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Morphometric analysis of gallbladder adenocarcinoma: discrimination between carcinoma and dysplasia

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Summary. To characterize the cytological features of well differentiated adenocarcinoma of the gallbladder, a comparative morphometric analysis was made using 35 histologically classified cases of invasive well differentiated adenocarcinoma, 13 cases of mild dysplasia, 19 cases of severe dysplasia, and 22 control gallbladders. The variables analyzed were nucleocytoplasmic (N/C) ratio and nuclear area (N.A.). Both the mean values of N/C ratio and N.A. demonstrated a progressive increase from control to mild dysplasia, to severe dysplasia and to carcinoma. The differences were statistically significantly different. Discriminant analysis was made with a set for learning and a set for testing, selected from the 89 lesions with random numbers. Using this discriminant function, all the cases except one carcinoma were discriminated as carcinoma, and all the cases of control, adenoma, and mild dysplasia were discriminated as benign lesions. However, cases of severe dysplasia were subdivided into benign or malignant. These results indicate that morphometric analysis clearly differentiates carcinomas from benign lesions, and that the dysplastic mucosal lesions can be divided into benign and malignant, although some difficult borderline lesions exist.

Key words: Morphometry – Gallbladder carcinoma – Dysplasia – N/C ratio – Nuclear area

Introduction

The development of new image-analysis techniques has facilitated detection of early carcinoma of the gallbladder. However, there are no definite criteria for carcinoma in situ or mucosal carcinoma of the gallbladder. Dysplastic mucosal lesions with various degrees of cellular atypia are often observed in the gallbladder with stones or adjacent to gallbladder carcinoma, and in some of them it is difficult to differentiate whether they are carcinomas or not.

Dysplastic lesions have been assumed to be one of the precursor lesions of gallbladder carcinoma (Albores-Saavedra et al. 1980, 1986; Laitio 1983a, 1983b; Dowling and Kelly 1986; Yamagiwa and Tomiyama 1986; Yamamoto et al. 1988). Therefore, the definition of carcinoma in situ and dysplasia is important for the diagnostic pathology and for the study of histogenesis of gallbladder carcinoma. Some authors have described the criteria for differentiating carcinoma in situ from dysplasia based on the degree of loss of architecture and nuclear atypia. However, these criteria are subjectively determined and differential diagnosis is not always easy (Laitio 1983a; Dowling and Kelly 1986; Albores-Saavedra et al. 1986). Morphometric analysis has been used to differentiate between dysplasia and carcinoma in situ of the uterine cervix (Foraker and Reagan 1956), colon (Eide 1986), liver (Kondo et al. 1987), ovary (Baak and Derley 1984), and nasal cavity (Boysen and Reith 1983). However, no morphometric data are available for the gallbladder. In the present study, a morphometric analysis was made for quantitating the cytological characteristics of invasive well differentiated adenocarcinoma in comparison with those of dysplastic and normal gallbladder mucosa in order to develop an objective criteria for differentiating carcinoma and dysplasia.

Materials and methods

Eighty-five surgically resected gallbladders comprising 38 cases of chronic cholecystitis with cholelithiasis, 12 cases of adenoma

