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Cytophotometric DNA Analysis of Esophageal Dysplasia and Carcinoma Induced in Rats by *N*-Methyl-*N*-amylnitrosamine

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Abstract—*A series of esophageal lesions, mild, moderate and severe dysplasia, carcinoma* in situ (*CIS*), *early and advanced squamous cell carcinoma were induced in rats with* N-*methyl*-N-*amylnitrosamine* (*MAN*). *To evaluate the biological nature of each lesion, the ploidy level was estimated by microspectrophotometrical measurement of cell nuclear DNA content. DNA distribution patterns were classified into types I, II, III and IV, according to the degree of dispersion and the peak modal value on the DNA histogram. The incidences of type III of high ploidy in early cancer, CIS and severe dysplasia were* 3/11 (27.3%), 5/13 (33.3%) *and* 4/16 (25%), *respectively. On the other hand, in moderate and mild dysplasia,* 15/16 (93.8%) *and* 20/21 (95.2%) *were low ploidy (types I and II), respectively. The mean DNA content of advanced and early cancer, CIS and severe dysplasia were* 3.88*c*, 3.34*c*, 3.24*c and* 3.13*c*, *respectively, while those of moderate and mild dysplasia were near diploid, showing* 2.67*c and* 2.51*c*, *respectively. These findings indicate that the biological nature of severe dysplasia may be considered as serious a lesion as cancer, in terms of DNA analysis. Cytophotometric DNA analysis aids the evaluation of various degrees of dysplasia and carcinoma of the esophagus.*

INTRODUCTION

PRECANCEROUS conditions of the esophagus have been known, since Druckrey *et al.* [1] reported the induction of esophageal carcinoma in rats by *N*-methyl-*N*-nitroanilin. There were two main opinions, one was that papilloma was the source of esophageal cancer [2] and the other was that a hyperplasia or dysplasia would progress to carcinoma [3, 4]. This was deduced from morphological and histological observations of lesions in the esophagus of rats, induced with various alkylnitrosamines. However, a valid definition of the precursors of esophageal cancer remains uncertain. Histological observations have limitations when attempting to estimate the biological nature of precancerous lesions and the sequence of a carcinogenic process.

Cytophotometric DNA analysis is a reproducible and useful method for estimating the degree of DNA aneuploidy of tumor cells and representation of the malignant potentiality of clinical specimens [5, 6]. We have determined the cell nuclear DNA content, microspectrophotometrically, in the cases of early and advanced esophageal carcinoma and found that patients with esophageal carcinoma with high ploidy (types III and IV) DNA distribution pattern usually died of a recurrence shortly after curative operation, and that patients with low ploidy (types I and II) had an uneventful clinical course [7, 8]. Thus, an analysis of the DNA pattern of esophageal carcinoma should facilitate assessments of the malignant potentiality of the tumor. In addition, DNA analysis can be used for investigation of carcinogenic processes in various experimental models, i.e. gastric tumors [9], colonic tumors [10] and hepatic tumors [11]. Such detailed studies using DNA analysis have not been documented in animal models of esophageal carcinoma. In the present study, the DNA content was measured in the series of lesions in the esophagus of rats, induced by *N*-methyl-*N*-amylnitrosamine (MAN). The biological nature of the dysplastic lesions is discussed in terms of nuclear aneuploidy.

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Abbreviations: MAN, *N*-methyl-*N*-amylnitrosamine; CIS, carcinoma *in situ*.

MATERIALS AND METHODS

Twenty-three, male, 4-week-old Wistar rats weighing about 100 g and purchased from Charles River Japan Inc., Kanagawa, were used in this study. They were fed standard pellet food (CE-2, CLEA Japan Inc., Tokyo). *N*-Methyl-*N*-amylnitrosamine (MAN), obtained from Iwai Kagaku Yakuhin Co. Ltd., Tokyo, was dissolved in deionized water at a final concentration of 0.003%. Sixteen rats were given this MAN solution for the first 12 weeks as drinking water followed by tap water for the next 16 weeks *ad libitum*. The control group of seven rats drank tap water only for the 28 weeks. All animals were observed for 28 weeks after initiation of the regimen and were autopsied at death or at 28 weeks. All organs were observed macroscopically and the entire esophagus and forestomach were resected, fixed in 10% neutral formalin solution, cut into 10–15 longitudinal strips and each strip was embedded in paraffin. A 5 μm-thick paraffin section from each block was stained with hematoxylin and eosin (H & E) and reviewed by the same pathologist. The histological diagnosis of mild, moderate and severe dysplasia, carcinoma *in situ* (CIS) was made according to the criteria of Enterline and Thompson [12], and early and advanced squamous cell carcinoma were also defined as follows:

