Expression of the GLUT1 and p53 Protein in Atypical Mucosal Lesions Obtained from Gastric Biopsy Specimens

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Background : The diagnosis of atypical mucosal lesions by performing hematoxylin-eosin staining is too subjective, and it is also subject to considerable inter-observer variation. There is a need for reliable immunohistochemical markers that can give reproducible results and that are not subject to individual interpretation. **Methods :** We reviewed a total of 199 cases of gastric biopsy specimens, which were all diagnosed as atypical mucosal lesions, and 124 cases of the adenocarcinomas specimens had been classified from category 1 (C1) to C5 according to the Vienna classification. We also examined the immunohistochemical expressions of the glucose transporter GLUT1 and the p53 protein in the gastric biopsy specimens to determine if they were useful markers for differentiatial diagnosis under the Vienna classifications. **Results :** None of the specimens in categories C1 to C3 showed GLUT1 expression, but 10.1% of the C4 specimens and 25.0% of the C5 specimens were GLUT1-positive ($p < 0.05$). The expression of p53 was undetectable in the C1 specimens, but this was expressed in 2.9% of the C2 specimens, 15.6% of the C3 specimens, 37.8% of the C4 specimens, and 65.3% of the C5 specimens ($p < 0.05$). **Conclusions :** The Vienna classification is very applicable to the gastric biopsy specimens of the atypical mucosal lesions, and the GLUT1 and p53 expressions are candidates as highly useful markers to differentiate the Vienna C4 lesions from the C3 and C5 lesions.

Key Words : Stomach; Biopsy; Glucose transporter type 1; Tumor suppressor protein p53

The histological diagnosis of gastric mucosal biopsy specimens is the definite method for identifying neoplastic lesions. However, diagnosing premalignant gastric lesions and adenocarcinoma has been complicated because Japanese pathologists and their counterparts in many Western countries have differences in some of their basic approaches and specific morphological interpretations. Because gastric carcinoma develops in the surface epithelium, identifiable structural or functional abnormalities must presumably be present before invasion can occur.[1] The diagnosis of gastric carcinoma in Western countries is conventionally based on invasiveness because of the ability of invasive cells to metastasize. However, Japanese pathologists diagnose carcinoma on the basis of the nuclear and structural changes that are seen in the gastric epithelial cells, irrespective of invasiveness. For example, gastric lesions that are considered to be high-grade adenoma/dysplasia by Western pathologists by using the conventional Western classification[1] are often diagnosed as carcinoma by Japanese pathologists by using the Japanese group classification.[2] To overcome these differences, the Padova classification,[3] the Vienna classification,[4] and a revision of the Vienna

classification[5] have recently been proposed. A large body of evidence from many studies has indicated that the Vienna classification resulted in the highest agreement scores, and that the Vienna system's categories best matched with the current post-diagnostic clinical practices.[1] However, there is great diversity seen among biopsy specimens: many are diagnosed as atypical lesions and they are difficult to classify into the categories as defined by the Vienna system. Biopsied tissues are frequently taken from the surface or the periphery of a lesion and these specimens are not always representative of the entire lesion site. A descriptive diagnosis of dysplasia, a suspicious for atypical lesion or atypical epithelium are now tentatively used for such controversial cases, and this system requires another biopsy in order to get the additional information needed for making a definite diagnosis.[6] Furthermore, it is not easy to make a differential diagnosis between every subcategory of the Vienna categories 4. The distinction between the subcategories of C4 is based on the classic hematoxylin-eosin stain. The differences are too subjective and this results in considerable interobserver variation. Consequently, there is a need for finding immunohis-

tochemical or molecular markers that are reproducible and distinctive, and that can be visualized or quantified without interpretative bias.