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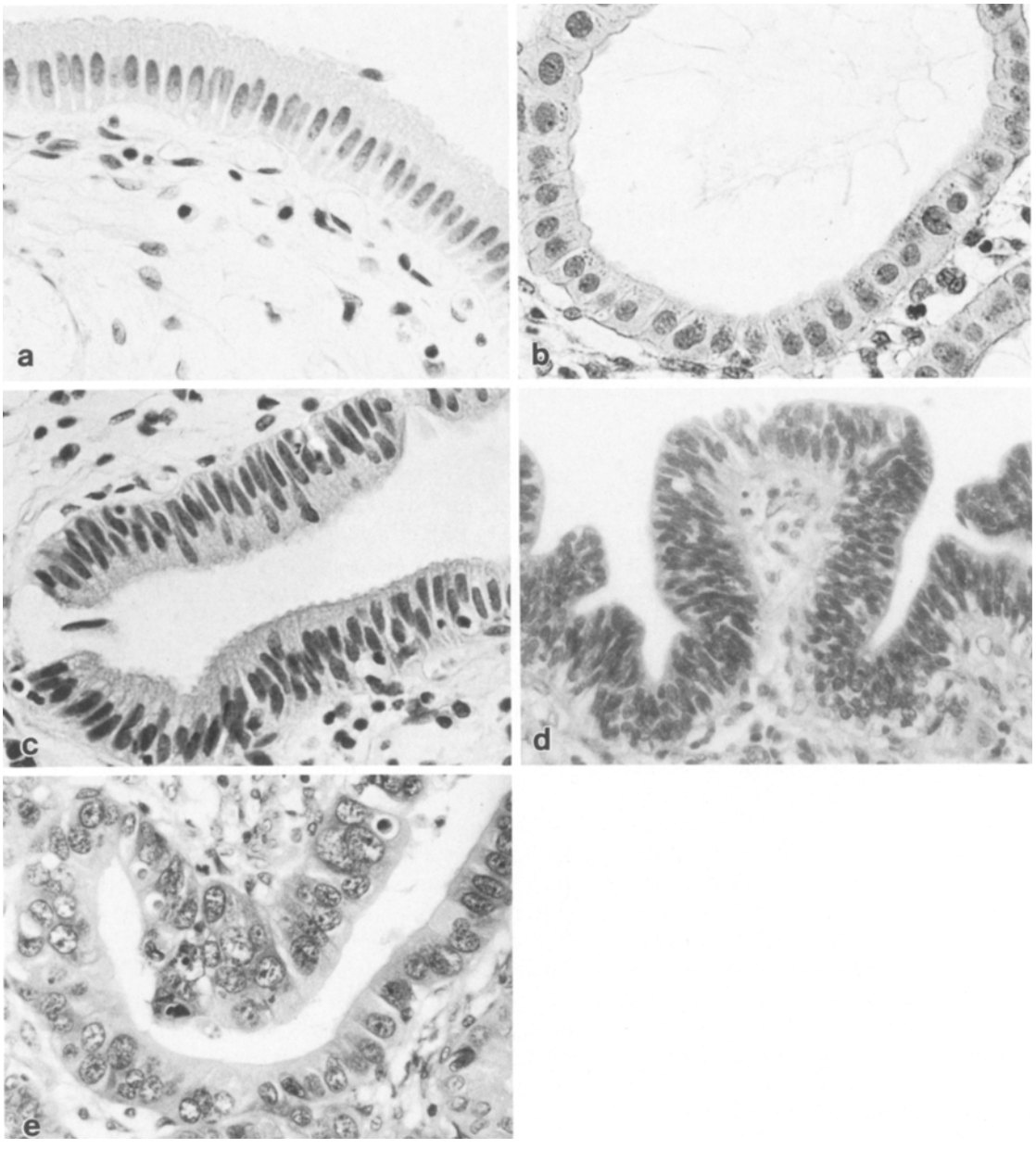

Fig. 1a–e. Photographs of various types of gallbladder epithelium. H & E, ×570. **a** Control. The mucosa consists of a layer of columnar cells with oval and uniform nuclei situated within the basal halves of the cells. **b** Adenoma. The proliferative glands consist of cuboidal or columnar cells with oval to round nuclei. N/C ratio and N.A. are increased. **c** Mild dysplasia with crowding and stratification of hyperchromatic elongated nuclei. Their nuclei are mostly within the basal halves of the cells. **d** Severe dysplasia. The cells are severely crowded and stratified and their nuclei are hyperchromatic and extended into the luminal halves of the cells. **e** Adenocarcinoma. The nuclei are large and irregular in shape and stratified. N/C ratio is increased

(Fig. 1b), and 35 cases of invasive well differentiated adenocarcinoma (Fig. 1e) were examined. The specimens were fixed in 10% buffered formalin, embedded in paraffin and cut into 4 μm sections. The sections were stained with haematoxylin and eosin.

