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Morphometrical analysis of gall-bladder adenoma and adenocarcinoma with reference to histogenesis and adenoma-carcinoma sequence

Susumu Nakajo[1], Masami Yamamoto[2], and Eiichi Tahara[1]

[1] Department of Pathology, Hiroshima University School of Medicine and
[2] Division of Anatomical Pathology, Hiroshima University Hospital, Hiroshima, Japan

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Summary. In order to examine whether our subdivision of gall-bladder adenoma and adenocarcinoma into non-metaplastic and metaplastic types is reasonable from the viewpoint of their cytological features, a morphometrical analysis was conducted on 17 adenomas and 59 adenocarcinomas. The morphometrical parameters used were nucleo-cytoplasmic ratio (N/C ratio) and nuclear areas (NA). N/C ratio in the metaplastic type of both adenoma and adenocarcinoma was significantly larger than that in the non-metaplastic. This result shows that the different tumour types are associated with a different N/C ratio. Continuous measurement of N/C ratio and NA in progressing from non-cancerous mucosa to the lesion was made and the data obtained were analysed by the Lowess method. In some adenomas the total area of polypoid lesions was serially measured and these data were also analysed by the Lowess method. The results showed different processes in non-metaplastic and metaplastic types of adenoma and adenocarcinoma from the standpoint of nuclear changes of N/C ratio and NA. These results indicate that our histogenetic classification of adenocarcinoma is reasonable in morphometrical nuclear analysis. We also investigated the adenoma-carcinoma sequence as a possible histogenesis for gall-bladder carcinoma. Eight (72.7%) of 11 metaplastic adenomas and two (33.3%) of six non-metaplastic adenomas had foci of atypical gland proliferation and were considered to be carcinomas. Moreover, these carcinomatous areas were surrounded by severe dysplasia. These findings indicate that adenoma-carcinoma sequence accounts for one of the histogeneses of gall-bladder carcinoma.

Offprint requests to: S. Nakajo, Department of Pathology, Hiroshima University School of Medicine, 1-2-3 Kasumi, Minami-ku, Hiroshima 734, Japan

Key words: Morphometry – Gall-bladder – Adenoma – Adenocarcinoma – Histogenesis – Adenoma-carcinoma sequence

Introduction

Our recent study has shown that there are two histogenetically different types of adenomas and adenocarcinomas of the gall-bladder, one being a non-metaplastic type derived from ordinary epithelium of the gall-bladder and the other being derived from metaplastic epithelium. This classification is based on the presence or absence of metaplastic changes in tumour tissue (Yamamoto et al. 1986, 1988, 1989a) and on the presence or absence of endocrine cells and/or lysozyme immunoreactivity. We have already examined the incidence of endocrine cells and lysozyme immunoreactivity and that of goblet cells, Paneth cells and pseudo-pyloric glands in the gall-bladder of the fetus, normal adult and patients with cholecystitis, and have found that these two markers are the most useful indicators of gastrointestinal metaplasia of the gall-bladder mucosa (Yamamoto et al. 1986). The metaplastic type was considered to be present in cases which contained at least one marker of endocrine cells or lysozyme immunoreactivity and the non-metaplastic type in cases which showed no markers. We have also shown that metaplastic carcinoma has a better prognosis than non-metaplastic carcinoma (Yamamoto et al. 1989b). Recently, we have made a morphometrical analysis of various gall-bladder lesions by using two parameters of nucleo-cytoplasmic ratio (N/C ratio) and nuclear areas (NA) and obtained a discriminant function