It's long been recognized that cancer cells have higher rates of glucose metabolism than do normal cells.[7] Glucose uptake is mediated by glucose transporters, and these transporters' expression and activity are regulated by oncogenes and growth factors.[8] Facilitative glucose transporters (GLUT) in the plasma membrane mediates the flux of glucose between blood and these tissues. Among the various facilitative glucose transporter isoforms GLUT1 is the basic, high-affinity glucose transporter. Researchers are recognizing that glucose transporters may be the key to understanding the enhanced glucose uptake in tumor cells, so several primarily immunohistochemical studies have been carried out. These studies have provided evidence that GLUT1 is overexpressed in malignant tumors, including stomach cancer.[9-15] However, to the best of our knowledge there have been no previous reports of GLUT1 immunostaining in the biopsy specimens from various gastric epithelial lesions. The p53 gene has a critical role in cell cycle regulation and tumor suppression as the guardian of the genome.[16] Adenocarcinomas express p53 protein at a high frequency in gastric lesions, but dysplastic lesions have a relatively low p53 expression rate.[17]

In the present study, we reclassified atypical gastric mucosal lesions from their biopsy specimens according to the Vienna classification. The selected specimens had been previously diagnosed as being suspicious for "atypical lesions", "atypical epithelium", and "atypical glands", or "suspicion of dysplasia", and "suspicion of carcinoma". In addition, we performed immunostaining for GLUT1 and p53 protein in the gastric biopsies, to test whether these proteins may be useful markers for making the differential diagnosis according to Vienna categories and the subcategories.

MATERIALS AND METHODS

The patients and tissue samples

The authors reviewed a total of 29,126 gastric mucosal biopsy samples that were obtained from the Kyung Hee University Hospital from 1998 to 2003. From these slides, we selected the difficult-to-diagnose cases, including those cases designated as "suggestive of malignancy or dysplasia", "suspicious atypia or dysplasia", "atypical glands or lesions", and "suspicious for carcinoma" and also the cases that required rebiopsy or a serial/deep recut. We reviewed the histological slides and then categorized them in accordance with the Vienna classification (Table 1). All of the original hematoxylin-eosin slides were freshly examined by three pathologists who were kept "blind" to the original diagnoses. After the individual assessments, we held a consensus meeting in which we reviewed all the biopsies that there was disagreement about, and the final diagnoses were determined by discussion. There was a total of 199 cases including 14 that were judged to be negative for neoplasia/dysplasia (Category 1; C1), 34 cases that were "indefinite for neoplasia/dysplasia" (C2), 32 cases of "low-grade adenoma/dysplasia" (C3) and 119 cases of "non-invasive high grade neoplasia" (C4), these 119 cases included 66 cases of high-grade adenoma/dysplasia (C4.1), 25 cases of carcinoma *in situ* (C4.2), and 28 cases of "suspicious for being invasive carcinoma" (C4.3). Also included were a total of 124 cases of invasive neoplasia (C5), of which 11 were papillary carcinomas, 57 were well differentiated tubular adenocarcinomas, 20 were moderately differentiated tubular adenocarcinomas, 18 were poorly differentiated tubular adenocarcinomas and another 18 were signet ring cell carcinomas.

Immunohistochemistry

Immunohistochemical staining was performed by using a streptoavidin-biotin immunoperoxidase method, according to the supplier's protocol (LSAB kit, DAKO, Carpenteria, CA, USA). In brief, the paraffin-embedded sections were deparaffinized in xylene and rehydrated with a graded series of ethanol solutions. After quenching the endogenous peroxidase activity in 0.3% hydrogen peroxide for 30 min and then incubating the slides with blocking reagents for 30 min, primary polyclonal rabbit anti-human GLUT1 (DAKO, Carpenteria, CA, USA) and primary monoclonal mouse anti-human p53 protein (DAKO, Carpenteria, CA, USA) were applied to the sections at

Table 1. Vienna classification of gastrointestinal epithelial neoplasia

Category 1	Negative for neoplasia/dysplasia
Category 2	Indefinite for neoplasia/dysplasia
Category 3	Non-invasive low grade neoplasia
	(low grade adenoma/dysplasia)
Category 4	Non-invasive high grade neoplasia
	4.1 High grade adenoma/dysplasia
	4.2 Non-invasive carcinoma (carcinoma in situ)
	4.3 Suspicious of invasive carcinoma
Category 5	Invasive neoplasia
	5.1 Intramucosal carcinoma
	5.2 Submucosal carcinoma or beyond

dilutions of 1:200 and the sections were next incubated in a moist chamber for 2 hr at room temperature. After washing out the excess complex, the localization of antibodies was visualized by incubating the sections for 10 minutes in 3,3′-diaminobenzidine tetrahydrochloride (DAKO, Carpenteria, CA, USA). The red blood cells present in each section served as a positive control for GLUT1. In the negative controls, the normal horse serum was substituted for the primary antibody.