According to the criteria for dysplasia described by Dowling and Kelly (1986), the 38 cases of chronic cholecystitis were classified into 22 cases without cellular atypia as controls (Fig. 1a), 13 cases with mild cellular atypia as mild dysplasia (Fig. 1c), and three cases with severe cellular atypia as severe dysplasia. Sixteen cases of invasive adenocarcinoma contained mucosal lesions with severe cellular atypia adjacent to the carcinoma, and these lesions were also used as severe dysplasia (Fig. 1d). Some of these severe dysplastic lesions might be designated carcinoma in situ in view of their nuclear atypia, but they showed neither structural atypia nor invasive growth. Therefore, we tentatively classified these lesions into severe dysplasia.

Morphometric analysis was made using a commercially available semiautomatic image analyzer (Cosmozone-s, Nikon,

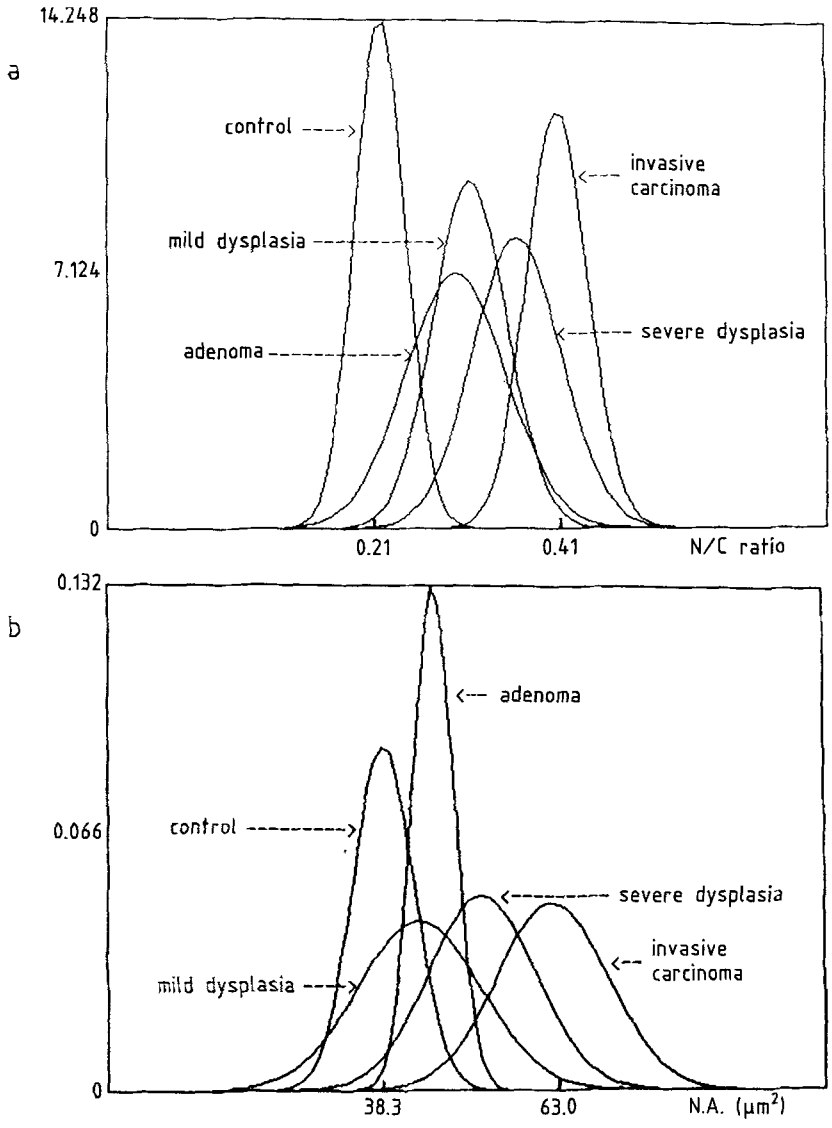

Fig. 2. Frequency distribution histograms of N/C ratio (**a**) and N.A. (**b**). The mean values of N/C ratio and N.A. increase according to the extent of cellular atypia. The mean values of N/C ratio and N.A. in adenomas are between those of mild and severe dysplasia