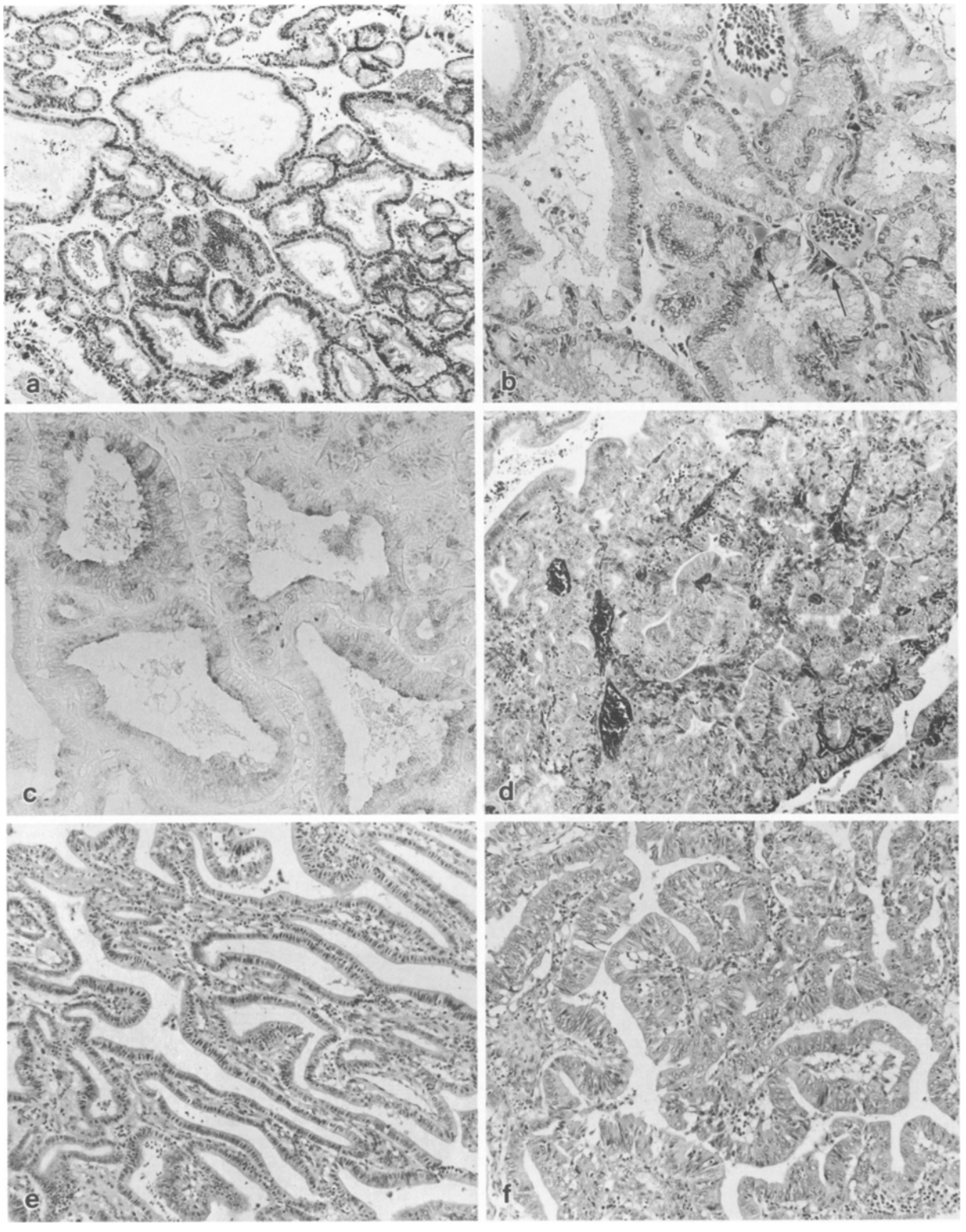

Fig. 1 a–f. Photomicrographs of two types of gall-bladder adenoma. **a** Metaplastic adenoma without atypical glands. Glandular proliferation of mucous cells with oval to round nuclei. H & E, ×188. **b** Metaplastic adenoma. Mucous glands contain argyrophil cells (*arrows*). Grimelius stain, ×375. **c** Metaplastic adenoma. Mucous glands show lysozyme immunoreactivity. PAP method, ×375. **d** Atypical lesion with structural and cellular atypia observed within a metaplastic adenoma, which is discriminated as carcinoma. H & E, ×188. **e** Non-metaplastic adenoma without atypical glands. The glands consist of a layer of columnar cells with oval to round nuclei resembling normal gall-bladder epithelium. H & E, ×188. **f** Atypical lesion observed within an non-metaplastic adenoma, which is discriminated as carcinoma. H & E, ×188

which differentiates carcinomas from other benign lesions (Nakajo et al. 1989).

The main purpose of this study was to examine whether the cytological features from a viewpoint of N/C ratio and NA are different between histogenetically different adenomas and adenocarcinomas of the gall-bladder.

Adenomas of the gall-bladder have long been studied from the point of view of their potential pre-malignancy. Foci of carcinoma-in-situ in adenomas of the gall-bladder have been noted by several authors (Tabah and McNeer 1953; Ochsner and Carrera 1956; Christensen and Ishak 1970; Araki and Tahara 1975; Kozuka et al. 1982; Yamamoto et al. 1988). These diagnoses of carcinoma-in-situ were made only on the basis of morphological features (Albores-Saavedra et al. 1980; Albores-Saavedra and Henson 1986; Dowling and Kelly 1986). However, there are no definite morphological criteria to differentiate carcinoma from dysplasia and a further purpose of this study was to clarify objectively the relationship of adenoma to carcinoma as a possible sequence in the gall-bladder, using morphometrical analysis.

Materials and methods

Surgically resected adenomas (17) from 15 patients and 59 well-differentiated adenocarcinomas of the gall-bladder, comprising 17 mucosal carcinomas and 42 invasive carcinomas were used in this study. The adenomas are lesions characterized by closely packed glands lined by cuboidal or columnar cells with slight increase of N/C ratio and NA (Albores-Saavedra and Henson 1986; Yamamoto et al. 1988; Nakajo et al. 1989). 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 and by Grimelius technique for argyrophil reaction.

