

## Flow Cytometric Analysis of DNA Ploidy in Primary Non-small Cell Carcinoma of the Lung in Korea

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*Flow cytometrically determined nuclear DNA content has been measured on 74 formalin-fixed, paraffin embedded specimens of non-small cell carcinoma of the lung. Of the 60 tumors that were successfully analyzed, 32 (53%) were diploid and 28 (47%) were aneuploid. The mean DNA index of aneuploid tumor was  $1.5 \pm 0.25$ , ranging from 1.1 to 2.0. There was no significant correlation between DNA ploidy and the patient's clinical characteristics, histology of tumor, nodal status or tumor stage. Tumor ploidy was not found as a prognostic determinant in non-small cell carcinoma of the lung in this study.*

**Key Words:** Flow cytometry, ploidy, non-small cell carcinoma, lung

Lung cancer is a heterogenous disease comprising a wide spectrum of tumors with different behavior biologically, morphologically and therapeutically. The causes of such heterogeneity are not well understood and could be related to the conditions in which tumors arise, develop, and interact with the host cell system. Lung cancer is characterized by a poor prognosis, despite recent improvements in therapeutic procedures. To advance therapeutic approaches, the natural history, biologic behavior, and prognosis of the disease should be understood. DNA content analysis of solid tumors has been studied for the last 10 years, but there was no unified view of the relationship between the

DNA content of the tumor and clinical phenomena and the clinical significance of these data remains in doubt. Evaluation of the DNA content and proliferative index have been found to be significant prognostic factors in a variety of tumors (Barlogie *et al.* 1983; Friedlander *et al.* 1984; Merkel *et al.* 1987). In general, tumors with abnormal DNA content or a high proliferative fraction appear to have a more aggressive biologic behavior and poorer outcome (Fallenius *et al.* 1988; Sigurdsson *et al.* 1990). Flow cytometric study can rapidly quantitate the DNA content of isolated nuclei by measuring their fluorescence after staining with dyes which bind stoichiometrically to DNA (Moran and Melamed 1984). This technique is now in widespread use, but there is very little basic data concerning lung cancer using flow cytometry in Korea.

So the aim of this study was to determine the frequency of aneuploidy in Korean patients with surgically removed non-small cell carcinomas of the lung, to relate ploidy to clinical and pathological characteristics, and

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to assess the importance of ploidy as a prognostic determinant.

## MATERIALS AND METHODS

### Review of medical record

We examined archival paraffin blocks of carcinoma of the lung from 74 patients diagnosed histologically and treated by surgical excision at the Yonsei University Severance Hospital between January, 1986 and December, 1989. Survival times were calculated from the date of diagnosis to the date of death, or until Dec 31, 1992, for living patients.

### Sample preparation

Thirty to fifty  $\mu\text{m}$  sections of the paraffin blocks of each tumor were cut from these archival paraffin blocks. These sections were dewaxed and rehydrated with progressively decreasing concentrations of ethanol, by the technique of Hedley *et al.* (1983). The specimen was washed in distilled water and digested in 0.5% pepsin (Sigma Co.) solution at pH 1.5 for 37°C, 60 min with frequent vortex mixing. The cell pellet was suspended in phosphate buffered saline (pH 7.2) and stained with propidium iodide (Sigma Co.) with ribonuclease (BRL) added. And the samples were filtered through a nylon mesh (45  $\mu\text{m}$ ).

