Objective Quantification of Grade of Atypia in Epithelial Tumors of the Stomach by Image Processing

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An image processing technique to extract tubular and nuclear areas from histological specimens stained with hematoxylin was established. Two indices representing tubular density and nucleus-to-cytoplasm ratio of glands were examined in intestinal-type tumor of the stomach. In specimens clearly stained with hematoxylin, significant differences of brightness among nuclei, cytoplasm and stroma can be obtained. Image processing by computer enabled us to identify nuclei, cytoplasm and stroma by utilizing the differences of brightness among them. Both indices tended to increase with severity of histological atypia and their values in the tubular adenoma group were significantly different from those in the tubular adenocarcinoma group ($P < 0.05$). Therefore, these two indices could be regarded as valid and objective measures of the grade of histological atypia. Eighty-four percent of tubular adenoma and tubular adenocarcinoma cases were classified correctly with the discriminant formula and critical value calculated from the two indices on the basis of measurement of the images magnified by 40 times. There is, however, still a non-negligible overlap of discriminant scores between the tubular adenoma group and tubular adenocarcinoma group. More indices representing histological atypia will be required to allow objective differential diagnosis between malignant and benign lesions.

Key words: Gastric tumor — Morphometry — Image processing

The histological differential diagnosis between tubular adenocarcinoma and tubular adenoma is made by so-called pattern perception of the complex histological picture. This involves assessment of various structural and cellular features such as tubular density, irregularity in shape of tubuli and nuclei, irregularity of tubular and nuclear size, N/C ratio³ and irregularity of nuclear arrangement. Differences of diagnosis by different pathologists often occur. This tends to happen especially in borderline cases between tubular adenocarcinoma and tubular adenoma. This is because the interpretation of structural and cellular atypia is subjective. The objective quantification of the degree of atypia is required so that such inconsistency in histological diagnoses can be avoided.

There have been many morphometrical studies quantifying the degree of histological atypia.¹⁻¹⁴ However, it would be difficult to utilize these methods in clinical diagnosis because input of the nuclei and tubuli to the image analyzer was done by hand, and thus, these methods were time-consuming.

In the present study, a new method was developed to extract tubular and nuclear areas more accurately and objectively by computer-processing of the images. We

also examined to what extent histological differential diagnosis between tubular adenocarcinoma and tubular adenoma of the stomach could be made with these two indices.

MATERIALS AND METHODS

Materials Ninety-three surgical specimens were examined histologically. According to the histopathological criteria for differentiating tubular adenocarcinoma from tubular adenoma of the stomach in The General Rules for the Gastric Cancer Study,¹⁵ these 93 cases were diagnosed as follows: 18 cases were diagnosed as tubular adenoma, 51 cases as tubular adenocarcinoma (36 cases as mucosal carcinoma and 15 cases as infiltrative carcinoma) and 24 cases as borderline between malignant and benign status. Intestinal metaplastic mucosae were examined as the control. Typical histological pictures of each group are demonstrated in Fig. 1a, 1b and 1c.

All specimens were fixed in 10% formalin for 2 or 3 days and embedded in paraffin. Sections 2 μ m thick were stained with Gill-hematoxylin. The tissue processing was done by a skillful technician under carefully controlled conditions. The time of staining was varied in each section to get an appropriate staining result.

Procedure of image processing The histological images observed with a microscope (Olympus, New Vanox-S AH2) were input to a color image analyzer (Olympus

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³ Abbreviations: N/C ratio, nucleus-to-cytoplasm ratio of glands; ITD, index of tubular density.

Fig. 1. Histological pictures of typical cases. a: Tubular adenoma ($\times 110$, H-E staining). Spindle-shaped nuclei are regularly arranged on the basement membrane side and many of the tubuli show a round or oval shape. b: Borderline case ($\times 130$, H-E staining). The nuclei are mildly pleomorphic and some of them are displaced toward the lumen of the tubuli. The tubuli show moderate abnormality with branching. c: Tubular adenocarcinoma ($\times 130$, H-E staining). The nuclei are pleomorphic and pseudostratified. The nuclear arrangement is irregular. Many of the tubuli are irregular in size and shape.

Fig. 2. a: Histological picture of a tubulus. b: Histogram of the brightness of the green element at the site designated by a white area in Fig. 2a. The abscissa represents the grading brightness of each pixel and the ordinate represents the percentage of pixels in each brightness. Peak A corresponds to the brightness segment of nuclei in tubuli and stromal lymphocytes, Peak B corresponds to that of cytoplasm and Peak C corresponds to that of stroma and lumen of tubuli.

color image analyzer recognizes each component of the images by grading brightness from 0 (darkest) to 255 (brightest).