Morphometrical analysis was performed on haematoxylin-and-eosin-stained 4-μm sections. The morphometrical parameters used were N/C ratio and NA which were measured using a digitizer (Nikon, Tokyo, Japan). In order to investigate the presence of field-to-field variations within 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. Fifty nuclei were sufficient to give a cumulative average within the 95% limits. To be on the safe side, 100 nuclei were measured at random. Ten areas were sufficient to reach this number. The sampling was systematic with a random start. NA 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 order to examine whether the processes of nuclear changes in the development of carcinoma are different among different types of carcinoma, continuous measurements of N/C ratio and NA from the non-cancerous mucosa to carcinoma were made using ten cases of metaplastic carcinoma and five cases of non-metaplastic carcinoma. Twenty-four to 60 points (average 30 points) were marked at 3-mm intervals on a glass slide, and ten cells in each point were measured. In these measurements, all the plotted values of N/C ratio and NA were analysed in each type by the Lowess method (locally weighted regression scatter plot smoothing; Cleveland 1979; Chambers et al. 1983). This method is one of exploratory data analyses (Tukey 1977) and is a good general statistical purpose tool for smoothing scatter plots; it can be used for making two distributional data comparisons. Lowess employs a robust procedure (one that is not distorted by a small fraction of outliers) and uses iterated weighted least squares.

Discrimination of carcinoma from benign lesions was made by the discriminant function, being $+0.2641 \times NA + 16.3522 \times N/C$

Table 1. Clinico-pathological data of gall-bladder adenomas

Type of adenoma	Case no.	Sex	Age	Size (mm)	Gall-stone	Lz	Arg	Atypical glands
I	1	F	64	10	−	+	−	+
	2	F	60	3	+	+	+	+
	3	F	72	12	−	+	+	+
	4	F	70	10	−	+	+	+
	5	F	58	13	?	−	+	+
	6	M	54	15	−	+	+	+
	7	M	67	6	+	+	+	+
	8	M	36	7	+	+	+	+
	9	M	61	20	+	+	+	+
	10	M	75	10	+	+	+	+
	11	F	48	10	+	+	+	+
II	12	M	39	2	−	−	−	−
	13	F	40	15	−	−	−	+
				6	−	−	−	−
				5	−	−	−	−
	14	F	55	5	−	−	−	−
	15	F	74	30	−	−	−	+

I, Metaplastic adenoma; II, non-metaplastic adenoma; Lz, lysozyme immunoreactivity; Arg, argyrophil cells

Table 2. Nucleo-cytoplasmic (N/C) ratio and nuclear areas (NA) of adenomas with or without atypical glands

Type of adenoma	Case no.	Adenoma		Atypical glands	
		N/C ratio	NA (μm^2)	N/C ratio	NA (μm^2)
I	1	0.345±0.027	47.43±7.02	0.371	52.98[a]
	2	0.324±0.024	41.75±6.08	0.383	49.91
	3	0.330±0.039	46.90±8.15	0.367	55.28[a]
	4	0.395±0.042	44.02±6.09	0.412	47.16
	5	0.338±0.021	48.85±7.95	0.368	55.42[a]
	6	0.366±0.032	46.49±6.09	0.406	47.26
	7	0.404±0.028	45.84±8.32	0.439	55.19[a]
	8	0.349±0.034	47.91±6.98	0.402	59.48[a]
	9	0.362±0.026	44.00±7.46	0.394	54.02[a]
	10	0.406±0.033	42.11±6.03	0.473	53.20[a]
	11	0.350±0.043	39.66±5.19	0.478	46.01[a]
Mean value		0.361±0.032*	45.00±6.82	0.408	52.36
II	12	0.277±0.023	38.62±6.57		
	13	0.253±0.022	50.27±7.63	0.285	57.75[a]
		0.213±0.036	44.38±5.50		
		0.209±0.054	45.03±5.52		
	14	0.240±0.046	46.09±5.41		
	15	0.311±0.043	51.88±9.66	0.347	63.79[a]
Mean value		0.251±0.037*	46.05±6.71	0.316	60.77

I, Metaplastic adenoma; II, non-metaplastic adenoma

[a] Atypical glands which were discriminated as carcinoma

* Significantly different by t-test ($p < 0.01$)

ratio $-19.8326=0.0$, obtained in our previous study (Nakajo et al. 1989).

When adenomas contained atypical glands, the measurement of N/C ratio and NA was made in the areas with and without atypical glands separately. For this purpose, NA of 30 nuclei and N/C ratio of 30 cells were measured in the areas with atypical glands.

In some adenomas, all the areas of polypoid lesions were measured serially. Twenty-five to 120 points (average 91 points) were marked at 1-mm intervals on the glass slide; ten cells in each point were measured. These measurements were made on three metaplastic adenomas and two non-metaplastic adenomas that contained atypical glands. In these cases, discrimination of severe from mild dysplasia was also made by the discriminant function, being $32.927 \times$ N/C ratio $+0.1660 \times$ NA $-18.7211=0.0$. Moreover, the changes of the obtained data of N/C ratio and NA on a scattered diagram were analysed by Lowess.

The peroxidase-antiperoxidase (PAP) method was applied in demonstrating lysozyme immunoreactivity as described in detail elsewhere (Yamamoto et al. 1986). Antisera were obtained from Dako (Copenhagen, Denmark). The specificity of immunoreactivity was confirmed by replacing the primary antibody with normal rabbit serum.

Chi-square test was used to examine the normality of the variables. Student's *t*-test was used to evaluate the significant differences among the tumour types.