### DNA analysis by flow cytometry

Analysis of DNA content of single nuclei

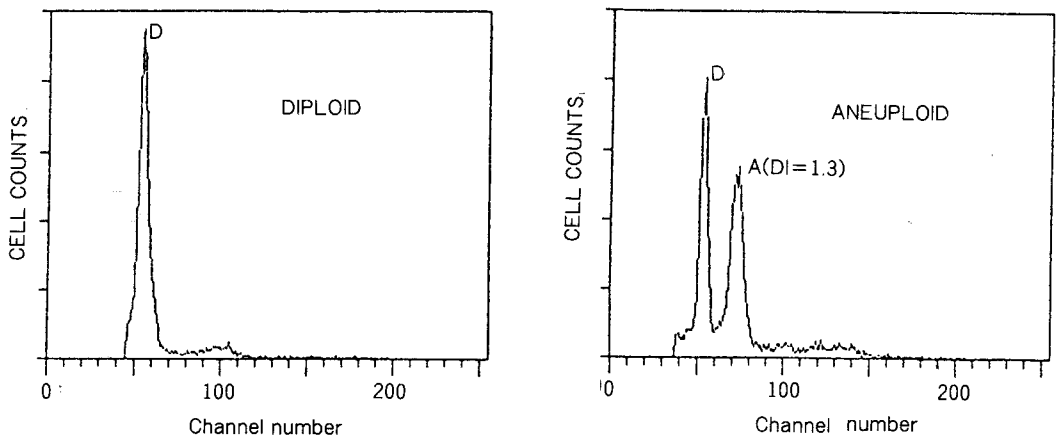
was done with FACStar (Fluorescent Activated Cell Sorter, Becton Dickinson Immunocytometry System, USA). Tumors in which the stemline DNA content is not measurably different from nonmalignant reference cells are referred to as diploidy and those with altered stemline DNA content as aneuploidy. The numerical ratio of the mean DNA content of phase G0-1 tumor cells to that of normal cells is the DNA index (DI). Histogram with a coefficient of variation exceeding 8 % were not used.

### Statistical analysis

The associations of tumor ploidy with histology and nodal status were analysed by chi-square test. The different groups were compared with the log rank test and survival curves were estimated by the Kaplan-Meier method.

## RESULTS

Of the 74 archival paraffin samples, 14, in which adequate DNA histograms were not obtained were excluded from analysis. A total of 60 patients that were successfully analyzed were evaluated for the study. The patient population consisted of 14 females and 46 males with an age range from 25 to 74 yr ( $58 \pm 11$  yr). Histologic types included 40 epidermoid carcinomas, 16 adenocarcinomas, 2

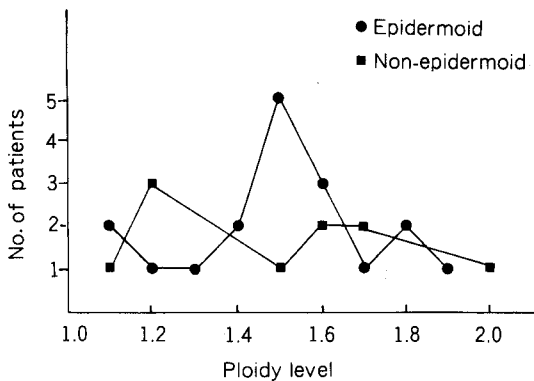


**Fig. 1.** DNA content histograms from patients with non-small cell lung cancer.

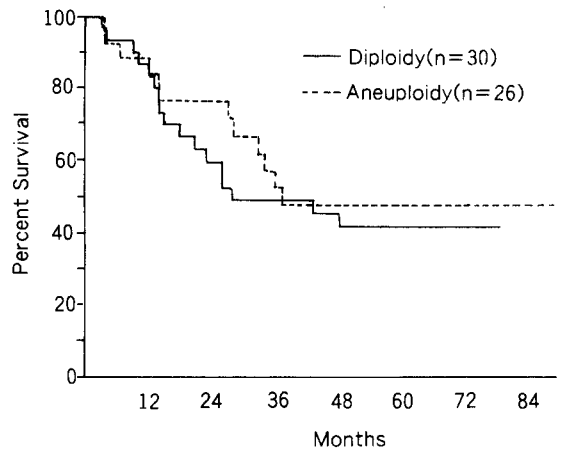
large cell carcinomas and 2 undifferentiated carcinomas. Twenty of 60 patients were evaluated as Stage I, 14 as Stage II, and 26 as Stage III. Analysis of the DNA content of the tumors revealed 32 (53%) diploid tumors and 28 (47%) aneuploid tumors. The typical example of diploid and aneuploid tumors are illustrated in Figure 1. The mean DNA index of aneuploid non-small cell carcinoma of the lung was  $1.5 \pm 0.25$ , ranging from 1.1 to 2.0. The mean CV and SD was  $5.4 \pm 1.22$ . The DNA indices of tumor clones observed in epidermoid carcinoma and non-epidermoid carcinoma are reported in Figure 2.

