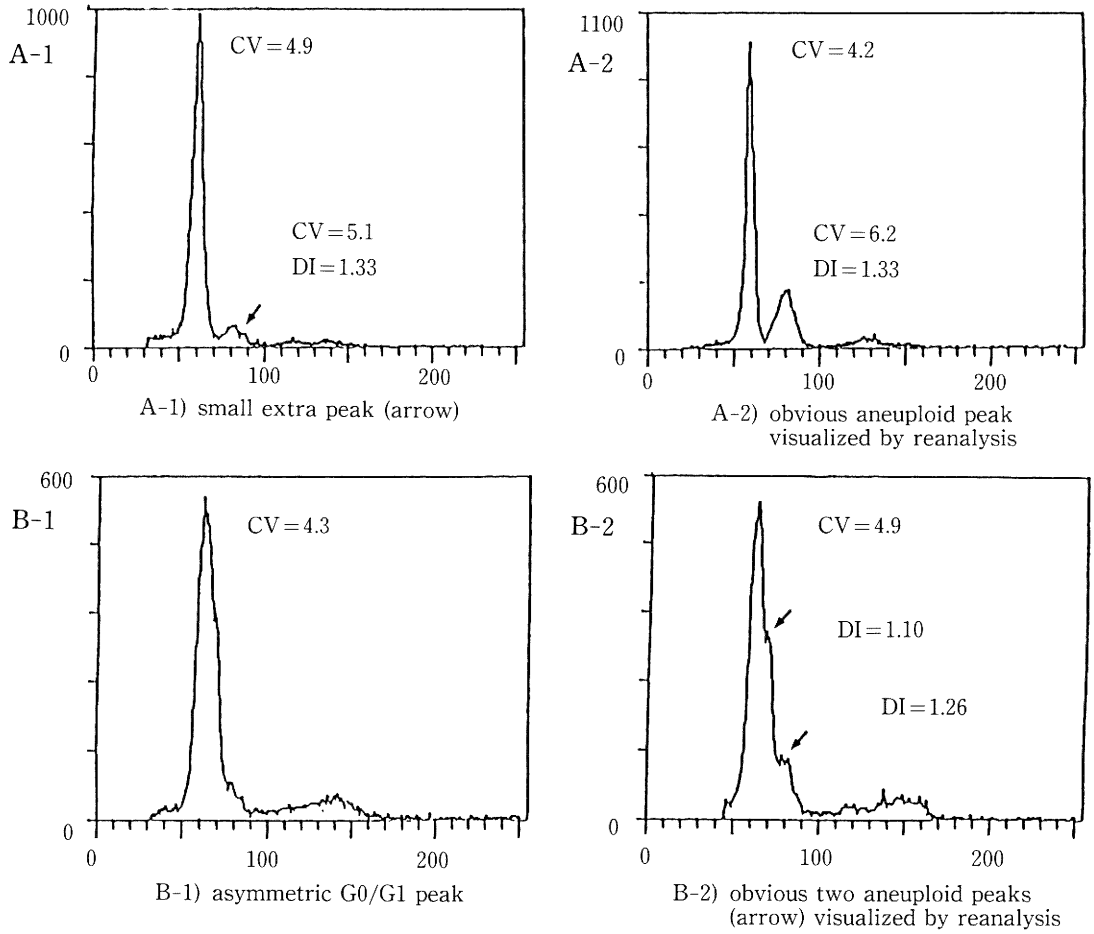


Fig. 2. Representative DNA histograms classified as DNA aneuploid by reanalysis of additional tumor blocks.

in survival were analyzed by the generalized Wilcoxon test.

RESULTS

Of all 87 lung cancer tissues, 17 (19.5%) showed DNA diploid and 70 (80.5%) DNA aneuploid (Table 1). Fifteen of 70 DNA aneuploid cases needed one to three reanalyses until the histograms were clearly classifiable. Of 80 non-small cell lung cancer tissues, 65 (81.3%) showed DNA aneuploid (Table 1). The coefficient of variation (CV) for the G0/G1 peaks for all cases ranged from 2.9 to 10.2 (median, 4.9). Four cases showed histograms with CV>8. They were all classified as DNA aneuploidy, because they had distinct aneuploid peaks.