A processed image of a specimen stained with hematoxylin is shown in Fig. 2a. Fig. 2b shows a brightness histogram of the green component image at the site designated by a white area in Fig. 2a. The brightness histogram consists of three peaks, A, B and C. Peak A represents the brightness segment of nuclei in tubuli and stromal cells consisting mainly of lymphocytes, peak B represents that of cytoplasm in tubuli and peak C represents that of stroma and lumen of tubuli. In a clearly stained specimen, the slope of each peak is sharper and overlap of each peak is smaller. The color image analyzer can, then, extract nuclei and tubuli by using the bright-

CIA system) via a TV camera (Ikegami ITC-370M). The color image was divided into three components, red, green and blue components, by the TV camera. The

Fig. 3. Procedure of image processing to extract tubuli and nuclei of tubuli from the specimen stained with hematoxylin. a: Original histological picture which is input into the image analyzer. b: Image obtained when the brightness segments of nucleus and cytoplasm are picked up. c: Image processed by thinning Image b. d: Image processed by labeling, masking and dilating Image b. Tubuli can be detected. e: Image obtained when the brightness segments of lumina and stroma are picked up. f: Image processed by matching Image d on Image e and labeling. The lumen can be extracted. g: Image obtained when the brightness segment of nuclei is picked up. h: Image processed by matching Image d on Image g. Tubular nuclei can be extracted.

ness difference in each segment. On the other hand, there is no significant difference between the brightness segment of nuclei and that of cytoplasm in the specimen stained with hematoxylin and eosin. Consequently, the color image analyzer can not accurately identify the nuclei.

The procedure of image processing is demonstrated in Fig. 3. Fig. 3a shows an original histological picture. Fig. 3b shows an image obtained when the brightness segments of nuclei and cytoplasm are picked up. Lymphocytes in stroma are extracted as well as nuclei and cyto-

plasm. Fig. 3c shows an image processed by thinning in order to detach the image of lymphocytes from that of tubuli. Fig. 3d shows an image processed by labeling, masking and dilating in order to exclude the detached image of lymphocytes. The expected tubuli are extracted accurately on the whole. Further, the extracted image can be modified by comparing it with the original histological picture.

The brightness segment of lumen of tubuli is subsequently picked up and lumen as well as stroma are extracted (Fig. 3e). Image f can be obtained when Image

d is matched on Image e and a labeling procedure is performed. Then the image of the lumen can be obtained. The brightness segment of nuclei is finally picked up. Nuclei of tubuli as well as stromal lymphocytes are picked up (Fig. 3g). The image of tubular nuclei can be obtained when Images d and f are matched on Image g and a labeling procedure is performed (Fig. 3h).

Formulas for ITD and N/C ratio The two indices of atypia were calculated according to the following formulas:

$$\text{ITD} = \frac{\text{area of tubuli}}{\text{area of (tubuli + stroma)}} \text{ per unit area} \times 100$$

N/C ratio

$$\begin{aligned} &= \frac{\text{area of nuclei}}{\text{area of cytoplasm of tubuli}} \text{ per unit area} \times 100 \\ &= \frac{\text{area of nuclei}}{\text{area of (tubuli - lumen of tubuli)}} \text{ per unit area} \\ &\times 100 \end{aligned}$$

In the procedure mentioned above, the areas of tubuli, lumen of tubuli, and nuclei of tubuli and stroma were obtained. Therefore, the two indices could be calculated easily.

Magnification and selection of measurements of the two indices In ITD analysis, measurement was done on images magnified by 40 times (objective 10 times and TV camera 4 times). In N/C ratio analysis, measurement was done on images magnified by 100 times (objective 20 times and TV camera 5 times). Specimens were selected at random from the lesions.

Objective discrimination between malignant and benign status by using the two indices The discriminant formula and critical value to discriminate malignant from benign status were calculated from ITD and N/C ratio values in the tubular adenoma group and tubular adenocarcinoma group. The linear discriminant function employing Mahalanobis' generalized distance was used.¹⁶⁾ One hundred and ninety-six samples were selected at random from the 93 cases magnified by 40 times, and classified into a malignant group and a benign group by using the discriminant formula and critical value.

Statistics Student's *t* test was used to evaluate the difference between mean values of groups, with $P < 0.05$ as the criterion of statistical significance (one-sided test). The F-test on variations of groups was also used, with $P < 0.05$ as the criterion of statistical significance. The discriminant function was calculated with Statistical Library software (IBC Corporation, Miyazaki).

RESULTS

Measurement error in the analysis of the two indices Measurement variability in the examination of ITD was

Fig. 4. Variation of N/C ratio obtained by 23 measurements on the same specimen. The mean value is 40.81 and the standard deviation is 1.38. The coefficient of variation of this measurement can be obtained as 3.37%.