(1) Mild dysplasia: irregular alteration of the first several layers of the basal epithelium with cellular atypism (Fig. 1).
(2) Moderate dysplasia: abnormal proliferative zone encompassing from a quarter to a half of the thickness of the epithelium (Fig. 2).
(3) Severe dysplasia: abnormal proliferative zone encompassing from half to three-quarters of the thickness (Fig. 3).
(4) Carcinoma *in situ* (CIS): abnormal proliferative zone encompassing the entire thickness of the mucosa (Fig. 4).
(5) Early squamous cell carcinoma: invasive proliferation of cancer cells restricted to the mucosal or submucosal layer (Fig. 5).
(6) Advanced squamous cell carcinoma: proliferation of cancer cells invading beyond the proper muscular layer (Fig. 6).

A 10 μm-thick section, just adjacent to the same H & E lesion, was stained by the Feulgen method, according to Naora [13] for DNA analysis. Cell nuclear DNA content was measured in these sections, by the two-wavelength method [14] using a microspectrophotometer (MMSP Olympus Co. Ltd., Japan) as reported previously [7, 8]. The DNA content was measured in 25 stromal lymphocytes and their mean DNA content was defined as diploid (2c). The relative DNA content, compared with the 2c value, was determined in 100 cells of each lesion on the same section and the DNA histograms were constructed using a personal computer (HE-85, U.S.A.). The distribution patterns of these histograms are shown in Fig. 7 and grouped into four types as follows:

Type I: a prominent peak in the 2c diploid region with a narrow range of dispersion to the 4c region.
Type II: a relatively high peak in the 2c–3c regions with a dispersion to the 6c region.
Type III: a low peak beyond the 3c region with less than 20% of cells over the 6c region.
Type IV: more than 20% of cells beyond the 6c region.

In addition, the mean DNA content of each lesion was also measured.

Statistical significance was determined by Student's *t* test. A P value of < 0.05 was considered to be statistically significant.

RESULTS

The incidence of lesions is summarized in Table 1. Macroscopically, there were two types of proliferation of lesions, flat and papillomatous. Histologically, various degrees of dysplasia and cancerous changes were identified in both types of proliferation. A series of lesions were classified histopathologically according to the criteria described above, irrespective of their macroscopic growth pattern. Squamous cell carcinoma of the esophagus was present in 15 rats (93.8%) treated with MAN and their total were 44 esophageal lesions, including 16 CIS in seven rats (43.8%), 11 early cancer in seven rats (43.8%) and 17 advanced cancer in 11 rats (68.8%) (Table 1). All CIS, early and advanced cancers were moderately or well differentiated squamous cell carcinoma and one advanced cancer had metastasized to the paratracheal lymph node. There was no evident macroscopic tumor in any other organ in the rats given MAN. In the control rats, no tumor developed in any organ.

Cell nuclear DNA content was cytophotometrically measured in 21 lesions of mild dysplasia, 16 moderate dysplasia, 16 severe dysplasia, 15 CIS, 11 early cancer and 17 advanced cancer. The DNA content of 11 normal esophageal epithelium was measured in seven control rats. As shown in Fig. 8, mean DNA content of the normal epithelium was diploid with 2.00c and those of mild and moderate dysplasia were near diploid with a mean of 2.51c and 2.67c, respectively. On the other hand, severe dysplasia, CIS, early and advanced cancer exhibited a greater than triploid DNA content of 3.13c, 3.24c, 3.34c and 3.88c, respectively. There was a statistical difference in the DNA content of normal mucosa and each type of lesion ($P < 0.001$). The mean DNA content of severe dysplasia was statisti-

