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## Differences in biological features of gastric dysplasia, indefinite dysplasia, reactive hyperplasia and discriminant analysis of these lesions

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### Abstract

**AIM:** To investigate the differences in biological features of gastric dysplasia (Dys), indefinite dysplasia (IDys) and reactive hyperplasia (RH) by studying the biomarker alterations in cell proliferation, cell differentiation, cell cycle control and the expression of house-keeping genes, and further to search for markers which could be used in guiding the pathological diagnosis of three lesions.

**METHODS:** Expressions of MUC5AC, MUC6, adenomatous polyposis coli (APC), p53, Ki-67, proliferation cell nuclear antigen (PCNA) and EGFR were studied by immunohistochemistry with a standard Envision technique in formalin-fixed and paraffin-embedded specimens from 43 RH, 35 IDys, 35 Dys and 36 intestinal type gastric carcinomas (IGC). In addition, Bayes discriminant analysis was used to investigate the value of markers studied in differential diagnosis of RH, IDys, Dys and IGC.

**RESULTS:** The MUC5AC and MUC6 antigen expressions in RH, IDys, Dys and IGC decreased gradually (MUC5AC: 86.04%, 77.14%, 28.57%, 6.67%; MUC6: 65.15%, 54.29%, 20.00%, 25.00%, respectively). The expressions of the two markers had no significant difference between RH and IDys, but were all significantly higher than those of the other two lesions (MUC5AC:  $\chi^2 = 27.607$ , 38.027 and 17.33, 26.092; MUC6:  $\chi^2 = 16.54$ , 12.665 and 9.282, 6.737,  $P < 0.01$ ). There was no significant difference between RH and IDys, Dys and IGC in MUC6 expression. The APC gene expression in the four lesions had a similar decreasing tendency (RH 69.76%, IDys 68.57%, Dys 39.39%, IGC 22.86%), and it was significantly higher in the first two lesions than in the last two ( $\chi^2 = 7.011$ ,

16.995 and 14.737, 19.817,  $P < 0.05$ ). The p53 expression in RH, IDys, Dys and IGC was 6.98%, 20%, 57.14% and 50%, respectively. There was no significant difference between RH and IDys or Dys and IGC, but the p53 expression in RH and IDys was significantly lower than that in Dys and IGC ( $\chi^2 = 7.011$ , 16.995 and 14.737, 19.817,  $P < 0.01$ ). The Ki-67 label index was significantly different among four lesions (RH:  $0.298 \pm 8.92\%$ , IDys:  $0.358 \pm 9.25\%$ , Dys:  $0.498 \pm 9.03\%$ , IGC:  $0.620 \pm 10.8\%$ ,  $P < 0.001$ ). Positive immunostaining of PCNA was though observed in all specimens, significant differences were detected among four lesions ( $F = 95.318$ ,  $P < 0.01$ ). In addition, we used Bayes discriminant analysis to investigate molecular pathological classification of the lesions, and obtained the best result with the combination of MUC5AC, Ki-67 and PCNA. The overall rate of correct classification was 67.4% (RH), 68.6% (IDys), 70.6% (Dys) and 84.8% (IGC), respectively.

**CONCLUSION:** Dys has neoplastic biological characteristics, while RH and IDys display hyperplastic characteristics. MUC5AC and proliferation-related biomarkers (Ki-67, PCNA) are more specific in distinguishing Dys from RH and IDys.

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**Key words:** Gastric dysplasia; Indefinite dysplasia; Reactive hyperplasia

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### INTRODUCTION

Gastric indefinite dysplasia (IDys) and reactive hyperplasia (RH) are common hyperplastic lesions of stomach mucosa in Padova international classification (1998)<sup>[1]</sup>. Gastric dysplasia (Dys) is a precancerous lesion, a non-invasive neoplasia or intraepithelial neoplasia (IEN), which is divided into low grade (LGD) and high grade (HGD). In Padova classification, morphological description is adopted for classification and diagnosis of these lesions. However, the description is simple and often poses difficulties for pathologists to differentiate RH from IDys or IDys from

Dys, and the differential diagnosis of IDys and LGD is especially difficult to make. Up to now, only a few systemic investigations have been made on the biological characteristics of these lesions. Therefore, it is necessary to precisely and clearly discriminate RH, IDys and Dys so as to diagnose them accurately and to treat them efficiently. The main purpose of this study was to investigate the biological differences among Dys, IDys and RH, and to further combine them with their morphological descriptions, to search for clear and external markers, which could be used in the pathological diagnosis of the three lesions.