Table I. ITD Values at High and Low Magnifications

Histological diagnosis	$\times 100$	$\times 40$
Tubular adenoma	76.9 ± 10.0	70.5 ± 7.2
Tubular adenocarcinoma	77.4 ± 8.4	75.0 ± 7.4
<i>t</i> Test ($P < 0.05$)	$t = 0.321$ (not significant)	$t = 2.541$ (significant)

good; the coefficient of variation (C.V.) was less than 5%. This was because the extracted image of tubuli could be modified easily by comparing it with the original histological image. As regards measurement variability in the examination of N/C ratio, Fig. 4 shows a histogram of the data from 23 measurements of a clearly-stained image obtained with hematoxylin. The C.V. was 3.37%, which is an acceptable value. The C.V. of data obtained from the specimen stained with hematoxylin and eosin was 10% or larger, which is unacceptably large.

Pilot study on measurement of the two indices The values of ITD and N/C ratio vary depending on the selection of the image and section of tubuli, even in the same specimen. A pilot study was required to determine which method showed the least variability.

Table I demonstrates the variability of data obtained from the image magnified by 40 times and those obtained from the image magnified by 100 times in the same case. Magnification by 40 times yielded a significant difference between the tubular adenoma group and tubular adenocarcinoma group. However, magnification by 100

times did not. Consequently, in analysis of ITD, measurement was done on the image magnified by 40 times in this study.

Two different measurements for the analysis of N/C ratio were examined in the same lesions: one is measurement of a single tubulus and the other is measurement of all tubuli contained in an image magnified by 100 times. Two hundred and ten tubuli were examined in the former measurement and 42 images in the latter. In the former measurement the N/C ratio was 35.07 ± 8.58 , and in latter it was 33.82 ± 4.33 . The standard deviation of the former measurement was significantly larger than that of the latter (F test: $P < 0.05$). Therefore, all tubuli contained in one image magnified by 100 times were measured for the analysis of N/C ratio in this study.

ITD As shown in Table II, the values tended to increase with severity of atypia and there were significant differences between groups. In the tubular adenocarcinoma group, there was no significant difference between intramucosal adenocarcinoma and infiltrative adenocarcinoma.

N/C ratio As shown in Table III, the values tended to increase with the severity of atypia and there were significant differences between groups. In the tubular adenocarcinoma group, there was no significant difference between mucosal carcinoma and infiltrative carcinoma.

Table II. ITD Values

Histological diagnosis	ITD value
Control	55.7 ± 11.8^a
Tubular adenoma	69.9 ± 7.8^b
Borderline lesion	71.7 ± 8.8^c
Tubular adenocarcinoma	74.5 ± 7.8^d
Mucosal carcinoma	74.4 ± 7.9^e
Infiltrative carcinoma	75.0 ± 5.5^f

t Test ($P < 0.05$): significant a): b), b): d); not significant b): c), c): d), d): e), e): f).

Table III. N/C Ratio Values

Histological diagnosis	N/C ratio value
Control	25.5 ± 6.2^a
Tubular adenoma	35.2 ± 5.0^b
Borderline lesion	47.6 ± 6.7^c
Tubular adenocarcinoma	50.1 ± 7.8^d
Mucosal carcinoma	50.3 ± 7.8^e
Infiltrative carcinoma	48.9 ± 6.7^f

t Test ($P < 0.05$): significant a): b), b): c), b): d); not significant c): d), d): e), e): f).

Discrimination between malignant and benign status by using the two indices The discriminant formula and critical value to discriminate malignant from benign status were obtained as follow:

$$F = 0.1098(\text{N/C ratio}) + 0.0168(\text{ITD}) - 5.7155$$

$$\text{Critical value} = 0.8826$$

Seventy-five percent of images in the tubular adenoma group were classified as showing benign status and 83% of images in tubular adenocarcinoma group were classified as showing malignancy with the discriminant formula and critical value. As a whole, 84% of images from tumors of the stomach were classified correctly (Table IV). Out of 66 images of borderline lesions, 35% were classified into the benign group and 65% into the malignant group.

A histogram of discriminant score in these groups was obtained (Fig. 5). The control group and malignant group did not overlap. On the other hand, the tubular

Table IV. Discrimination of the Tumors of the Stomach by Two Indices

Histological diagnosis	Discrimination		Total
	Benign	Malignant	
Control	37 (100%)	0 (0%)	37
Tubular adenoma	39 (75%)	13 (25%)	52
Tubular adenocarcinoma	15 (17%)	74 (83%)	89
Borderline lesion	23 (35%)	43 (65%)	66

Fig. 5. Histogram of discriminant values of each group. The abscissa represents discriminant score and the ordinate represents percentage of images. Dotted bar: control group. Black and white bar: tubular adenoma group. Black bar: tubular adenocarcinoma group.

adenoma group overlapped greatly with the tubular adenocarcinoma group.