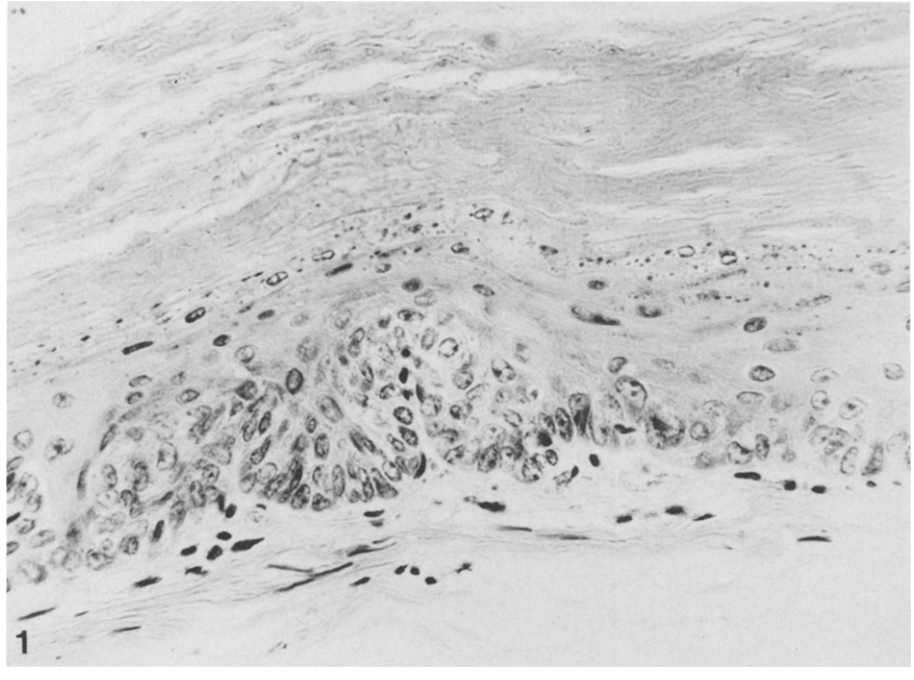

Fig. 1. *Mild dysplasia* (*H* & *E*, *original magnification* ×540).

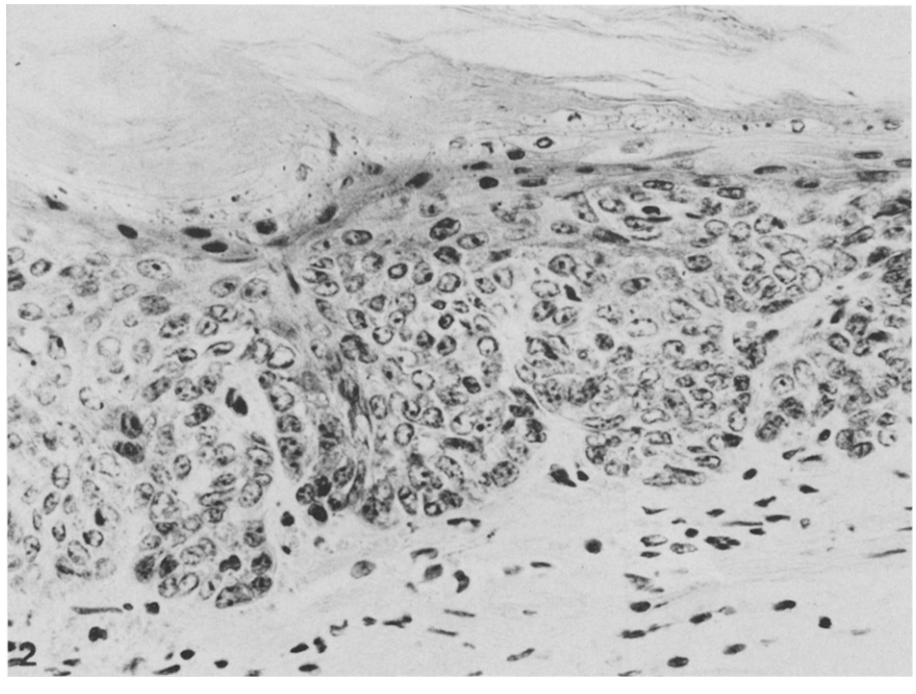

Fig. 2. *Moderate dysplasia* (*H* & *E*, *original magnifification* ×540).