Results

Morphometric analysis of adenoma

The patients ranged in age from 36 to 75 years with a mean of 58.2 years. Six were males and nine were females. Stones were present in 6 of 14 patients. One patient had three adenomas.

Of the 17 adenomas, 10 contained argyrophil cells and 10 showed lysozyme immunoreactivity. Based on the presence or absence of these metaplastic markers in the tumour tissue, these 17 adenomas were divided into 6 non-metaplastic adenomas showing no metaplasia and 11 metaplastic adenomas showing at least one of these markers of metaplasia (Fig. 1).

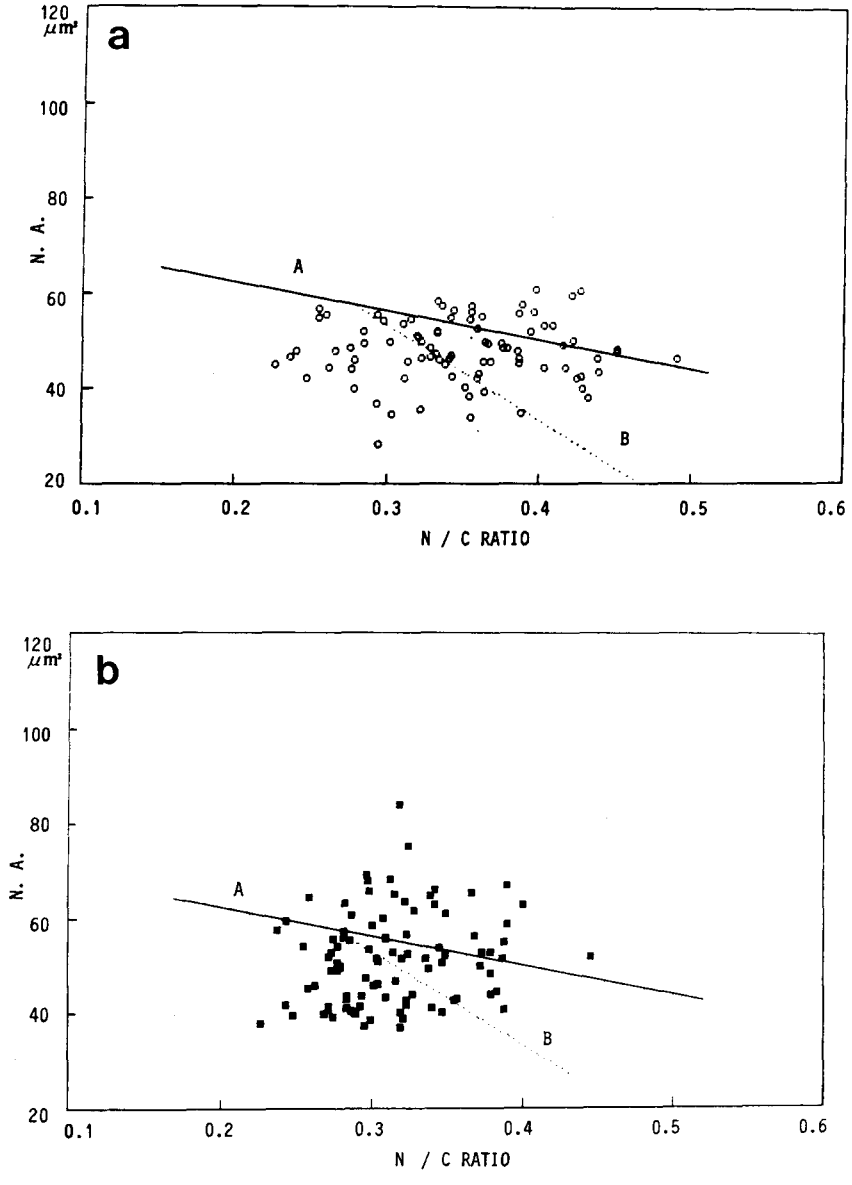

Fig. 2a, b. Scattered plot diagrams of nuclear areas (NA) and nucleo-cytoplasmic (N/C) ratio in
a metaplastic adenoma (case 9) and
b non-metaplastic adenoma with atypical glands (case 15). *A*, Discriminant line between carcinoma and other benign lesions. *B*, Discriminant line between severe dysplasia and mild dysplasia