As seen in Table 1, there was no significant correlation between the DNA index and histology of the tumor, or nodal status. The

56 cases excluding 4 post-operative mortality cases could be enrolled in the prognostic study, in which the median follow-up period was 30 months (3~90 months). Overall survival at 1, 3 and 5-year for all patients were 84%, 51% and 44%, respectively. The 1, 3 and 5-year survival rates among patients with diploid tumors were 83%, 49% and 42%, respectively, whereas they were 84%, 53% and 48% in patients with aneuploid tumors ( $p > 0.1$ ) (Fig. 3). There was no correlation between the survival status and DNA index. The result of this analysis was the same in the epidermoid group ( $p > 0.1$ ) (Fig. 4).



**Fig. 2.** Distribution of DNA Ploidy levels of tumor clones observed in epidermoid carcinoma and Non-epidermoid carcinoma.



**Fig. 3.** Comparison of survivals according to DNA ploidy for all patients.

**Table 1.** Ploidy related to tumor histology and nodal status

	Diploidy	Aneuploidy	Total	X <sup>2</sup>	DNA index*
Epidermoid ca	22	18	40		$1.5 \pm 0.29$
Adenocarcinoma	9	7	16		$1.4 \pm 0.24$
Large cell ca	1	1	2		1.7
Undifferentiated	0	2	2	2.39	$1.6 \pm 0.56$
N0	17	11	28		$1.5 \pm 0.25$
N1~3	15	17	32	0.42	$1.5 \pm 0.25$
Total	32	28	60		$1.5 \pm 0.25$

\*DNA index of aneuploid tumor (mean  $\pm$  SD)

†coefficient of variation (mean  $\pm$  SD):  $5.4 \pm 1.22$

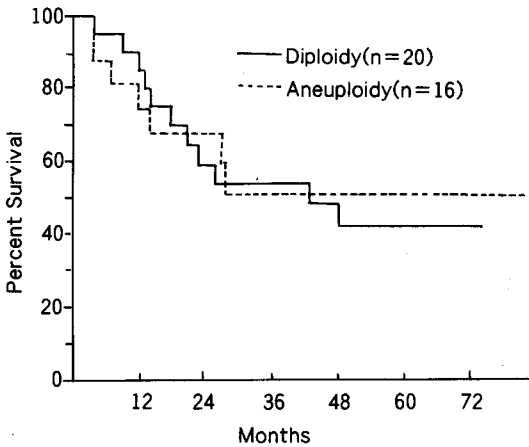


Fig. 4. Comparison of survivals according to DNA ploidy in epidermoid carcinoma.

## DISCUSSION

Though it is well known that chromosomal aneuploidy takes part in the development and progression of a tumor, there are many divergences of opinion as to its causal relation (Nasiell *et al.* 1978; Saccomanno *et al.* 1974; Tsutsui *et al.* 1983; Vindelov *et al.* 1980). The numeral and structural change of chromosomes can stimulate oncogene and cause overexpression or amplification of it (Knudson 1985). Flow cytometry permits a rapid, accurate, quantitative evaluation of the DNA content (ploidy) in large numbers of individual tumor cells.

The incidence of DNA aneuploidy in previous flow cytometric studies of carcinoma of the lung has been variable, ranging from 45% to 96% (Bunn *et al.* 1983; Salvati *et al.* 1989; Ten Velde *et al.* 1988; Tirindelli-Danesi *et al.* 1987; Volm *et al.* 1985; Zimmerman *et al.* 1987) and the highest value was obtained by Tirindelli-Danesi *et al.* (1987) who analyzed multiple samples. Isobe *et al.* (1990) reported that the proportion of DNA aneuploidy was statistically higher in adenocarcinoma than in epidermoid carcinoma. Moran and Melamed (1984) reported that mean DNA indices for adenocarcinoma were higher than those for