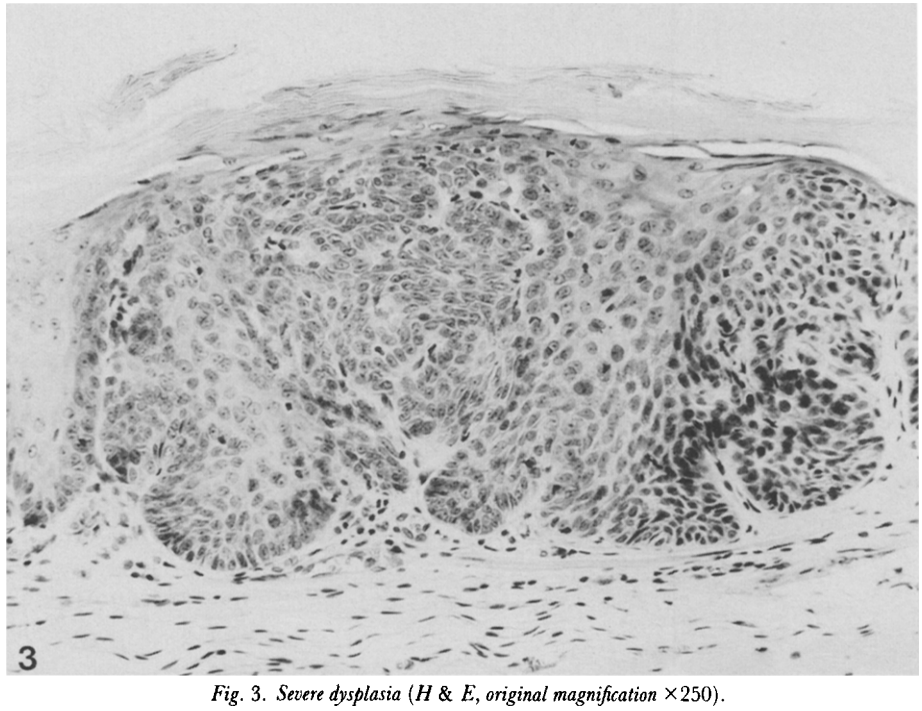

Fig. 3. *Severe dysplasia* (*H & E, original magnification* ×250).

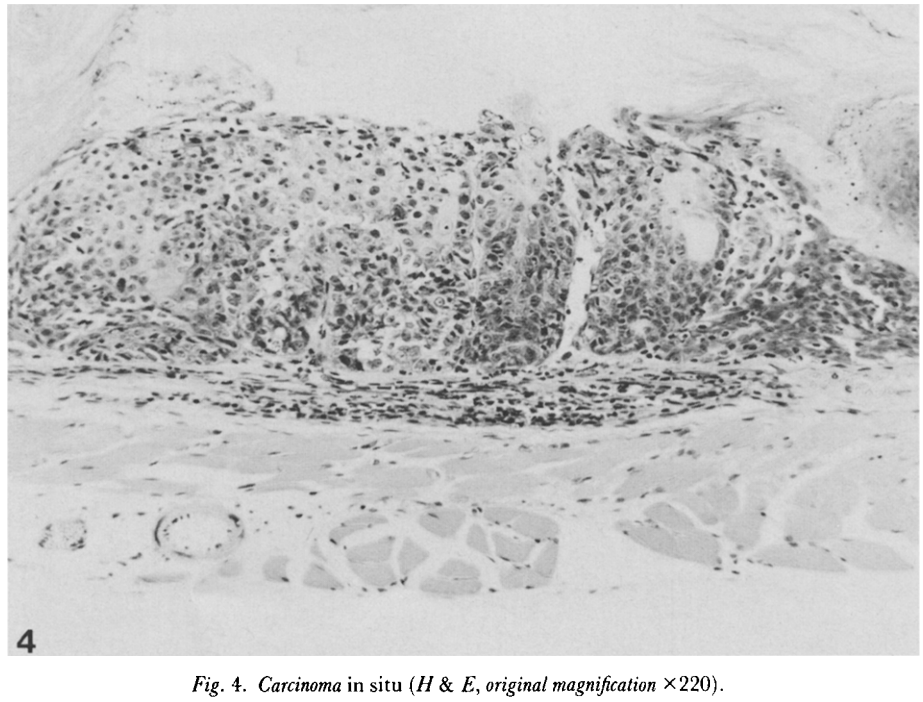

Fig. 4. *Carcinoma* in situ (*H & E, original magnification* ×220).