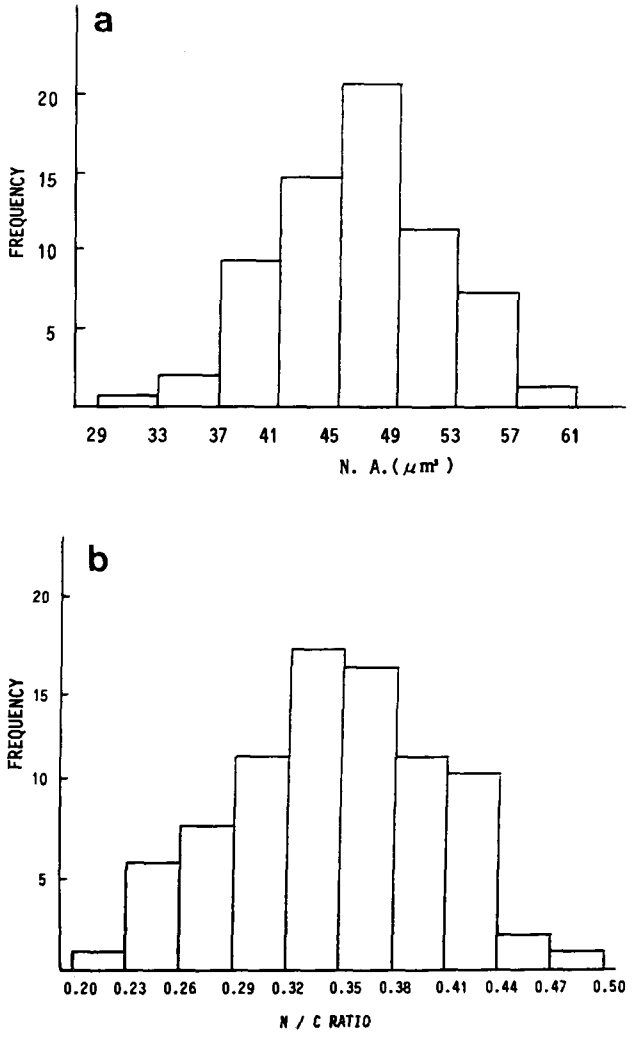

Fig. 3a, b. Frequency distribution diagrams of NA (**a**) and N/C ratio (**b**) in an adenoma (case 9). The normality of these distributions was proved by chi-square test

The clinicopathological findings of 17 adenomas which were divided into metaplastic and non-metaplastic are shown in Table 1. All the three adenomas observed in a single patient were the non-metaplastic type. Gallbladder stones were present in six of ten cases of metaplastic adenomas, whereas no case of non-metaplastic adenoma had stones. Atypical glands were present in all of the metaplastic adenomas and in two of the non-metaplastic adenomas (Fig. 1). Moreover, these atypical glands in metaplastic adenomas also showed metaplastic changes and those in non-metaplastic adenomas showed no metaplastic changes.

The data of N/C ratio and NA are shown in Table 2. The mean value (0.361) of N/C ratio in metaplastic adenomas was larger than that (0.251) in non-metaplastic adenomas with a statistically significant difference ($p < 0.01$). There was no significant difference with respect to NA.

As to the areas with atypical glands, the discrimination between carcinoma and benign lesion was made by using the discriminant function, being $+0.2641 \times \text{NA} + 16.3522 \times \text{N/C ratio} - 19.8326 = 0.0$. Eight (72.7%)

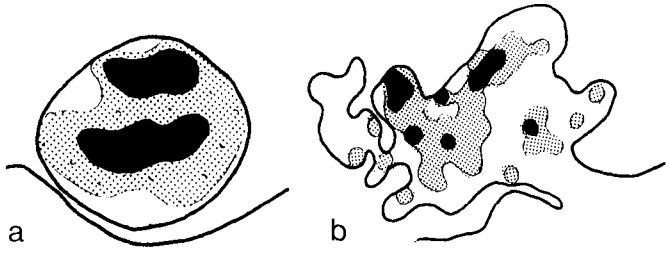

Fig. 4a, b. Distributions of various lesions in two types of adenoma containing atypical glands. **a** Metaplastic adenoma. **b** Non-metaplastic adenoma. *Black area*, Atypical lesions discriminated as carcinoma by discriminant formula; *shaded area*, atypical lesions discriminated as severe dysplasia by discriminant formula

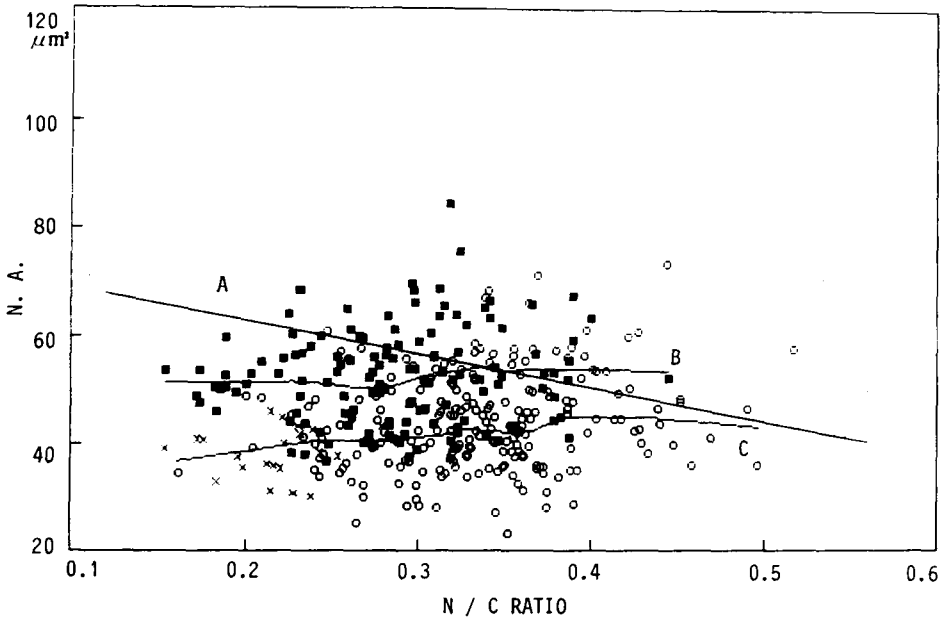

Fig. 5. Scattered plot diagram of NA and N/C ratio with discriminant line and curves calculated by Lowess method (adenoma cases). *Open circles*, Plots of metaplastic adenoma; *shaded squares*, plots of non-metaplastic adenoma. *A*, Discriminant line between carcinoma and other benign lesions. *B*, A curve derived from the plots of non-metaplastic adenoma by applying Lowess method. *C*, A curve derived from the plots of metaplastic adenoma by applying Lowess method