The proportion of DNA ploidy in each TN factor (TNM classification), pStage, and histopathological features are shown in Tables 1 and 2. The incidence of DNA aneuploidy was higher in larger T factor and in less differentiated carcinomas, although it was not statistically significant. There was no correlation between the incidence of DNA aneuploidy and TN factor, pStage and histopathological features.

Patients with DNA aneuploid tumors had significantly shorter survival times than those with DNA diploid tumors ($p<0.001$) (Fig. 3). The survival time of patients with DNA aneuploid tumors was shorter than that of patients with DNA diploid tumors in pStage I+II and III+IV (Fig. 4). However, a significant difference in survival time was noticed only in pStage III+IV ($p<0.05$). The same relationship was also noticed in patients with adenocarcinoma and those with squamous cell carcinoma, though not significant (Fig. 5).

Table 1. Relation between TNM classification and DNA ploidy

	Diploid (%)	Aneuploid (%)	Total	
All patients	17(19.5)	70(80.5)	87	
Non-small cell ca.	15(18.8)	65(81.3)	80	
T factor is	1(50.0)	1(50.0)	2	NS
1	8(34.8)	15(65.2)	23	
2	7(19.4)	29(80.6)	36	
3	1(5.9)	16(94.1)	17	
4	0(0)	9(100)	9	
N factor 0	10(20.8)	38(79.2)	48	NS
1	3(21.4)	11(78.6)	14	
2	4(16.7)	20(83.3)	24	
3	0(0)	1(100)	1	
pStage I	10(27.0)	27(73.0)	37	NS
II	2(33.3)	4 (66.7)	6	
IIIA	4(12.9)	27(87.1)	31	
IIIB	0(0)	9(100)	9	
IV	1(25.0)	3(75.0)	4	
I+II	12(27.9)	31(72.1)	43	NS
III+IV	5(11.4)	39(88.6)	44	

Table 2. Relation between histopathologic features and DNA ploidy

	Diploid (%)	Aneuploid (%)	Total	
Histologic Type				
Adenocarcinoma	6 (15.0)	34(85.0)	40	NS
Squamous cell ca.	8 (22.9)	27(77.1)	35	
Large cell ca.	1 (20.0)	4(80.0)	5	
Small cell ca.	2 (28.6)	5(71.4)	6	
Differentiation of Adenocarcinoma				
Well or Moderate	5 (17.9)	23(82.1)	28	NS
Poor	1 (8.3)	11(91.7)	12	
Differentiation of Squamous cell ca.				
Well or Moderate	7 (24.1)	22(75.9)	29	NS
Poor	1 (16.7)	5(83.3)	6	

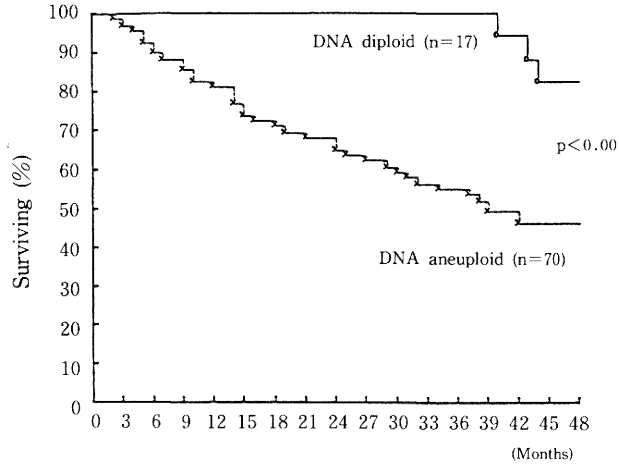


Fig. 3. Survival curves of all patients subdivided according to DNA ploidy.