epidermoid carcinoma. In the present study using archival paraffin embedded tissue of surgically resected non-small cell carcinoma of the lung, the percentage of diploid tumors was 53% and that of aneuploidy was 47%. The mean DNA ploidy level of aneuploid tumors was  $1.5 \pm 0.25$ , ranging from 1.1 to 2.0. A cluster distribution appeared to be present at a DI value around 1.5 and it showed no difference between epidermoid and non-epidermoid carcinomas (Fig. 2). The frequency of aneuploid tumors in our study was less than that of most other studies using fresh tissues, but, similar to the value of Zimmerman *et al.* (1987) and Ten Velde *et al.* (1988) using paraffin embedded tissues. Zimmerman performed DNA flow cytometry on 100 surgically resected archival paraffin sections of non-small cell carcinoma of the lung and reported that 45% of tumors were aneuploid and 55% were diploid. Ten Velde reported that DNA aneuploidy was present in 44/67 cases (65%). In our opinion, this lower value is probably due to the sampling modality, to the paraffin embedded material used or to the technical and methodological inadequacies leading to an overestimation of diploidy incidence and an underestimation of aneuploidy. Carey *et al.* (1990) emphasized the importance of intratumor variation of DNA ploidy as a biologic parameter, reporting that they analyzed 208 individual tumor blocks from the 20 cases in which only one case was homogeneously diploid and one case was uniformly multiploid. They concluded that 95% of the tumor had evidence of aneuploidy and that 90% had intratumor variation in ploidy status. So it seems likely that the discrepancies observed between different studies result from different sampling techniques in a cancer which is markedly heterogenous with respect to DNA content.

Ploidy has no consistent relation to the clinical and pathological characteristics examined (Table 1). Some studies on carcinoma of the lung have also shown a possible relationship between DNA ploidy and biologic behavior of these tumors (Blondal and Lindgren 1982; Teodori *et al.* 1983; Volm *et al.* 1985). However, other reports provide no such evidence (Bunn *et al.* 1983). Although several authors have reported an inferior quality of DNA histograms in paraffin embedded tis-

sues compared with fresh tissues (Haag *et al.* 1987; Hedley *et al.* 1985), the advantage of paraffin embedded tissue is that survival data are retrospectively available. Though the data reported in the literature record a large variability among tumors of a different site, in terms of time free of disease and/or survival time, several authors have observed a better prognosis in diploid tumors than in aneuploid tumors. Recently, studies in a variety of tumors have suggested that analysis of cellular factors including tumor cell DNA content and proliferative growth fraction may correlate with cell growth and risk of tumor relapse (Barlogie *et al.* 1983; Friedlander *et al.* 1984; Merkel *et al.* 1987). This has been well studied in breast carcinoma in which the DNA content and proliferative fraction are significant independent prognostic variables (Fallenius *et al.* 1988; Sigurdsson *et al.* 1990). Fallenius *et al.* (1988) reported that patients with diploid, node-negative tumors were found to have an excellent prognosis of 95% probability of a 10-year survival and patients with aneuploid, node-positive tumors were shown to have an extremely bad prognosis with only 31% probability of a 10-year survival. Clark *et al.* (1989) also reported that the prognosis was particularly good in patients with diploid tumors and low S-phase-fraction value. The value of tumor ploidy and proliferative fraction analysis in bronchogenic carcinoma is less well established. Several studies have reported DNA content to be a significant prognostic variable in a variety of non-small cell types and stages. Zimmerman's (1987) group has reported that patients with aneuploid tumors had significantly shorter survival than those with diploid tumors and a subset of patients without node involvement at operation and with diploid tumors had a particularly long survival rate. Asamura *et al.* (1989) reported that nuclear DNA content of the recurrent group was significantly larger than that of the nonrecurrent group in the well differentiated and T1 subgroups, so the tumor DNA content accurately predicts prognosis in stage I adenocarcinoma of the lung. In contrast, other studies did not find tumor DNA content to be a significant independent prognostic variable (Cibas *et al.* 1989; Van Bodegom *et al.* 1989). For example, Bunn *et al.* (1983) found no evi-

dence to support the hypothesis that the degree of aneuploidy can provide prognostic information. In our 5-year follow-up study, no significant correlation was found between DNA ploidy and the patient's survival and there was no evidence that DNA ploidy provides prognostic information (Fig. 3). According to the current study of Cibas *et al.* it is possible that ploidy has no effect on the survival of patients with adenocarcinoma but has a great effect with epidermoid carcinoma. But in our epidermoid carcinoma group, there was no evidence that the DNA content was a prognostic factor for survival, either (Fig. 4). In this study, analysis of the S-phase fraction in the DNA histogram was not performed because of suboptimal histograms.

Therefore, it is concluded that 47% of Korean non-small cell carcinoma of the lung was aneuploid. There was no significant correlation between DNA ploidy and clinical characteristics, tumor histology, nodal status or tumor stage. Tumor ploidy was not found as a prognostic determinant in non-small cell carcinoma of the lung in this study. But considering the intratumoral variation of ploidy, we must take multiple site sampling into account in this kind of study.

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