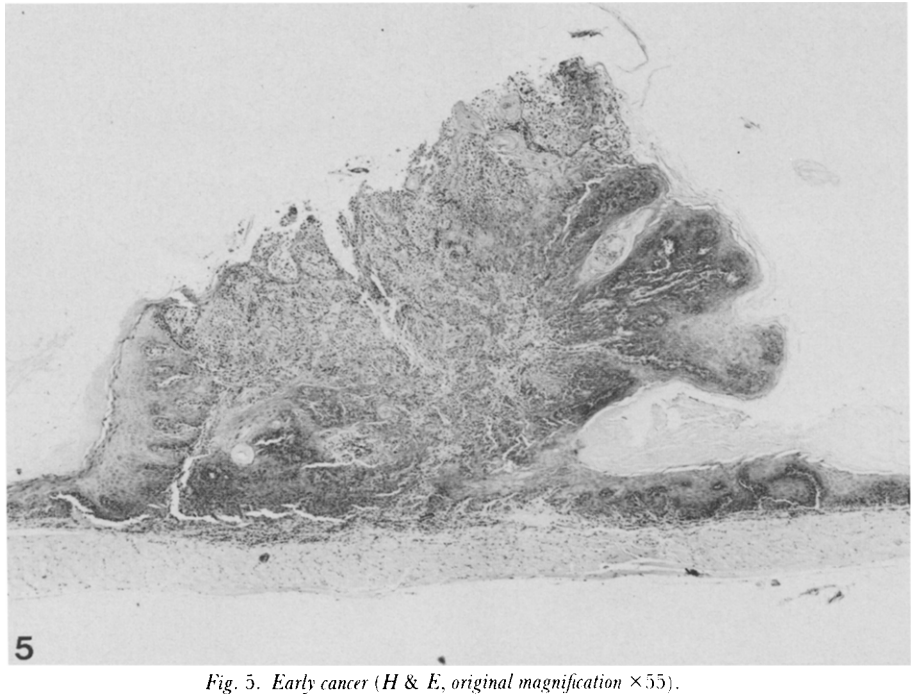

Fig. 5. Early cancer (H & E, original magnification ×55).

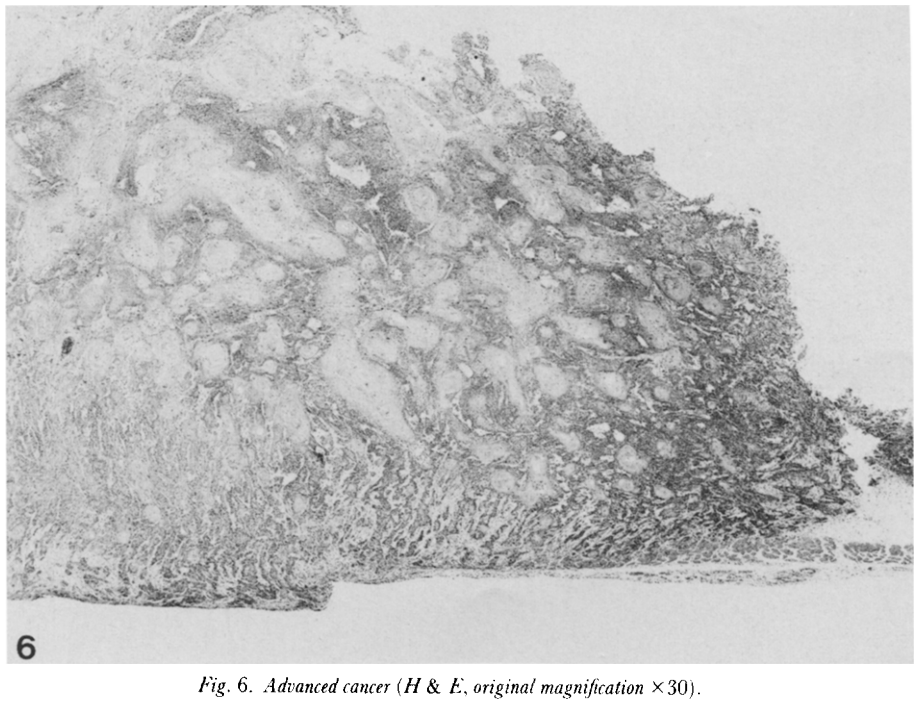

Fig. 6. Advanced cancer (H & E, original magnification ×30).