DISCUSSION

Flow cytometry (FCM) can rapidly and objectively analyze the nuclear DNA content or DNA ploidy of large numbers of cells. The analysis of DNA ploidy on paraffin-embedded tissue blocks[9] is possible using this technology. It also enables the retrospective study of many tumor tissue blocks from patients whose clinical course has clearly been evaluated.

Table 3 shows previous studies of DNA ploidy in paraffin-embedded specimens of non-small cell lung cancer. The incidence of DNA aneuploidy varies from 45% to 81.3%. This variation may be attributed to the difference in sampling procedures[10] or the disagreement in DNA histogram classification.[11] Multiple sampling is extremely important for FCM analysis because of the high degree of heterogeneity of lung cancer.[11,12] The present study showed a high incidence of DNA aneuploidy, compared with the result of other studies. In the present study, 15 of 70 DNA aneuploid cases required reanalyses until the definite aneuploid peaks were revealed. Although 2 of 15 cases needed two or three reanalyses, the other cases required only one. When a DNA histogram could not be identified with certainty, it was necessary to reexamine additional tumor blocks.

It has been known that fresh unfixed materials reveal a higher incidence of DNA aneuploidy than that of paraffin-embedded materials. Tirindelli-Danesi et al. found DNA aneuploidy in 97 (96%) of 101 fresh samples of lung cancer.[13] Formalin fixation may reduce DNA fluorescence of stained nuclei.[14] In paraffin-embedded material, tumor tissues with near-diploid aneuploid population might be misinterpreted as DNA diploidy because of a high coefficient of variation.

Several studies have shown that DNA aneuploidy is a reliable factor predicting a poor prognosis in lung cancer patients (Table 3).[1-5] This study confirmed the prognostic implication of DNA ploidy. Consider-

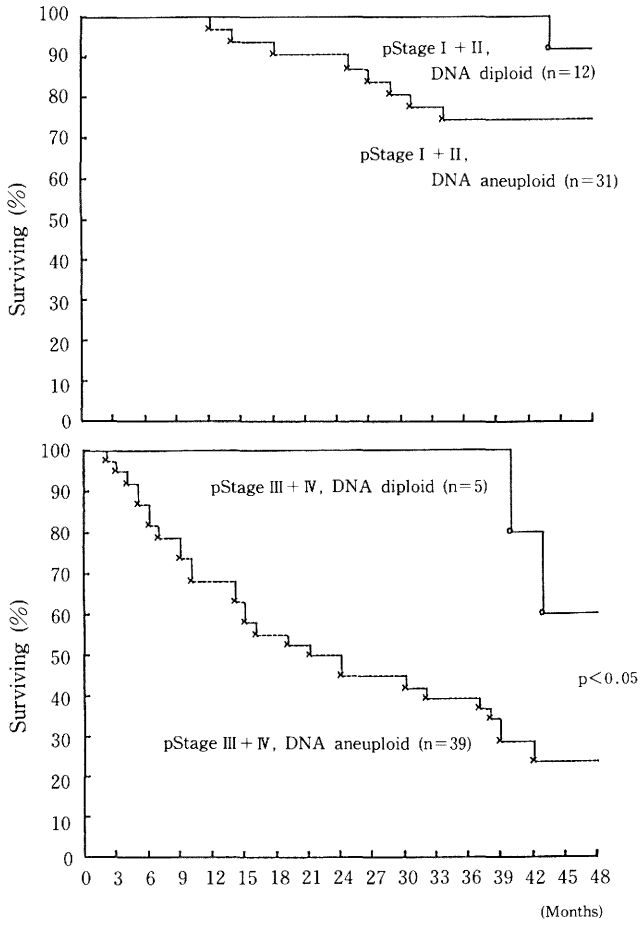


Fig. 4. Survival curves of patients with pStage I+II and pStage III+IV according to DNA ploidy.