Tokyo, Japan) consisting of a digitizer connected with a microcomputer (NEC PC9801). The sections were observed by means of a microscope. The image of the microscopic field was analogue-digital converted and projected into the monitor screen (final enlargement, ×1570). Using the digitizer, N/C ratio and N.A. were measured. Sampling numbers were determined according to Baak et al. (1985). The sampling was systematic with a random start. N.A. was calculated in measuring the outlines of longitudinally sectioned nuclei. N/C ratio was calculated by measuring the outline of an area containing an average of 20 cells and the outline of each nucleus included in this area. In each case, ten areas were measured at random. In cases of invasive adenocarcinoma, N/C ratio and N.A. of both the mucosal portion and invasive portion were individually measured. In the measurement of mucosal portion of carcinoma, central area of mucosa of the tumour tissue was selected.

The numerical taxonomy of all the lesions was attempted by cluster analysis (Ward 1963; Hartigan 1975) using only the evaluated mean values of N/C ratio and N.A. The correlation between the grouping of cluster analysis and morphological grouping such as control, mild dysplasia, severe dysplasia, adenoma, and carcinoma was examined.

Discriminant analyses were made with a set for learning and a set for testing, selected from 89 lesions (containing 35 adenocarcinomas, 32 dysplasias and 22 controls) with random numbers (Baak and Oort 1983). Linear discriminant function was used to compare the groups (Lachenbruch 1975). The 16 mucosal carcinomas which were diagnosed histologically were also discriminated with the obtained discriminant function. In these cluster and discriminant analyses, the library software developed by Tanaka et al. (1984) was employed.

In order to determine whether the changes in N/C ratio and N.A. were continuous or not between neoplastic and non-neoplastic lesions, the N/C ratio and N.A. of ten cells were measured at 3 mm intervals on the slide glass with the maximum diameter of the tumour. These measurements were made on 13 mucosal carcinomas and 10 invasive carcinomas.

In order to investigate the presence of field-to-field variations wihtin one section, analysis of variance in each field was made according to Baak et al. (1985), and showed no significant differences. To find the number of nuclei to be measured, the running mean procedure (Baak et al. 1985) was applied. 50 nuclei were sufficient to have a cumulative average within the 95% limits. To be on the safe side, 100 nuclei were measured

at random and 10 areas were sufficient to reach this number. Using this procedure, the intra- and inter-observer variation was assessed. Student's t-test was applied to determine whether there was consistent intra- or inter-observer difference. No significant difference was detected between the observers (S.N. and M.Y.)

The Chi-square test was used to investigate the normality of the variables. The test failed to reject the hypotheses of normality. Student's t-test was used to evaluate the significant differences among the groups.

Results

N/C ratio and N.A. in various lesions are summarized in Tables 1, 2. The frequency distribution histograms of N/C ratio and N.A. in various lesions are shown in Fig. 2. The mean values of N/C ratio and N.A. increased according to the extent of cellular atypia. N/C ratio and N.A. were significantly different ($P<0.01$) between the groups except for the difference between adenoma and mild dysplasia. In carcinoma cases, the difference of mean N/C ratio and N.A. between invasive portion and mucosal portion was statistically significant.