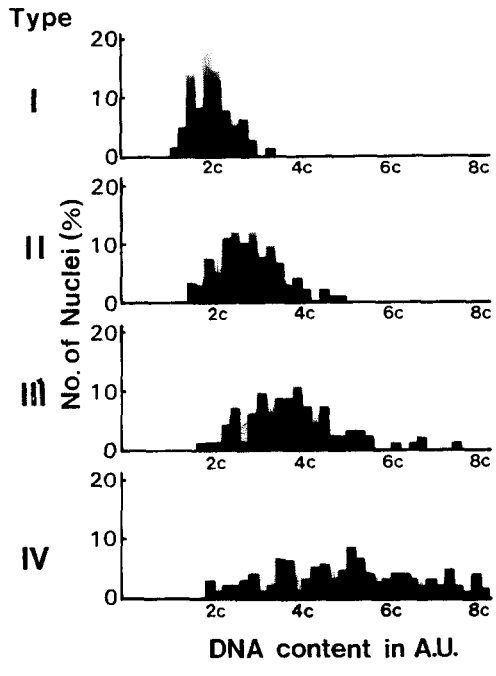

Fig. 7. Histogram of nuclear DNA content. Type I: peak: 2c (diploid) region; dispersion: to 4c region; Type II: peak: 2c–3c regions; dispersion: to 6c region; Type III: peak; beyond 3c region; over 6c region: less than 20% of cells. Type IV: over 6c region: more than 20% of cells.

cally higher than those of mild and moderate dysplasia ($P < 0.01$). There were no statistical differences among severe dysplasia, CIS and early cancer.

The proportion of the types of DNA pattern in each lesion is shown in Table 2. All normal esophageal mucosae were type I and mild dysplasia consisted of 20 lesions of type I and II (low ploidy) and only one lesion of type III of high ploidy. In moderate dysplasia, 15 lesions (93.8%) were types I and II and one lesion (6.3%) was type III. On the other hand, severe dysplasia consisted of four of type III of high ploidy (25%) and 12 of types I and II (75%). In CIS, there were five of type III (33.3%) and 10 of type II (66.7%). Furthermore, early cancer also included three of type III (27.3%) with a similar proportion to those of severe dysplasia and CIS. Nine of 17 advanced cancers were high ploidy including seven of type III (41.2%) and two of type IV (11.8%).

The DNA distribution pattern of a metastatic lymph node was type IV, arising from an advanced primary cancer of type III.

DISCUSSION

The behavior of clinical precancerous lesions in various organs has been studied, i.e. the uterine cervix [15, 16], stomach [17] and breast [18]. Dysplastic lesions of the uterine cervix are considered to be precursors of cervical cancer [15, 16]. In clinical studies of the esophageal lesions, esophagitis [19] and dysplasia [20] were suggested to be precursors of cancer. In a study in a high-incidence area of esophageal cancer in the Republic of China [20], the incidence of dysplasia coexistent with esophageal cancer was found to be high. Furthermore, esophageal cancer developed from severe dysplasia, determined in prospective studies on patients with severe dysplasia. Thus, dysplasia both in the cervix and in the esophagus was closely correlated with malignancy. However, there is considerable argument about classifying intraepithelial neoplastic lesions into dysplasia and carcinoma *in situ*, by clinical and histological observations, as the definition of these two terms is not agreed universally by pathologists.

Cytophotometric and flow cytometric DNA analyses provide information on the malignant potentiality or biological nature of the lesions. It was found that the neoplastic progression correlated with the DNA aneuploidy. Jakobsen *et al.*, measuring the DNA content of cervical lesions, reported that the ploidy level increased with the degree of dysplasia of the cervical mucosa and that severe dysplasia appeared to be a precancerous lesion of cervical cancer [21]. In our studies on esophageal models in rats, the mean DNA content in severe

Table 1. Incidence of esophageal lesions induced by MAN

Histologic findings	Incidences of lesions		Total number of lesions
Carcinoma	15 rats	(93.8)	44
Carcinoma *in situ*	7	(43.8)	16
Early cancer	7	(43.8)	11
Advanced cancer	11	(68.8)	17
Dysplasia	16	(100)	265
Mild dysplasia	16	(100)	87
Moderate dysplasia	16	(100)	82
Severe dysplasia	15	(93.8)	96

Percentage indicated in parentheses.