## MATERIALS AND METHODS

### *Patients and samples*

From the pathologic files at the Pathology Department of Beijing Cancer Hospital, 36 intestinal type gastric carcinomas (IGCs) surgically resected at Beijing Cancer Hospital between 1998 and 2002 were collected. Other lesions including RH, IDys and gastric Dys came mostly from mucosae near the carcinomas in 60 patients. Twenty-two gastric Dys lesions were collected from the People's Hospital of Linqu County in Shandong Province by endoscopic biopsy between 1996 and 2001. A total of 149 specimens from 88 patients were used in our study. Among these specimens, 43 had RH (27 males and 16 females, age range 27-84 years), 35 had IDys (21 males and 14 females, age range 38-81 years), 35 had Dys (24 males and 11 females, age range 40-70 years) and 36 had IGC (25 males and 11 females, age range 36-75 years). All specimens were checked by at least two pathologists. To rule out any effect of sex and age on the biological behaviors of the lesions, we did statistical analysis and found that sex and age had no effect on the biological features.

### *Antibodies and other chemicals*

MUC5AC (NeoMarkers, MS-145-P0 45M1), MUC6 (NeoMarkers, MS-1153-S0 MCN6.01/CLH5) and p53 (MAB-0142 DO-7) were purchased from Maxim Biotech, Inc. APC (EMM43) was obtained from Novacastra Laboratories Ltd. EGFR (H11 M3563) was purchased from Dako (1:60 dilution) and Ki-67 (MAB-0129 MIB-1) from Maxim Biotech Inc. Proliferation cell nuclear antigen (PCNA) (ZM-0213 PC10) (1:100 dilution) was from Zhongshan Biotechnology Co., Ltd. Secondary antibody of IgG anti-Fab-HRP and PV-6000 PicTure™ Kits were from Zymed Laboratory Co., Ltd.

### *Immunohistochemical staining*

Five-micrometer-thick tissue sections were dewaxed with xylene and rehydrated with graded alcohol, then briefly immersed in water. Endogenous peroxidase activity was blocked by incubating the sections with 3% hydrogen peroxide for 10 min. For all biomarkers, except for MUC5AC, heat-mediated antigen retrieval was performed by heating the sections (immersed in 0.01 mol/L citrate buffer, pH 6.0) in a microwave oven (450 W) for 10 min. The slides were then washed with phosphate-buffered saline (PBS) before incubation with respective primary antibody overnight at 4 °C. After being washed with PBS, the slides were incubated

for 1 h with the secondary antibody, and then further washed for 3 min×5 min with PBS. Peroxidase reaction was developed in PBS using hydrogen peroxide as a substrate and DAB as a chromogen. Sections were counterstained with hematoxylin, dehydrated, and evaluated under a light microscope. Negative control sections were processed immunohistochemically after the primary antibody was replaced with PBS.

### *Evaluation of immunohistochemical staining*

To study the biological features, we used MUC5AC and MUC6 for cell differentiation, p53 for cell cycle control, Ki-67, PCNA and EGFR for proliferation, APC for house-keeping gene abnormality. MUC5AC was highly expressed in foveolar epithelium and mucous neck cells of antrum and body regions. Expression of MUC6 was detected in the glands of the antrum and mucopeptic cells in the neck zone of the normal gastric body region<sup>[2]</sup>. Only nuclear immunostaining was considered as positive for p53 protein accumulation<sup>[3-6]</sup>. EGFR and APC positive staining were located on cytoplasm and cell membranes. Sections were classified according to the immunostaining into negative group (less than 10% of positive cells) and positive (positive in more than 10% of positive cells)<sup>[4,7]</sup>. With regard to the Ki-67 labeling index (LI), only nuclear staining was evaluated as positive. We randomly selected four high-power (400×) microscopic fields and counted 500 cells in each section, and then determined the average Ki-67 LI<sup>[8-11]</sup>.