Table 1. N/C ratio of various lesions in the gallbladder

Histology	Number of cases	N/C ratio (Mean ± SD)	
Control	22	0.217 ± 0.028	* *
Adenoma	12	0.301 ± 0.056	
Dysplasia mild	13	0.311 ± 0.035	*
severe	19	0.373 ± 0.046	*
Invasive carcinoma mucosal portion	35	0.405 ± 0.034	* *
invasive portion		0.414 ± 0.038	

* $P<0.01$; ** $P<0.05$, Student's t-test

Table 2. Nuclear area of various lesions in the gallbladder

Histology	Number of cases	Nuclear area (Mean ± SD) μm^2	
Control	22	38.3 ± 4.4	* *
Adenoma	12	45.6 ± 3.0	
Dysplasia mild	13	43.5 ± 9.0	*
severe	19	52.9 ± 7.9	*
Invasive carcinoma mucosal portion	35	68.4 ± 9.3	**
invasive portion		63.0 ± 8.1	

* $P<0.01$; ** $P<0.05$, Student's t-test

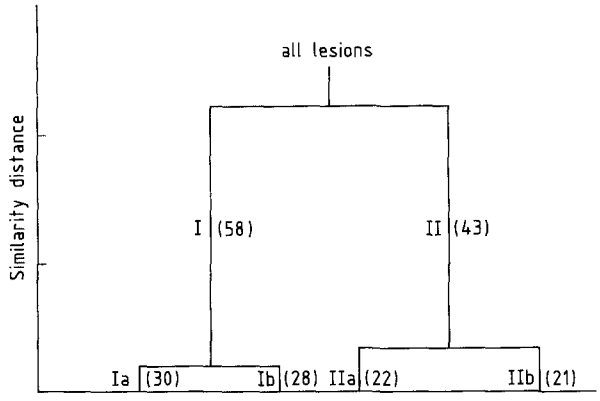

Fig. 3. Dendrogram of cluster analysis. All the lesions are divided by numerical taxonomy using the evaluated mean N/C ratio and N.A. into two groups (I and II), and each group is further subdivided into two groups (Ia, Ib and IIa, IIb). Y-axis means the degree of similarity. The larger distance of Y-axis, the smaller similarity of N/C ratio and N.A. X-axis shows the distribution of lesion number. Parentheses mean the number of lesions

Table 3. Comparison between cluster analysis and histological group

Clustered group	Histological group	
Ia	Control	21 cases
	Adenoma	5 cases
	Mild dysplasia	4 cases
Ib	Control	1 case
	Adenoma	7 cases
	Mild dysplasia	9 cases
	Severe dysplasia	10 cases
	Carcinoma	1 case
IIa	Severe dysplasia	5 cases
	Carcinoma	17 cases
IIb	Severe dysplasia	4 cases
	Carcinoma	17 cases

The results of cluster analysis are shown in Fig. 3. The dendrogram showed that all the lesions were divided broadly into two groups, clustered group I (cluster I) and clustered group II (cluster II). In comparing these two groups by cluster analysis with the histological groups, cluster I contained most of the cases of control, adenoma and mild dysplasia, and half of the cases of severe dysplasia, whereas cluster II contained most of the cases of carcinoma and half of the cases of severe dysplasia (Table 3). The exceptional example of carcinoma belonging to cluster I was highly well differentiated carcinoma with prominent mucin production and was histologically difficult to diag-