The slides were evaluated independently by three examiners who were not given access the pathological findings. After the individual assessments, we held a consensus meeting in which we reviewed all the slides for which there was disagreement, and the final diagnoses were assigned following a discussion. The GLUT1 immunostaining was quantified by grading the proportion of cells that were GLUT1 positive. Those cells showing strong and distinctive membrane-localized immunoreactivity for GLUT1 were considered positive. Cells with cytoplasmic or nuclear staining were considered negative. The grading system was as follows: from zero to 5% of the immunoreactive cells=0; from 5% to less than one third of the immunoreactive cells=1+; one third to two thirds of the immunoreactive cells=2+; and more than two thirds of the immunoreactive cells=3+. Strong and distinctive nuclear immunoreactivity was considered positive for the cells stained with antibody to p53 and cytoplasmic or membranous staining was considered negative. The grading system for the percentage of positive cells was the same as that used for GLUT1.

Statistical analysis

We used a x^2 test and Fisher's exact test with SPSS (version 11.0; SPSS Inc., Chicago, IL, USA) to compare the expression of GLUT1 and p53 with the Vienna categories. Statistical significance was defined as p value <0.05.
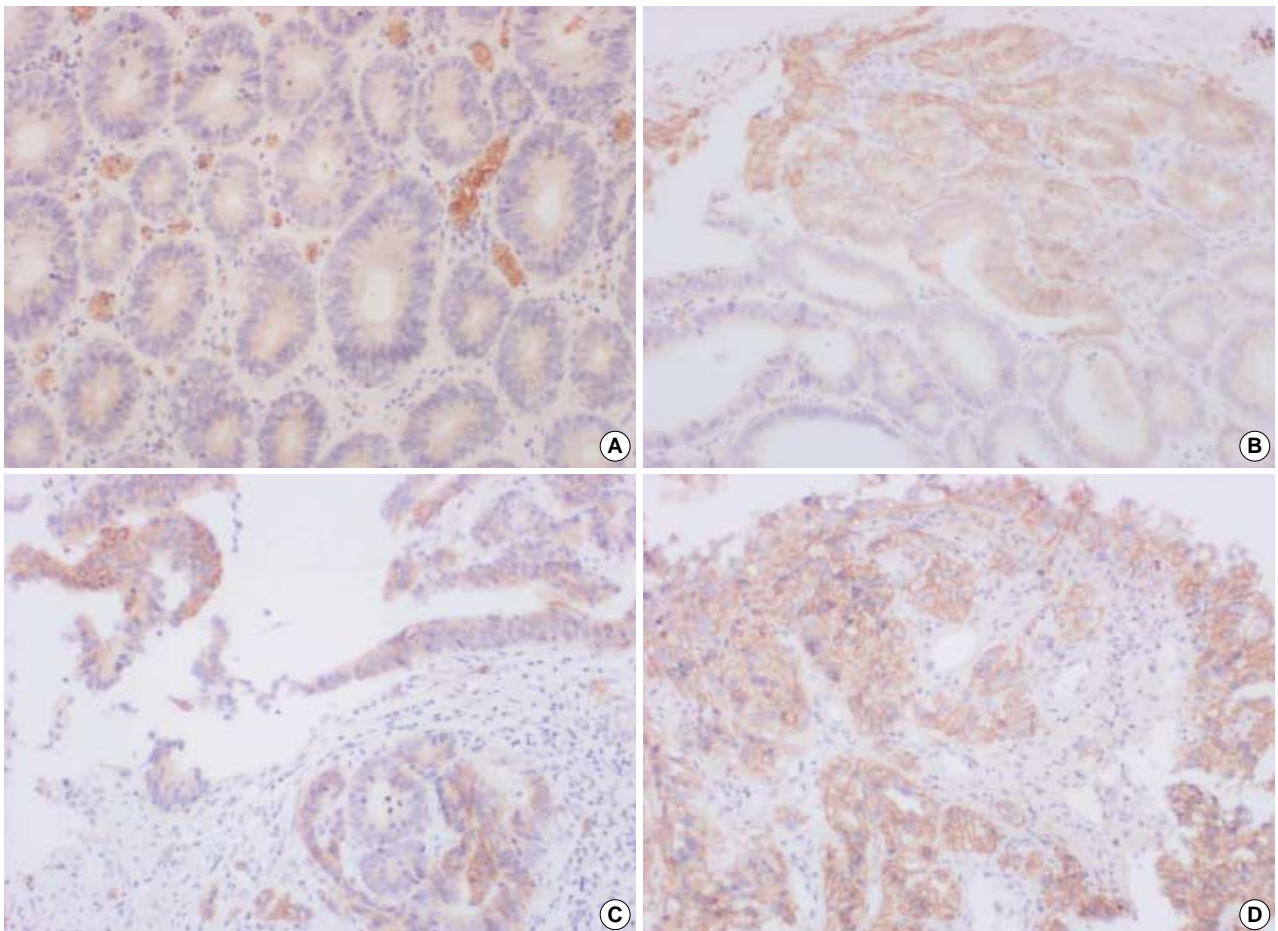