Only cells showing nuclear staining were considered to be positive for PCNA and graded as negative (-) (no positive cells); weak positive (+), positive cells accounted for 1-25%; moderately positive (++), the positive cells accounted for 26-50%; strong positive (+++), the positive cells accounted for 51-75%; very strong positive (++++) , the positive cells accounted for greater than >75%<sup>[12]</sup>.

### *Statistical analysis*

The  $\chi^2$  test and one-way ANOVA test were used to compare the frequencies by SPSS 10.0 for Windows.  $P < 0.05$  was considered statistically significant. In addition, we used Bayes discriminant analysis to investigate the molecular pathological classification of RH, IDys, Dys and IGC. Bayes discriminant analysis was used to identify the combined effect of variables important for discrimination of different lesions. Misclassification was evaluated by the leave-one-out cross-validation technique. Analyses were performed using SPSS 10.0.

Using a set of seven variables, discriminant analysis correctly classified lesions into one of the following three groups: left-sided RH, right-sided, Dys.

## RESULTS

### *Immunohistochemical study*

MUC5AC positive expression in each kind of lesions was 86.4% (37/43) for RH, 77.14% (27/35) for IDys, 28.57% (10/35) for Dys and 16.67% (6/36) for IGC. The percentage of RH and IDys with positive staining for MUC5AC was significantly higher than that in other lesions ( $\chi^2 = 27.607$ , 38.027 and 17.33, 26.092,  $P < 0.01$ ) (Figures 1 and 2).

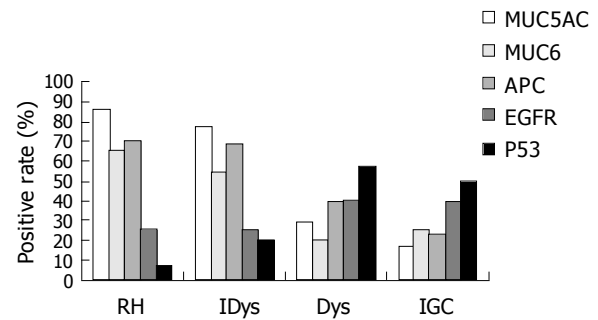
MUC6 positive expression in each kind of lesion

was 65.15% (28/43) for RH, 54.29% (19/35) for IDys, 20.00% (7/35) for Dys and 25.00% (9/36) for IGC. MUC6 positive expression had no difference in RH and IDys, but was significantly higher than that in the other two lesions ( $\chi^2 = 16.54, 12.665$  and  $9.282, 6.737, P < 0.01$ ), and no significant difference was observed between Dys and IGC (Figures 1 and 2).

APC positive expression in each kind of lesions was 69.76% (30/43) for RH, 68.57% (24/35) for IDys, 39.39% (13/33) for Dys and 22.86% (8/35) for IGC. APC positive expression had no difference in RH and IDys, but was significantly higher than that in the other two lesions ( $\chi^2 = 7.011, 16.995$  and  $14.737, 19.817, P < 0.05$ ), and no significant difference were observed between Dys and IGC (Figure 1).

EGFR positive expression in each kind of lesions was 26.19% (11/42) for RH, 25.00% (8/35) for IDys, 40.00% (14/35) for Dys and 38.89% (14/36) for IGC. EGFR positive expression had no difference in each group (Figure 1).