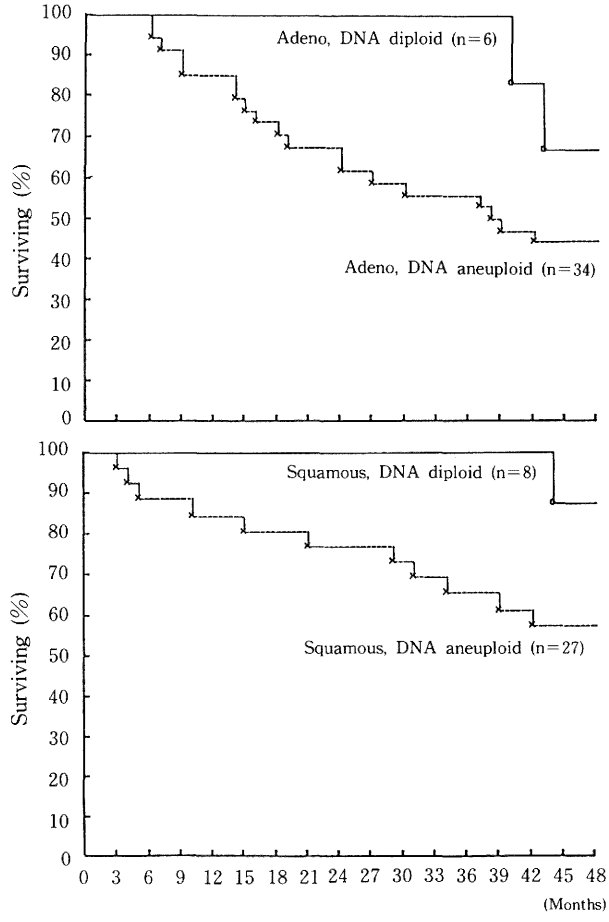


Fig. 5. Survival curves of patients with adenocarcinoma and squamous cell carcinoma according to DNA ploidy.

Table 3. Studies of flow cytometric DNA content in paraffin-embedded tissue of non-small cell lung cancer

Author	No. of patient	Aneuploid (%)	Correlation between Survival and DNA ploidy
Zimmerman et al.[1] (1987)	100	45	(+)
Ten Velde et al.[6] (1988)	67	65	(−)
Sahin et al.[2] (1990)	146	58	SCC* (+), non-SCC (−)
Isobe et al.[3] (1990)	125	76.8	(+)
Yamaoka et al.[4] (1990)	144	77.1	(+)
Shiota et al.[5] (1990)	216	57.4	(+)
Current study	80	81.3	(+)

*SCC : Squamous cell carcinoma.

ing the very high incidence of DNA aneuploidy in fresh samples, all lung cancer tissues may naturally possess DNA aneuploidy. If so, it is meaningless to divide lung cancer tissue into DNA diploid and aneuploid tumors. However, the present FCM cannot detect all DNA aneuploidy in paraffin-embedded specimens of lung cancer tissue. Van Bodegom et al. reported that the DNA content, either DNA diploidy or aneuploidy, did not predict survival time, and that, however, the percentage of aneuploid tumor cells was

correlated with the prognosis.[15] It can be assumed that the DNA diploid tumor may have only a few aneuploid tumor cells rather than none.

Normal tissues, inflammatory lesions, and benign tumors of the lung always showed DNA diploidy.[16] On the other hand, primary lung cancer tissues revealed DNA aneuploidy with very high incidence. In addition to this, patients with DNA aneuploid tumor had a significantly poorer prognosis than those with DNA diploid tumor. Thus, DNA aneuploidy can serve as a useful marker indicating the degree of malignancy in lung cancer.