Fig. 1. Immunostaining for GLUT1: (A) Negative staining in low-grade adenoma/dysplasia. (B) Positive staining in high-grade adenoma/dysplasia. (C) Suspicious for invasive carcinoma. (D) Well differentiated adenocarcinoma.

RESULTS

We performed the immunohistochemical staining for GLUT1 and p53 on 323 cases of gastric biopsy specimens. The normal gastric mucosa did not express GLUT1 protein, but GLUT1 staining was observed at the cell membrane of the atypical, dysplastic and neoplastic cells. The expression of GLUT1 varied widely among the biopsy specimens and also among the assigned Vienna categories (Fig. 1). No GLUT1 expression was observed in any of the sections that were classified as negative or indefinite for neoplasia/dysplasia, or in those sections diagnosed as low-grade dysplasia/adenoma. By contrast, positive staining for GLUT1 occurred to the extent listed in Table 2, in the sections diagnosed as being high grade dysplasia/adenoma, carcinoma in situ, suspicious for carcinoma, papillary carcinoma, well differentiated tubular adenocarcinoma, moderately differentiated tubular adenocarcinoma, poorly differentiated tubular adenocarcinoma and signet ring cell carcinoma. With respect to the Vienna classification, no GLUT1 staining was observed in sections diagnosed as C1, C2, or C3, but it was observed in 10.1% of the cells in the sections classified as C4 and in 25.0% of the cells in the C5 sections (Table 3). These differences were statistically significant. The positive C4 sections had an intensity of

1+, while the positive C5 sections had intensities ranging from 1+ to 3+. However, there was no significant correlation between the GLUT1 expression and the subcategories of C4.

The same as for GLUT1, the normal gastric mucosa did not express p53 protein, and the p53 expression differed markedly among both the biopsy specimens and the assigned Vienna categories. Table 4 summarizes the percentage of the sections that showed p53 expression in each of the diagnosis categories. While no detectable p53 staining occurred in sections that were negative for neoplasia/dysplasia, the p53 expression ranged from 2.9% of the cells in the sections classified as indefinite for neoplasia/ dysplasia to much as 75.0% of the cells in the moderately differentiated adenocarcinomas (Fig. 2). Vienna category C1 showed no observable expression of p53, but this expression was observed in increasing percentages of cells in the C2 through C5 categories, and the results were statistically significant (Table 5). The p53 expression in cells of the C2 and C3 biopsies was generally 1+, C4 biopsies ranged from 1+ to 3+ and C5 biopsies were all 2+ to 3+. Unfortunately, the same as was seen for GLUT1, there was no correlation between the p53 expression and the subcategories of C4.