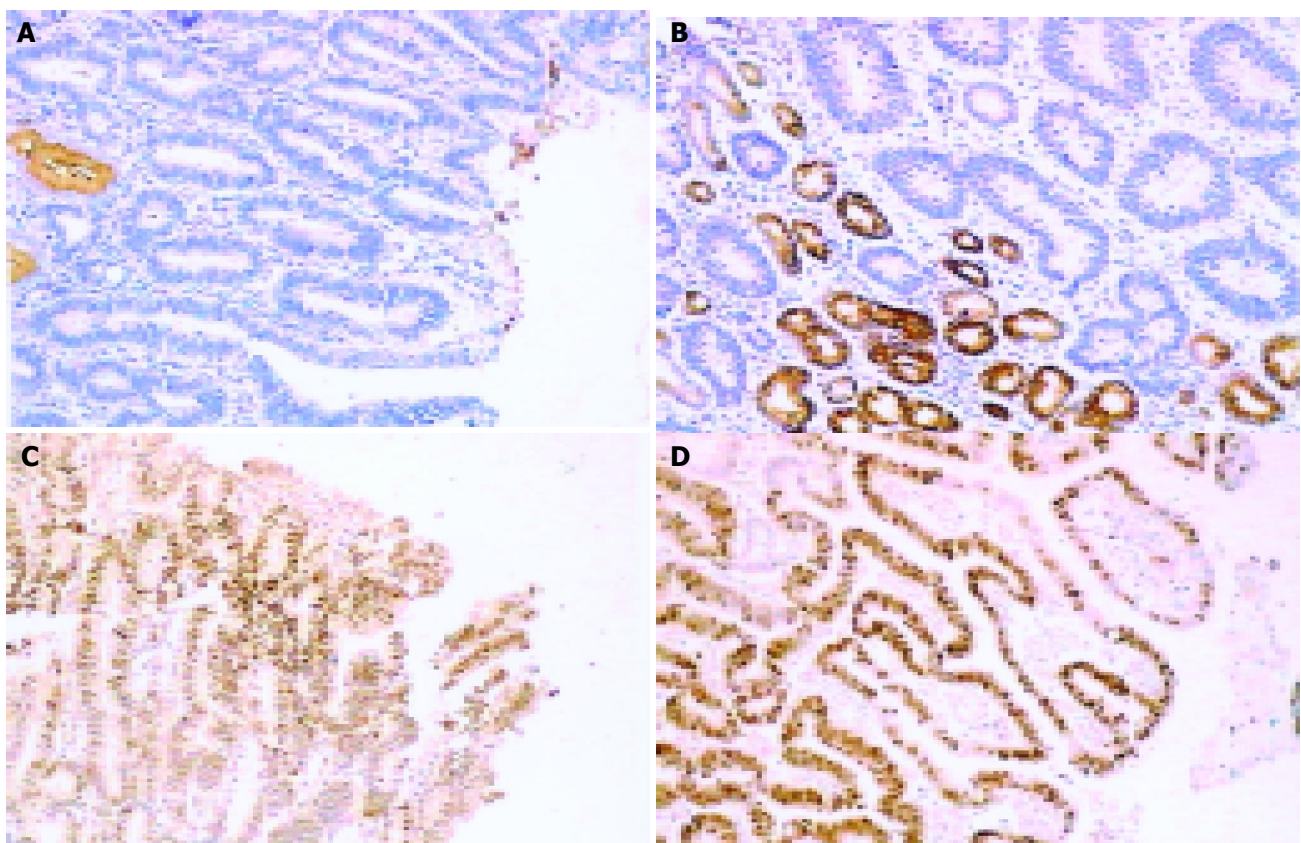
p53 positive expression in each kind of lesions was 6.98% (3/43) for RH, 20.00% (7/35) for IDys, 57.14% (20/35) for Dys and 50.00% (18/36) for IGC. For p53 expression, there was no relationship between RH and IDys. The p53 expression observed between Dys and IGC had no significant difference either, but both of these lesions had a significantly higher p53 expression than that detected in the nuclei of RH and IDys ( $\chi^2 = 7.011, 16.995$  and  $14.737, 19.817, P < 0.05$ ) (Figures 1 and 2).



**Figure 1** MUC5AC, MUC6, APC, EGFR, and p53 expressions in different gastric lesions.

In normal gastric mucosa, Ki-67 immunoreactivity was mainly found in foveolar hyperproliferative zone. The mean Ki-67 index (mean $\pm$ SE) was  $0.298 \pm 8.92\%$  for RH,  $0.358 \pm 9.25\%$  for IDys,  $0.498 \pm 9.03\%$  for Dys, and  $0.620 \pm 10.8\%$  for IGC. The Ki-67 index for IGC was significantly higher than that of the other three lesions ( $P < 0.001$ ). There was also a significant difference in RH, IDys and Dys ( $P < 0.01$ ) (Figure 3). In addition, there was no locus restriction in Dys and IGC (Figure 2).

All lesions showed a different extent of positive immunostaining of PCNA protein (Table 1). In normal gastric mucosa, it was mainly in foveolar hyperproliferative zone.