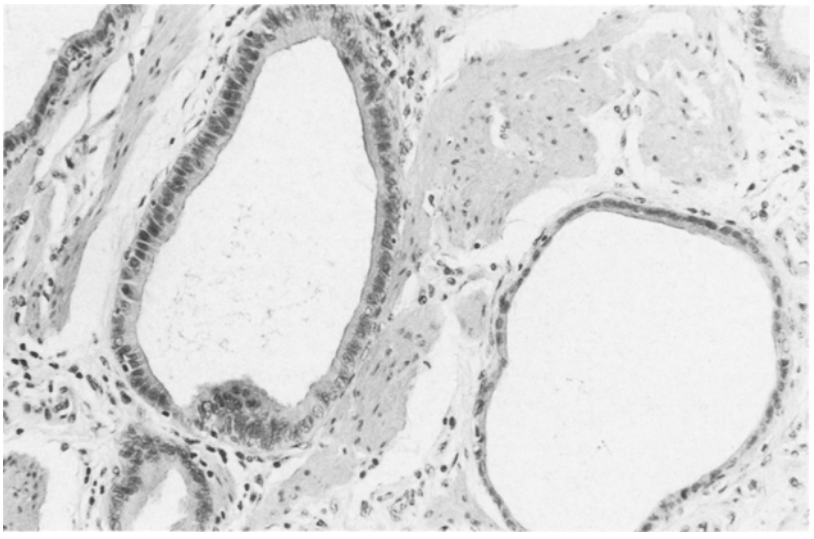

Fig. 4. An exceptional example of well differentiated adenocarcinoma which is not discriminated as carcinoma according to the derived discriminant function. The gland invades the muscular layer. H & E, ×285

Table 4. Confusion matrix of morphometry with discriminant analysis

	Total	Malignant	Benign	Correct discrimination
Invasive carcinoma	35	34	1	97.1%
Other lesions	54	9	45	83.3%

Table 5. Confusion matrix of morphometry with discriminant analysis in severe dysplasia and mild dysplasia

	Total	Severe dysplasia	Mild dysplasia	Correct discrimination
Severe dysplasia	19	16	3	84.2%
Mild dysplasia	13	4	9	69.2%

nose as carcinoma without the finding of invasive growth (Fig. 4). Moreover, these two clustered groups were subdivided into two groups, respectively (Fig. 3). Cluster Ia was mainly composed of controls, and cluster Ib contained the groups showing various dysplastic changes but not the group of carcinoma (Table 3). Cluster II was subdivided into IIa and IIb. Cluster IIa contained more cases of severe dysplasia than cluster IIb. As for cases of severe dysplasia, they were distributed separately in three groups of clusters, Ib, IIa, and IIb.

Discriminant analysis was made between carcinoma and other lesions, such as control, mild dysplasia, and severe dysplasia. The discriminant formula was as follows:

$$+0.2641 \times \text{N.A.} + 16.3522 \times \text{N/C ratio} - 19.8326$$

The lesions with a value greater than or equal to zero were regarded as malignant. In the learning set, all the 18 lesions of invasive carcinoma were discriminated as malignant, and 23 (85.2%) out of other 27 lesions were discriminated as benign. However, in the testing set, 16 (94.1%) of 17 lesions of carcinoma were discriminated as malignant, and 22 (81.5%) of other 27 lesions were discriminated as benign. Separate consideration of a set for learning and testing gave similar results. The confusion matrix of this morphometry is shown in Table 4. The discriminant analysis was also made between severe dysplasia and mild dysplasia, though the number of samples was slightly small (Table 5). All 16 cases of mucosal carcinoma were discriminated as malignant with the obtained discriminant function.

Scattered plot diagram of all lesions with discriminant line and clustered groups is shown in Fig. 5. The derived discriminant line between carcinoma and other lesions existed nearly between clusters Ib and II. According to this formula, all the lesions of control, adenoma and mild dysplasia were differentiated from carcinoma. All the lesions except one of invasive carcinoma were discriminated as carcinoma. The exceptional example of carcinoma was that described above. For severe dysplasia, 9 lesions in cluster Ib were discriminated as benign, and one lesion in cluster Ib, 5 lesions in cluster IIa and 4 lesions in cluster IIb were discriminated as carcinoma.