of 11 adenomas of metaplastic type and two (33.3%) of six adenomas of non-metaplastic type contained atypical foci being discriminated as carcinoma. In comparing the mean values of N/C ratio and NA in the carcinomatous area between non-metaplastic and metaplastic type, the non-metaplastic had a larger NA value, but a smaller N/C ratio than each of the metaplastic type. Scattered plot diagrams of all the serially measured N/C ratio and NA in metaplastic and non-metaplastic types together with discriminant lines are shown (Fig. 2). One of the frequency distribution diagrams of all the measured N/C ratio and NA in metaplastic type is shown in Fig. 3. According to these diagrams, frequency distributions of measured N/C ratio and NA showed a normal distribution, which was also proved by chi-square test.

The distributions of various lesions such as adenoma, severe dysplasia and carcinoma in the cases measured serially at 1-mm intervals are shown in Fig. 4. This diagram shows that carcinomatous area is surrounded by severe dysplasia and that severe dysplasia is surrounded by adenoma without atypical glands. That is to say, changes from adenoma to carcinoma were not abrupt but continuous. The scattered plot diagram of measured N/C ratio and NA of all the five cases measured serially with the results of 22 cases of normal epithelia is shown in Fig. 5. Moreover, all the plotted values of N/C ratio and NA were analysed in each adenoma type by the Lowess method and the resulting smoothed curves are depicted on the same diagram (Fig. 5). The smoothed curves of each type were almost parallel, but NA of the non-metaplastic type was larger than that of the metaplastic type.

Morphometric analysis of adenocarcinoma

The patients ranged in age from 41 to 85 years with a mean of 60.5 years. Fourteen were males and 45 were females. Stones were present in 34 of 59 patients.

Of the 59 adenocarcinomas, 34 contained argyrophil cells and 35 showed lysozyme immunoreactivity. Based on the presence or absence of these metaplastic markers in the tumour tissue, 17 mucosal carcinoma were divided into 5 non-metaplastic carcinomas showing no metaplasia and 12 metaplastic carcinomas showing at least one