**Figure 2** A: MUC5AC immunostaining cells in one representative Dys specimen (standard Envision, peroxidase method  $\times 100$ ); B: MUC6 immunostaining cells in one representative Dys specimen (standard Envision, peroxidase method

$\times 100$ ); C: p53 immunostaining cells in one representative Dys specimen (standard Envision, peroxidase method  $\times 100$ ); D: Ki-67 immunostaining cells in one representative Dys specimen (standard Envision, peroxidase method  $\times 100$ ).

Positive immunostaining was observed in the cell nuclei of tumor and epithelial tissues. There were significant differences among RH, IDys, Dys, and IGC ( $P < 0.01$ ). Additionally, PCNA expression had no locus restriction in Dys and IGC.

PCNA expression was graded as negative (-) (no positive cells); weak positive (+), positive cells accounted for 1-25%; moderately positive (++), positive cells accounted for 26-50%; strong positive (+++), positive cells accounted for 51-75%; very strong positive (++++) , positive cells accounted for greater than 75%.

We made discriminant analysis for seven parameters to find out which variables would best classify the lesions and found that the best result was obtained with the combination of MUC5AC, Ki-67 and PCNA. The overall rate of correct classification was 67.4% (RH), 68.6% (IDys), 70.6% (Dys) and 84.8% (IGC) respectively (Table 2).

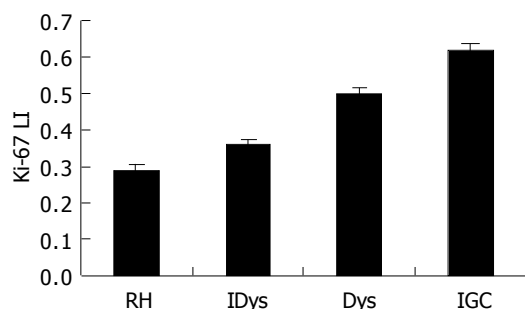


Figure 3 Ki-67 LI in different gastric lesions.

Table 1 Expressions of PCNA in different lesions

PCNA expression	RH	IDys	Dys	IGC
-	0	0	0	0
+	25	9	0	0
++	17	24	9	1
+++	1	2	15	11
++++	0	0	11	24
Total	43	35	35	36

## DISCUSSION

### Reactive hyperplasia

Compared with the other three lesions, RH preserved most characteristics of normal gastric mucosa (Figure 4A), and its cell differentiation, house-keeping gene abnormality, cell cycle control and proliferation had minimal abnormalities. This was supported by the fact that the expression rates of MUC5AC<sup>[13]</sup>, MUC6<sup>[14,15]</sup> (two biomarkers for cell differentiation) and APC (a house-keeping gene) were the highest, its p53 (a biomarker for cell cycle control) expression rate was the lowest, and its Ki-67 LI and PCNA expressions (two biomarkers for cell proliferation) were the lowest. We also saw that RH was significantly different from Dys and IGC in expression of all biomarkers except EGFR, suggesting that RH might be of an infectious nature. Therefore, taking its morphological characteristics into consideration, it is an inflammatory lesion.

### Indefinite dysplasia

This category was reserved in cases in which a pathologist was unable to decide for certain if the lesion could represent neoplastic or non-neoplastic cells. This situation might arise because the biopsy materials were inadequate or because the architectural distortion and nuclear atypia were present at the point of creating doubts about the dysplastic nature of proliferating cells. In such cases the doubts might be solved with more adequate new biopsies or after possible sources of cellular hyperproliferation or atypia were removed, such as *H. pylori* or nonsteroidal anti-inflammatory drugs<sup>[16,17]</sup>. In Padova classification, it emphasizes that the "gradient of maturation" must be seen in IDys, which means that the structural and cellular alterations tend to decrease from the bottom to the most superficial mucosal layers (Figure 4B).