Table 2. GLUT1 expression in different gastric lesions

Diagnosis	Number of cases	(+)	(++)	(+++)	Number of GLUT (+) cases
Reactive atypia	14	0	0	0	0 (0%)
Indefinite for dysplasia	34	0	0	0	0 (0%)
Low grade dysplasia	32	0	0	0	0 (0%)
High grade dysplasia	66	7	0	0	7 (10.6%)
Carcinoma in situ	25	0	1	0	1 (4.0%)
Suspicious for carcinoma	28	4	0	0	4 (14.3%)
Papillary carcinoma	11	6	1	1	8 (72.7%)
WD carcinoma	57	6	3	1	10 (17.5%)
MD carcinoma	20	4	4	1	9 (45.0%)
PD carcinoma	18	1	0	2	3 (16.7%)
Signet ring cell carcinoma	18	0	0	1	1 (5.5%)

WD, well-differentiated; MD, moderately differentiated; PD, poorly differentiated.

Table 4. p53 Expression in different gastric lesions

Diagnosis	Number of cases	(+)	(++)	(+++)	Number of p53(+) cases
Reactive atypia	14	0	0	0	0 (0%)
Indefinite for dysplasia	34	1	0	0	1 (2.9%)
Low grade dysplasia	32	3	2	0	5 (15.6%)
High grade dysplasia	66	4	8	7	19 (28.8%)
Carcinoma in situ	25	1	2	7	10 (40.0%)
Suspicious for carcinoma	28	3	3	8	14 (50.0%)
Papillary carcinoma	11	3	2	6	8 (72.7%)
WD carcinoma	57	2	11	21	34 (59.6%)
MD carcinoma	20	1	3	11	15 (75.0%)
PD carcinoma	18	3	2	8	13 (72.2%)
Signet ring cell carcinoma	18	4	2	2	8 (44.4%)

WD, well-differentiated; MD, moderately differentiated; PD, poorly differentiated.

Table 3. GLUT1 expression according to Vienna classification

Category	Number of cases	Number of GLUT1 (+) cases
I	14	0 (0%)
II	34	0 (0%)
III	32	0 (0%)
IV	119	12 (10.1%)
V	124	31 (25.0%)

Table 5. p53 expression according to Vienna classification

Category	Number of cases	Number of p53 (+) cases
I	14	0 (0%)
II	34	1 (2.9%)
III	32	5 (15.6%)
IV	119	45 (37.8%)
V	124	81 (65.3%)

DISCUSSION

In the Vienna classification, the diagnoses of high-grade adenoma/dysplasia, carcinoma in situ and "suspicious for invasive carcinoma" were clustered into one category, that of C4, which was called non-invasive high-grade neoplasia. This grouping was made because it was apparent that these three diagnoses could be reproducibly distinguished, and also that the recommended treatment would be the same for all three subcategories. In 2000, the World Health Organization (WHO) classification of tumors of the digestive system,[18] adopted the concept of the Vienna category C4 by introducing the term, high-grade intraepithelial neoplasia. This was defined as a mucosal change with the cytologic and architectural features of malignancy, but without any evidence of invasion into the stroma. High-grade intraepithelial neoplasia includes those lesions termed "severe dysplasia" and "carcinoma *in situ*". The same as with the Vienna classification, the WHO classification into five major categories should

suffice for clinical purposes. However, for research purposes, the subcategories of category 4 may still be important.