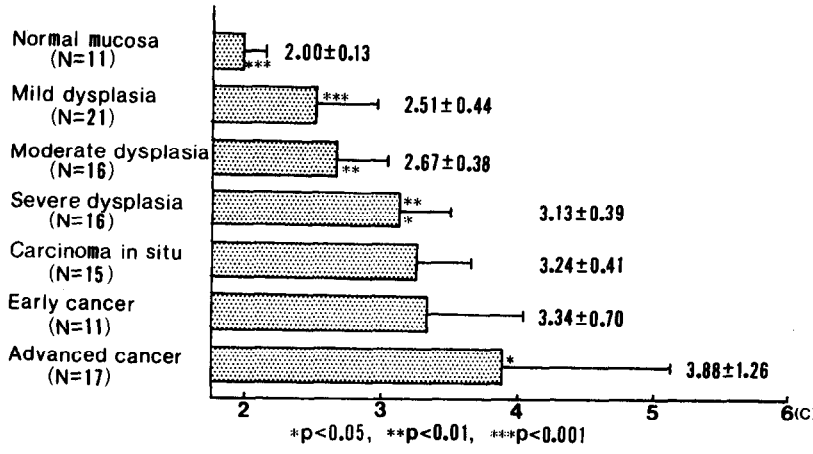

Fig. 8. In mean DNA content of a series of lesions, there are statistical differences between normal mucosa and mild dysplasia ($P < 0.001$), *between moderate and severe dysplasia* ($P < 0.01$) *and between severe dysplasia and advanced cancer* ($P < 0.05$), *while no difference is recognized between severe dysplasia and CIS or early cancer.*

Table 2. DNA distribution pattern in MAN-induced esophageal lesions in rats

Histological findings		DNA distribution pattern			
		Low ploidy		High ploidy	
		Type I	Type II	Type III	Type IV
Normal mucosa	(n = 11)	11 (100)	0	0	0
Dysplasia					
Mild dysplasia	(n = 21)	14 (66.7)	6 (28.6)	1 (4.8)	0
Moderate dysplasia	(n = 16)	7 (43.8)	8 (50.0)	1 (6.3)	0
Severe dysplasia	(n = 16)	1 (6.3)	11 (68.8)	4 (25.0)	0
Carcinoma					
Carcinoma *in situ*	(n = 15)	0	10 (66.7)	5 (33.3)	0
Early cancer	(n = 11)	0	8 (72.7)	3 (27.3)	0
Advanced cancer	(n = 17)	0	8 (47.1)	7 (41.2)	2 (11.8)

Percentage indicated in parentheses.

dysplasia was statistically higher than that of mild and moderate dysplasia and similar to findings in case of CIS and early cancer. Moreover, severe dysplasia, CIS and early cancer included similar proportions of type III distribution of high ploidy, while mild and moderate dysplasia mainly consisted of types I and II patterns (low ploidy). Our findings strongly suggest that severe dysplasia has a biological nature similar to that of CIS and even to early invasive carcinoma. In advanced cancer, the highest level of DNA aneuploidy suggested a highly malignant potentiality.

Early histopathological studies on experimental esophageal cancer in rats suggested that papilloma was the source of carcinoma [2]. Few papilloma-related lesions occur clinically [22] and papillomatous lesions also show various degrees of dysplasia [23]. Thus, we classified all lesions, whether they were papillomatous or flat macroscopically, into various degrees of dysplasia and carcinoma according to their histopathologic features.

A metastatic lymph node showed type IV of high ploidy, that is similar to the high ploidy of type III in the primary esophageal cancer, in the same rat. These findings are compatible with our view that clinical tumors with high ploidy more frequently metastasize to the lymph nodes than do those with low ploidy [8]. Moreover, findings that the ploidy levels between primary tumor and metastatic lymph node were similar are compatible with the hypothesis that the ploidy level of cancer cells is fairly stable [24, 25].

Little is known of the relationship between precancerous and malignant lesions or the mechanism of carcinogenesis. Whether or not a cancer arises as a continuous process through dysplasia and CIS or progresses through a different mechanism remains unknown. Our conclusion, from DNA analysis, is that severe dysplasia can be considered as serious a lesion as CIS.

It is important, of course, to detect the carcinoma of the esophagus of an early stage. Thus, when a

severe dysplasia of the esophagus presents, we follow it carefully by frequent biopsies observing both the histopathological features and DNA distribution.

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