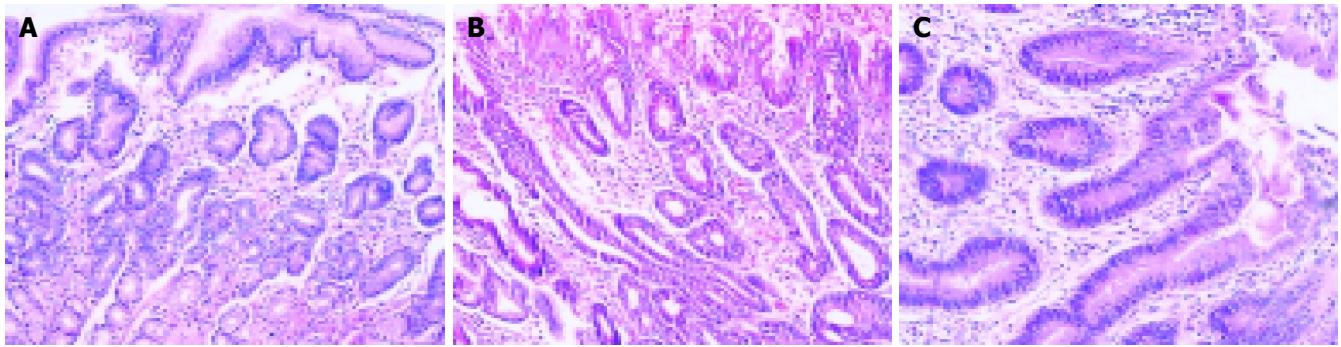
IDys has two subtypes, namely, foveolar hyperproliferation and hyperproliferative intestinal metaplasia. In our study, the p53 and PCNA positive expression rates were just a little bit higher than those of RH, but significantly lower than those of Dys and IGC ( $P < 0.05$ ). Its Ki-67 LI level was lower than that of Dys and IGC ( $P < 0.01$ ), though still higher than that of RH ( $P < 0.01$ ). The MUC5AC, MUC6 and APC positive expression rates were a little bit lower than those of RH, but significantly higher than those of

Table 2 Results of discriminant analysis and its cross-validated various lesions

Membership	Predicted group					Total
	PATH	RH	IDys	Dys	IGC	
Original Count	RH	29 (67.4)	13 (30.2)	1 (2.3)	0 (0.0)	43
	IDys	9 (25.7)	24 (68.6)	1 (5.7)	0 (0.0)	35
	Dys	0 (0.0)	3 (8.8)	24 (70.6)	7 (20.6)	34
	IGC	0 (0.0)	1 (3.0)	4 (12.1)	28 (84.8)	33
	Ungrouped cases	1 (50.0)	1 (50.0)	0 (0.0)	0 (0.0)	2
Cross-validated Count	RH	28 (69.8)	12 (32.6)	1 (2.3)	0 (0.0)	43 (100.0)
	IDys	14 (40.0)	20 (54.3)	1 (5.7)	0 (0.0)	35 (100.0)
	Dys	0 (0.0)	4 (11.8)	24 (67.6)	7 (20.6)	35 (100.0)
	IGC	0 (0.0)	3 (3.0)	19 (55.2)	47 (81.8)	69 (100.0)

The discriminant functions are as follows:  $Y_{RH} = -11.354 + 4.573MUC5AC + 30.432Ki-67 + 5.027PCNA$ .  $Y_{IDys} = -15.404 + 3.895MUC5AC + 39.669Ki-67 + 6.032PCNA$ .  $Y_{Dys} = -31.031 + 0.823MUC5AC + 60.027Ki-67 + 9.605PCNA$ .  $Y_{IGC} = -45.525 - 0.322MUC5AC + 74.916Ki-67 + 11.542PCNA$ .





**Figure 4** A: RH. The general architecture of the mucosa is well preserved. The foveolae are tortuous. Hyperplastic epithelial alterations become progressively less prominent and gradually disappear from the hyperplastic mucosa to the adjacent normal areas. Besides, we can see inflammatory infiltrate, edema, reflecting the underlying cause of the hyperproliferative state (HE,  $\times 100$ ); B: IDys. In the context of an atrophic gastritis, the intestinalized glands show back-to-back architecture. But the structural and cellular alterations tend to decrease from the bottom to the most superficial mucosal layers. This “gradient of maturation”

may be helpful in distinguishing the true noninvasive neoplastic (HE,  $\times 100$ ); C: LGD. Low-grade disorganized architecture consists of metaplastic glandular structures. Most of the glandular cells show mucus depletion. Nuclei are elongated (sometime with cigar-like appearance) and irregular in size. They are most frequently located at the basal pole and show slight pseudostratification. Atypical (rounded or cigar-like) nuclei are also seen at the surface epithelial layer. Both the architectural abnormality and the cytohistologic alterations are consistent with a noninvasive neoplastic lesion (i.e., low-grade Dys) (HE,  $\times 200$ ).