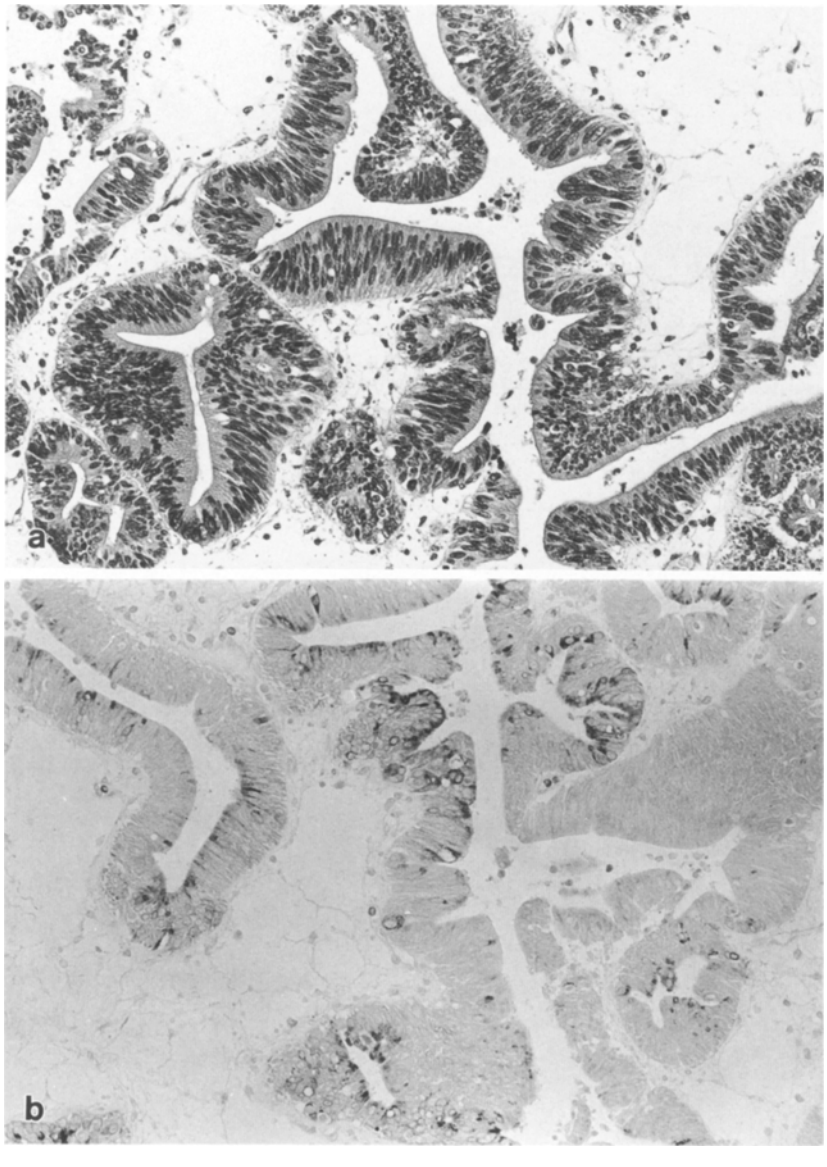

Fig. 6a, b. Histological (**a**) and immunohistochemical (**b**) features of well-differentiated adenocarcinoma of metaplastic type. ×280.
a The nuclei are large and irregular in shape and severely stratified. When compared with non-metaplastic carcinoma (**Fig. 7**), the nuclei are slightly smaller and more stratified. H & E.
b Cancer cells show diffuse lysozyme immunoreactivity. PAP method

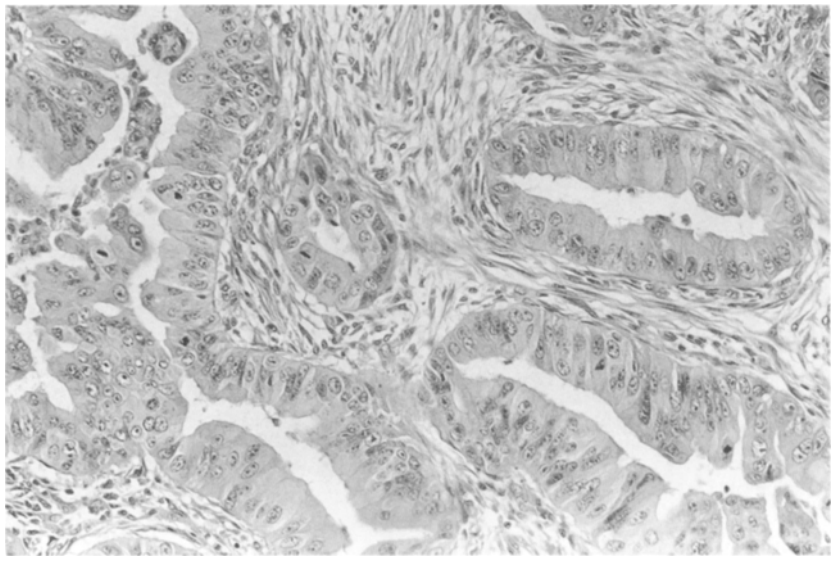

Fig. 7. Histological feature of non-metaplastic carcinoma. H & E, ×280. The nuclei are larger and less stratified than that of metaplastic type

Table 3. N/C ratio and NA in two types of gall-bladder adenocarcinoma

Tumour type	Mucosal carcinoma (mean ± SD)		Invasive carcinoma (mean ± SD)	
	N/C ratio	NA (μm^2)	N/C ratio	NA (μm^2)
Metaplastic	0.411 ±0.044*	65.15 ±13.4	0.414 ±0.037**	63.29 ±5.51
Non-metaplastic	0.361 ±0.028*	66.90 ±12.7	0.360 ±0.032**	63.49 ±8.54

* $p<0.05$; ** $p<0.01$; significantly different by t-test

of these markers of metaplasia, while 42 invasive carcinomas were classified into 32 metaplastic carcinomas (Fig. 6) and 10 non-metaplastic carcinomas (Fig. 7).

N/C ratio and NA are shown in Table 3. All the 59 carcinomas were discriminated as carcinomas by applying our previous discriminant function (Nakajo et al. 1989). The mean value of N/C ratio in metaplastic type of both mucosal and invasive carcinomas was larger than that in non-metaplastic type carcinomas with a statistically significant difference. The mean value of NA showed no significant difference between metaplastic carcinomas and non-metaplastic carcinomas. The scattered plot diagram of all the serially measured data of N/C ratio and NA from non-cancerous mucosa to cancer lesion in ten metaplastic carcinomas and five non-metaplastic carcinomas is shown in Fig. 8. Moreover, all the plotted values of N/C ratio and NA were analysed in each carcinoma type by the Lowess method and the resulting smoothed curves are depicted on the same diagram (Fig. 8). The smoothed curves of each type were almost parallel and did not intersect. Although both N/C ratio and NA gradually increased from non-cancerous mucosa to cancer lesion, there was a tendency for N/C ratio to be higher in metaplastic carcinoma and for NA to be larger in non-metaplastic carcinoma.