The continuous measurement of N/C ratio and N.A. in cancer cases from non-cancerous mucosa to cancer lesion revealed that there were two types

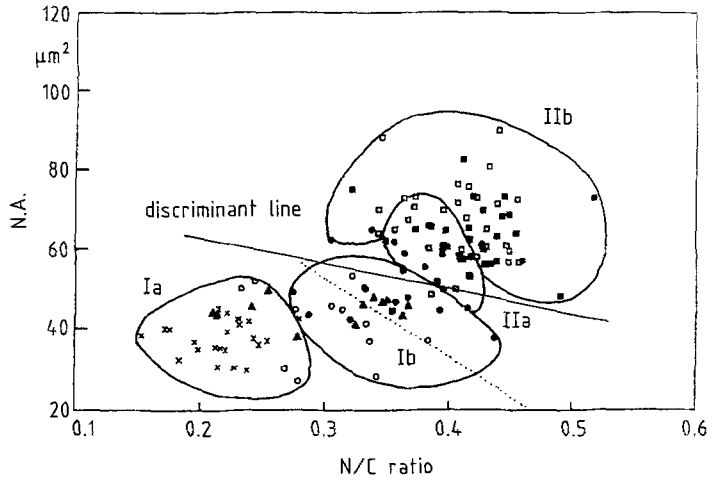

Fig. 5. Scattered plot diagram of N/C ratio and N.A. with discriminant lines and clustered groups. *Solid line*: discriminant line between carcinoma and other lesions, *Dotted line*: discriminant line between severe dysplasia and mild dysplasia, ■: invasive portion of carcinoma, □: mucosal portion of carcinoma, ●: severe dysplasia, ○: mild dysplasia, ▲: adenoma, X: control

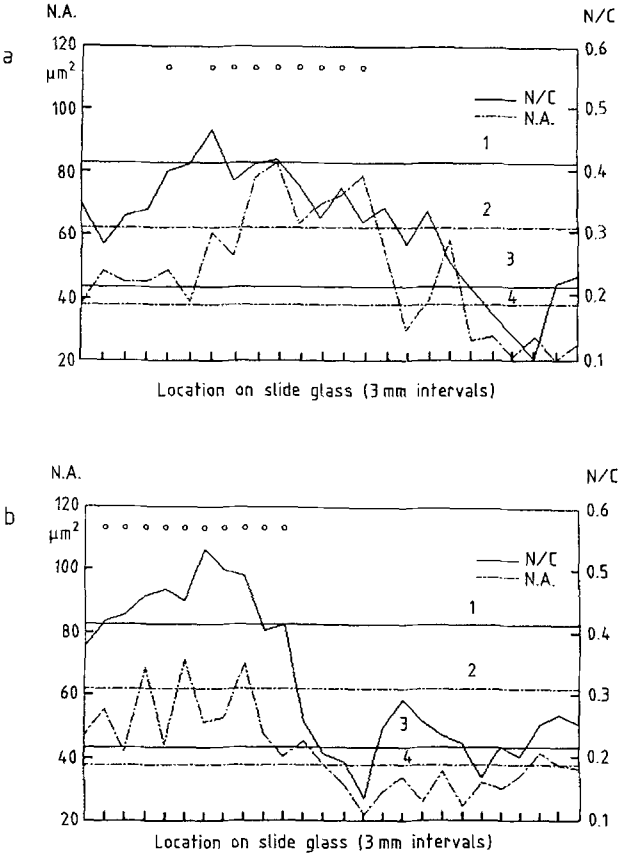

Fig. 6a, b. Two patterns of continuity of cellular atypia by continuous measurement of N/C ratio and N.A. in cancer cases from non-cancerous mucosa to cancer lesion. This diagram shows the means of N/C ratio (–) and N.A.(–·–·–) measured at 3 mm intervals on the slide glass with maximum diameter of the tumour. **a** A case whose cellular atypia gradually changed between carcinomatous area and adjacent non-neoplastic mucosa. **b** A case showing abrupt change in cellular atypia. Line 1, 3: mean values of N/C ratio in carcinomas and controls, respectively; Line 2, 4: mean values of N.A. in carcinomas and controls, respectively; o: carcinomatous area

of carcinoma from the standpoint of continuity of cellular atypia. In one type was that the change of cellular atypia was continuous and that the boundary between carcinoma and dysplasia was poorly defined (Fig. 6a). This type was observed in 15 cases of adenocarcinoma. In the other type (observed in 8 cases), cellular atypia changed abruptly so it was easy to determine the boundary between carcinoma and non-cancerous mucosa (Fig. 6b).