Dys and IGC ( $P < 0.05$ ). Results showed that in IDys, although the ability of cell proliferation was increased, it was still relatively low. IDys still kept many characteristics which were similar to those of normal gastric mucosa, such as cell differentiation and proliferation, cell cycle control and APC gene expression. It was significantly different from Dys and IGC in these areas, but was similar to RH. Therefore, IDys essentially belongs to benign hyperplastic lesions.

Commonly seen in clinics, this lesion was often difficult to diagnose and could lead to diverging opinions. Consequently, clinicians had difficulties in treating it. In Padova classification, no further illumination is provided to this lesion's essence either. Based on our study, we conclude that IDys is a benign hyperplastic lesion. Therefore, when we see the “gradient of maturation” in morphology, we should consider the lesion as a benign hyperplastic one.

### Dysplasia

Dysplastic cells extended to the surface epithelium, a feature absent in non-neoplastic lesions. Because it was frequently absent in cell maturation, we could not see the “gradient of maturation” in Dys. Dys was divided into two subcategories: LGD and HGD (Figure 4C).

Our study revealed that there were obvious abnormalities in cell differentiation and proliferation, and APC gene expression in Dys. These abnormalities were similar to those of IGC, but distinct from those in RH and IDys. In our study, its MUC5AC<sup>[18-20]</sup>, MUC6<sup>[21-23]</sup> and APC<sup>[24]</sup> positive expression rates were significantly lower than those of RH and IDys, but were not significantly different from those of IGC. p53 positive expression rate of Dys was significantly higher than those of RH and IGC, but not significantly different from that of IGC. Its Ki-67 LI and PCNA positive expression rates were significantly higher than those of RH and IDys, but significantly lower than those of IGC. Dys had the highest EGFR positive expression rate in the four lesions, but had no significant difference in other lesions ( $P > 0.05$ ). Although Dys was similar to IGC in its biological characteristics, the proliferation activity of IGC was higher

than that of Dys, and was invasive to deeper layers of gastric mucosa. Thus, it is reasonable to categorize Dys as a non-invasive neoplasia or an IEN.

### Intestinal gastric carcinoma (IGC)

In our study, IGC was used as a positive control for the other three lesions. Its MUC5AC, MUC6 and APC<sup>[26]</sup> positive expression rates were significantly lower than those of RH and IDys and its p53 positive expression rate was significantly higher than that of RH and IDys, but the activity of four markers in IGC had no significant differences when compared with Dys. The Ki-67 LI and PCNA positive expression rates in IGC were significantly higher than those in the other three lesions. All these indicate that cell differentiation in IGC was the poorest, the abnormality in cell cycle control was the greatest and the proliferative activity was the highest in the four lesions. Its EGFR positive expression rate was higher than that of RH and IDys, lower than that of Dys. But there were no significant differences in each group ( $P > 0.05$ ). EGFR positive expression was low in all the four lesions.

### Discriminant analysis

It is well known that the use of discriminant analysis on a single data set could establish a model. That is, “over-fit” and use of the same discriminant functions on a new set of data usually produced much lower correct classification percentage. Thus, we assessed the misclassifications by leave-one-out cross validation. Bayes stepwise discriminant analysis could overcome the shortcomings of the general discriminant function by selecting variables, which have a significant influence on the functions. Thus, it could classify one case effectively. In clinical application, as data may vary greatly, that the functions are considered to be effective when the correct rate of discriminant functions is higher than 70%.

RH, IDys and Dys are different in their biological nature, and it is important to combine their biological nature with morphologic features in their diagnosis and differential diagnosis.

In the seven indexes used in our study, MUC5AC, PCNA

and Ki-67 could best differentiate the four lesions. Besides, these markers were helpful in understanding the biological nature of these lesions. The correct rates of classification were 67.4% (RH), 68.6% (IDys), 70.6% (Dys), and 84.8% (IGC), respectively. The misclassifications mainly occurred in RH and IDys, or in Dys and IGC. The correct rate of the discriminant functions for Dys and IGC were higher than 70%. Therefore, if we encounter a gastric lesion which is difficult to diagnose, MUC5AC, Ki-67 and PCNA immunohistochemical assay and the discriminant model might be helpful for distinguishing Dys from RH and IDys.

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