The data on the expression or significance of GLUT1 in resected gastric cancer specimens has been recently reported.[12,13] However, to the best of our knowledge, the expression and significance of GLUT1 in the atypical lesions of biopsy specimens has not been reported on. In our study of gastric biopsy specimens, none of the C1, C2 and C3 cases showed GLUT1 expression, but 10.1% of the C4 cases and 25.0% of the C5 cases showed GLUT1 immunostaining. From the studies of resected gastric cancers, Noguchi *et al*.[12] have reported that 19% (13/70) of the specimens were GLUT1-positivie. Kawamura *et al*.[13] have reported that none of the 50 adenoma cases (with no distinction being made between low or high grade dysplasia) expressed GLUT1, whereas 182 of 617 carcinomas (21.9%) were GLUT1-positive. In the latter study, signet ring cell carcinomas and mucinous carcinomas were rarely positive (2.0% and 6.3%, respectively), and the frequency of GLUT1-positive papillary carcinomas was
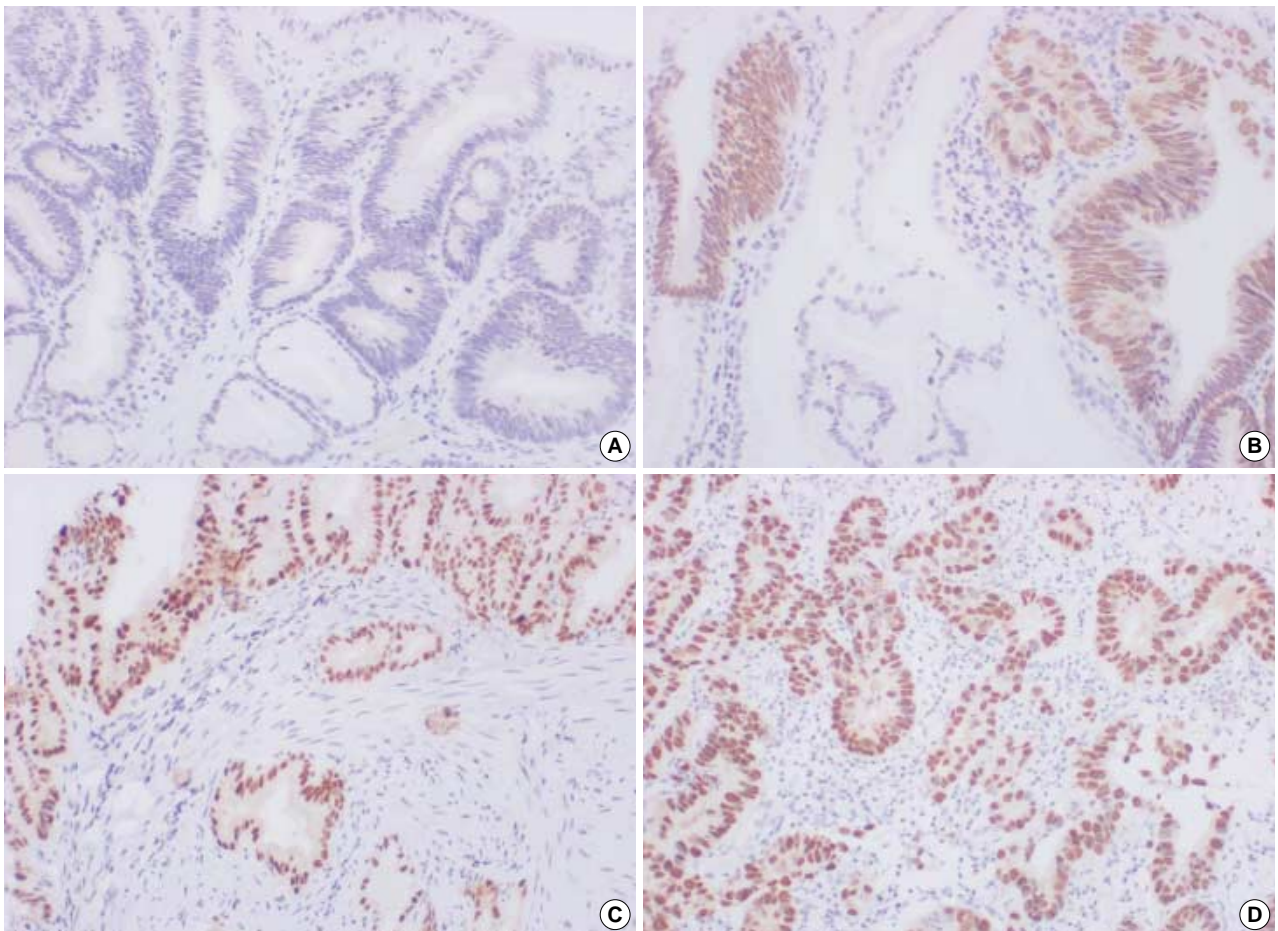