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REFERENCES

1) Zimmerman PV, Hawson GAT, Bint MH, Parsons PG: Ploidy as a Prognostic Determinant in Surgically Treated Lung Cancer. *Lancet* **5**: 530-533, 1987.
2) Sahin AA, Ro JY, El-Naggar AK, Lee JS, Ayala AG, Teague K, Hong WK: Flow Cytometric Analysis of the DNA Content of Non-Small Cell Lung Cancer: Ploidy as a Significant Prognostic Indicator in Squamous Cell Carcinoma of the Lung. *Cancer* **65**: 530-537, 1990.
3) Isobe H, Miyamoto H, Shimizu T, Haneda H, Hashimoto M, Inoue K, Mizuno S, Kawakami Y: Prognostic and Therapeutic Significance of the Flow Cytometric Nuclear DNA Content in Non-Small Cell Lung Cancer. *Cancer* **65**: 1391-1395, 1990.
4) Yamaoka N, Uchiyama Y, Azuma K, Yamaguchi H, Akama T, Tsuji K: Flow Cytometric Nuclear DNA Analysis in Resected Primary Non-Small Cell Lung Carcinomas and its Relationship to Malignant Intensity. *Haigan* **30**: 9-18, 1990. (in Japanese)
5) Shiota T, Konishi T, Kosaba S, Mitsuoka A, Matsubara Y, Hatakenaka R, Funatsu T, Ikeda S, Matsumoto H, Ishigami F: Flow Cytometric Analysis of Cellular DNA Content of Lung Cancer with Reference to Survival. *Nippon-Kyobu-Geka-Gakkai-Zasshi* **38**: 2364-2369, 1990. (in Japanese)
6) Ten Velde GPM, Schutte B, Vermeulem A: Flow Cytometric Analysis of DNA Ploidy Level in Paraffin-Embedded Tissue of Non-Small Cell Lung Cancer. *Eur J Cancer Clin Oncol* **24**: 455-460, 1988.
7) Schutte B, Reynders MJJ, Bosman FF: Flow Cytometric Determination of DNA Ploidy Level in Nuclei Isolated from Paraffin-Embedded Tissue. *Cytometry* **6**: 26-30, 1985.
8) Vindeløv LL, Christensen IJ, Nissen NI: A Detergent-Trypsin Method for the Preparation of Nuclei for Flow Cytometric DNA Analysis. *Cytometry* **3**: 323-327, 1983.
9) Hedley DW, Friedlander ML, Taylor IW, Ruggy CA, Musgrove EA: Method for Analysis of Cellular DNA Content of Paraffin-embedded Pathological Material Using Flow Cytometry. *J Histochem Cytochem* **31**: 1333-1335, 1983.
10) Carey FA, Lamb D, Bird CC: Intratumoral Heterogeneity of DNA Content in Lung Cancer. *Cancer* **65**: 2266-2269, 1990.
11) Joensuu H, Kallioniemi OP: Different Opinions on Classification of DNA Histograms Produced From Paraffin-Embedded Tissue. *Cytometry* **10**: 711-717, 1989.
12) Teodori L, Tirindelli-Danesi D, Mauro F, De Vita R, Uccelli R, Botti C, Modini C, Nervi C, Stipa S: Non-Small-Cell Lung Carcinoma: Tumor Characterization on the Basis of Flow Cytometrically Determined Cellular Heterogeneity. *Cytometry* **4**: 174-183, 1983.
13) Tirindelli-Danesi D, Teodori L, Mauro F, Modini C, Botti C, Cicconetti F, Stipa S: Prognostic Significance of Flow Cytometry in Lung Cancer: A 5-Year Study. *Cancer* **60**: 844-851, 1987.
14) Coon JS, Weinstein RS: Evaluation of Solid Tumors by Flow Cytometry: Methods and Interpretation. In: Coon JS, Weinstein RS (eds). Diagnostic Flow Cytometry United States and Canadian Academy of Pathology Inc., 1991, pp. 115-134.
15) Van Bodegom PC, Baak JPA, Stroet-Van Galen C, Schipper NW, Wisse-Brekelmans ECM, Vanderschueren RGJRA, Wagenaar SSC: The Percentage of Aneuploid Cells is Significantly Correlated With Survival in Accurately Staged Patients With Stage 1 Resected Squamous Cell Lung Cancer and Long-Term Follow Up. *Cancer* **63**: 143-147, 1989.
16) Salvati F, Teodori L, Gagliardi L, Signora M, Aquilini M, Storniello G: DNA Flow Cytometric Studies of 66 Human Lung Tumors Analyzed Before Treatment: Prognostic Implication: *Chest* **96**: 1092-1098, 1989.