Discussion

As the rate of detection of early carcinomas or small carcinomas of the gallbladder increases, the distinction between dysplasia and carcinoma becomes an important problem in the histopathology of the gallbladder. Some authors have classified dysplasia subjectively using certain criteria. Laitio (1983a) divided dysplasia into three types, mild, moderate and severe, based on morphological features such as epithelial pseudostratification, nuclear hyperchromatism, N/C ratio, nuclear size, shape and location of nucleus, and complexity of glandular branching. Dowling and Kelly (1986) classified dysplasia into two types, low grade and high grade, based large by on the accepted criteria for dysplasia in colonic adenomata and in chronic inflammatory bowel disease. According to their classification, high-grade dysplasia includes cases which could be designated as carcinoma in situ. These criteria are subjectively determined, and the changes in the dysplasia of different degrees are often gradual without morphologically well defined borders (Laitio 1983a) and the distinction between severe dysplasia and carcinoma in situ is often arbi-

trary and not always possible (Albores-Saavedra et al. 1986). The application of objective and quantitative methods is therefore necessary for the differential diagnosis between dysplasia and carcinoma.

We have examined whether morphometric analysis, a more objective technique, is useful in differentiating gallbladder carcinoma from dysplasia. There are many variables to examine which indicate cellular atypia. Among them two, N/C ratio and N.A. were selected because they are the most fundamental parameters of cellular atypia. Our preliminary examination showed that morphological factors such as nuclear ellipsoidity and nuclear perimeter which are indicators of regularity of the nuclear boundary are less reproducible discriminators.

Both the mean values of N/C ratio and N.A. demonstrated a progressive increase from control to mild dysplasia, to severe dysplasia, and to carcinoma and were statistically significantly different between each lesion by the *t*-test. According to the discriminant function obtained in this study, 34 cases (97.1%) out of the 35 invasive carcinomas were discriminated as carcinoma. The one exceptional case of adenocarcinoma was so well differentiated, with prominent mucin production, that N/C ratio and N.A. were not large enough to be discriminated as cancer. Moreover, all the lesions of control, adenoma, and mild dysplasia were clearly discriminated as benign lesion.

It is of interest to compare groupings based on morphological features with those based on a numerical taxonomy by cluster analysis using only the evaluated values of N/C ratio and N.A. A good correlation was observed between two groupings. Most cases of control belonged to cluster Ia, adenoma and mild dysplasia to clusters Ia and Ib, and carcinoma to clusters IIa and IIb. Comparing these clustered groups with the discriminant line, the boundary between benign and malignant was situated nearly between clusters Ib and II. Therefore, these results indicate that benign and malignant lesions of the gallbladder can be well differentiated by morphometric analysis form the viewpoint of cellular atypia. The cases of severe dysplasia, however, were subdivided into clusters Ib, IIa, and IIb. According to the discriminant function, most lesions of severe dysplasia in cluster Ib were discriminated as benign, and most lesions in cluster II were discriminated as carcinoma. In view of these results, it is reasonable retrospectively to consider that the lesions of severe dysplasia which were situated far above the discriminant line should be designated carcinoma in situ, whereas lesions of severe dysplasia which were situated far below the discriminant line were designated as benign lesions. For those lesions of severe dysplasia which were situated close to the discriminant line, it is difficult to determine whether they are benign or malignant. Moreover, the continuous measurements of N/C ratio and N.A. in cancer cases showed that there were some cases showing a continuous increase of N/C ratio and N.A., and in these cases it was difficult to establish a boundary between carcinoma and a benign lesion. It is thus likely that some true borderline lesions exist and lesions situated close to the discriminant line may be considered to be in this category.

Morphometric analysis appears to be useful in differentiating carcinomas from other benign lesions. Although some difficult lesions exist, morphometric analysis provides more objective information and contributes to the differential diagnosis of borderline lesions of the gallbladder.

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