Fig. 2. Immunostaining for p53: (A) Negative staining in low-grade adenoma/dysplasia. (B) Positive staining in high-grade adenoma/dysplasia. (C) Suspicious for invasive carcinoma. (D) Well differentiated adenocarcinoma.

slightly higher (44%) than that of tubular (32%) and poorly differentiated adenocarcinomas (28%). They concluded that for human gastric carcinoma, GLUT1 is expressed late during carcinogenesis and this increases with the progression of disease.[13] Our result are similar to those of Kawamura et al.,[13] in that, 72.7% of the papillary carcinomas, 17.5% of the well differentiated tubular adenocarcinomas, 45.0% of the moderately differentiated tubular adenocarcinomas, 16.7% of the poorly differentiated adenocarcinomas, and 5.5% of the signet ring cell carcinomas were GLUT1-positive. However, in our present study, there were no significant correlations between the GLUT1 expression and the subcategories of C4. The frequencies of GLUT1 positive C4.1, C4.2 and C4.3 were 10.6%, 4.0%, and 14.3%, respectively. Our present results for adenoma are also slightly different from those of Kawamura et al.[13]

Many lesions that are currently interpreted as being low-grade neoplasia by Western pathologists are regarded as high-grade neoplasia by the Japanese pathologists. Previous studies have shown that there are some nuclear and structural features that Japanese pathologists judge to be clues for the diagnosis of carcinomas, such as rounded nuclei, variably sized or enlarged nuclei, enlarged prominent nucleoli, and the variable shape of glands. However, most Western pathologists consider these to be less important. Furthermore, there are lesions for the diagnosis of low grade versus high-grade adenoma/dysplasia such as whether the neoplastic nuclei are primarily limited to the lower or upper halves of the cells in the glandular mucosa that are given much more weight by Western pathologists than by Japanese pathologists. Our results suggests that GLUT1 immunostaining might be a reliable diagnostic tool to differentiate between Vienna categories for gastric biopsy specimens.

In the present study, 0% of the C1 biopsies, 2.9% of the C2 biopsies, 15.6% of the C3 biopsies, 37.8% of the C4 biopsies and 65.3% of the C5 biopsies showed p53 expression. These differences were statistically significant ($p<0.05$). However, there was no significant correlation between the p53 expression and the subcategories of C4. Ito et al.[6] found that none of their cases of C4.1 showed p53 expression, whereas 45% (9/20) of their C4.2 and C4.3 and 41.7% (10/24) of their C5 specimens were positive for p53. Our findings of the p53 expression in 28.8% of C4.1 biopsies suggests that most Japanese pathologists may be overdiagnosing C4.1 to C4.2 or more.

The newly proposed classifications should be used with caution for biopsy specimens as sampling error may result in an underestimation of the neoplastic grade or the depth of invasion. When biopsies were classified by invasiveness according to the Vienna classification, about one-quarter of all assessments by the Western and Japanese pathologists incorrectly diagnosed noninvasive neoplasia (lower than C4) from the biopsy specimens, and invasive neoplasia (C5) from the corresponding resected specimens.[1]

In conclusion, the Vienna classification is very applicable to the gastric biopsy specimens from atypical mucosal lesions. In addition, GLUT1 and p53 are highly useful protein expression markers for differentiating Vienna category 4 lesions from the Vienna category 3 and 5 lesions. Yet the GLUT1 and p53 expressions are not usable to differentiate among the subcategories of C4 lesions.

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