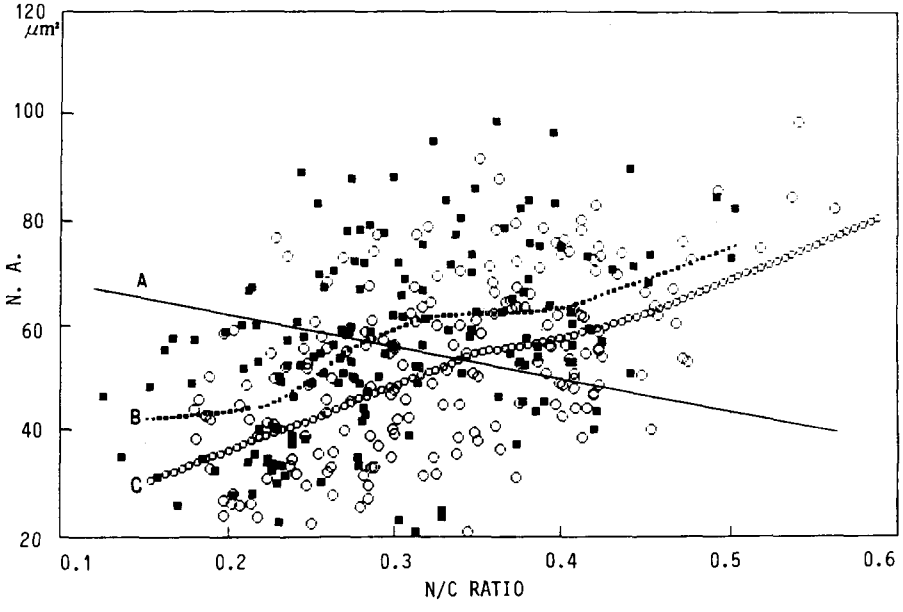

Fig. 8. Scattered plot diagram of NA against N/C ratio with discriminant line and curves calculated by Lowess method (carcinoma cases). *Open circles*, Plots of metaplastic type; *shaded squares*, plots of non-metaplastic type. *A*, Discriminant line between carcinoma and benign lesions. *B*, A curve derived from the plots of non-metaplastic type by applying Lowess method. *C*, A curve derived from the plots of metaplastic type by applying Lowess method. In this diagram, curve B is almost parallel to curve C, but curve B is shifted to the direction of NA axis compared with curve C

Discussion

We recently proposed a histogenetic classification of gall-bladder adenoma and adenocarcinoma into two types: metaplastic and non-metaplastic (Yamamoto et al. 1988, 1989a). We examined whether these two types of adenoma and adenocarcinoma could be discriminated and whether our classification was reasonable from the viewpoint of morphometrical analysis of the nucleus. In our previous report, we examined gall-bladder adenocarcinoma and dysplasia morphometrically by using two parameters of N/C ratio and NA and showed that gall-bladder adenocarcinoma could be well discriminated from other lesions by these two parameters (Nakajo et al. 1989). Therefore, in the present study, we examined the cytological features in these two types of adenoma and adenocarcinoma by using these two parameters.

N/C ratio in metaplastic type of both adenoma and adenocarcinoma was significantly larger than that in non-metaplastic type, with a statistically significant difference. This result showed that the different tumour types were associated with a different N/C ratio.

Adenomas of the gall-bladder have long been the subject of much study concerning their malignant potential. In our present cases, atypical glands within adenomas were not infrequently observed. The present study was made in order to clarify the possible adenoma-carcinoma sequence in the gall-bladder by using a morphometrical method. In applying our formula, 8 (72.7%) out of 11 metaplastic adenomas and 2 (33.3%) out of 6 non-metaplastic adenomas had foci of atypical gland proliferation which could be discriminated as carcinoma. Moreover, these carcinomatous areas were surrounded by severe dysplasia, i.e. nuclear atypia progressed gradually from adenoma to carcinoma. Moreover, the carcinomatous areas seen in metaplastic adenomas also showed metaplastic changes and those seen in non-metaplastic adenomas showed no metaplastic changes. It was also interesting to note that there were two main processes from normal epithelium to adenoma and to carcinoma from the viewpoint of nuclear changes of N/C and NA. The most important difference was observed in the process from normal epithelium to adenoma. One process was that N/C ratio predominantly increased and this pattern was often seen in metaplastic adenomas. The other was characterized by the predominant increase of NA and this pattern was seen in non-metaplastic adenomas. However, the curves from adenoma to carcinoma in these two types were almost parallel, that is, both N/C ratio and NA gradually increased.

Moreover, continuous measurement of N/C ratio and NA from non-cancerous mucosa to cancer lesion in carcinoma cases clearly demonstrated the processes to be different between non-metaplastic and metaplastic type carcinoma. There was a tendency for N/C ratio to be higher in metaplastic carcinoma and for NA to be larger in non-metaplastic carcinoma.

These results led to two important conclusions. Firstly, our histological classification of gall-bladder adenoma and adenocarcinoma into metaplastic and non-metaplastic was confirmed to be reasonable by morphometrical analysis. The second important conclusion is that there is an adenoma-carcinoma sequence as one of the histogeneses of gall-bladder carcinoma.

Our present results suggest the possibility that tumours originating from normal gall-bladder mucosa show predominant increase of NA, whereas tumours originating from metaplastic mucosa are characterized by an increase of N/C ratio. Our results pose an important problem that needs to be resolved; why do nuclear changes differ between histogenetically different types of tumours? Further studies on cell kinetics are indicated.

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