DISCUSSION

There have been many morphometric studies quantifying the degree of atypia in many organs including the gastrointestinal tract.¹⁻¹⁴⁾ Some of the studies were successful at the research level, but it is difficult to utilize such studies in clinical diagnosis, because nuclei and tubuli must be input manually into a computerized image analyzer.

Recently, region segmentation by using an image processor has become popular in various fields. Funakubo reported that tubuli could be extracted from silver-stained specimens of large intestine.¹⁷⁾ However, the nuclei of tubuli could not be extracted. In this study a new method was established to extract not only tubuli but also nuclei of tubuli from paraffin sections stained with hematoxylin, one of the easiest stains to use, with an image processor.

Stereology was used to quantify structural atypia in gastric dysplasia.^{1,2)} However, stereology can be used only in organs whose structures are solids of uniform quality in three dimensions and it cannot be applied to the tubular structure of the stomach, which has polarity.¹⁸⁾ Therefore, ITD has been developed to represent structural atypia in the stomach.

In the analysis of ITD the magnification of the measured images had to be chosen carefully. This is because as the magnification becomes higher, the unit area of microscopic features becomes smaller and more variant data are obtained. Comparison between data obtained at magnifications of 40 times and 100 times indicated statistically that we had to observe the specimen at the lower magnification in order to interpret histological atypia. The magnification at which measurement is carried out is one of the most important factors in quantifying structural atypia.

The values of ITD tended to increase with severity of atypia and there were statistically significant differences ($P < 0.05$) between groups, even though the images were selected at random. Therefore, ITD measured at a magnification of 40 times was proved to be objective and valid.

The value of N/C ratio in histological specimens varies because of measurement error and it is also affected by selection of the section. Therefore, in order to establish a more objective measurement in assessing cellular atypia, both measurement errors and variations depending on the section must be minimized.

A sharp slope of each peak and minimum overlap are required in the brightness histogram to minimize the measurement error, as shown in Fig. 2b. For that pur-

pose, the resolving power of color image analyzer must be high and specimens must be stained clearly. In order to obtain specimens clearly stained with hematoxylin, all the steps for preparation, time from resection to fixation, fixation time, embedding, sectioning and staining, must be strictly specified.

Specimens stained with hematoxylin and eosin were used in analysis of N/C ratio with an image analyzer in colon cancer.^{13,14)} However, it is very difficult to obtain accurate data because there is no significant difference of brightness between nuclei and cytoplasm in specimens stained with hematoxylin and eosin. Therefore, specimens clearly stained with hematoxylin should be used in analysis of the N/C ratio with the image analyzer.

Baak *et al.* reported that variations in tissue processing affected chromatin quantification, but did not significantly affect nuclear area, volume percent epithelium or surface density of glands in endometrium.⁹⁾ In this study, variation in processing was not regarded as having a significant effect on the two indices, because data obtained by measurement were used as relative figures instead of absolute figures in calculation.

Selection of the sections of the histological specimen, however, yielded variable measurement data in analysis of the N/C ratio. In other words, if only a few tubuli were selected for measurement, the selection of the tubuli was significant. Nakayama *et al.* calculated the N/C ratio of colonic tubuli by measuring only a particular section.⁵⁾ Such a measurement may be useful in research, but is not appropriate in the clinical setting, in which tubuli are taken at random for histological examination. We concluded that the measurement of all tubuli contained in one image magnified by 100 times is appropriate to minimize the variation of data in a particular section.

The N/C ratio tends to increase with the severity of atypia and there were significant differences ($P < 0.05$) between groups although the specimens were selected at random. Consequently, the N/C ratio obtained by measuring all tubuli contained in one image magnified by 100 times was proved to be objective and valid.

The use of two indices can allow us to make differential diagnosis between malignancy and benignancy more correctly. Eighty-four percent of 178 images were classified accurately with the discriminant formula and critical value (Table IV). As shown in Fig. 5, however, the overlap of discriminant values in the tubular adenoma group and tubular adenocarcinoma group was not negligible. In order to minimize the overlaps and to improve the accuracy of diagnosis, more indices representing histological atypia must be developed.

Image processing in this study could facilitate extraction of tubuli and nuclei from the histological pictures and this method may be useful to develop new indices representing other histological findings.

ACKNOWLEDGMENTS

We wish to thank Mr. T. Mogi (Research Cooperation, University of Tsukuba) for his excellent technical assistance,

Mr. H. Tanaka (Institute of Agriculture, University of Tsukuba) and Mr. M. Yamaguchi (Olympus Engineering Corporation) for their advice on image processing, and Ms. T. Urabe for her help in the preparation of the manuscript.

(Received August 3, 1990/Accepted November 13, 1990)

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