Usefulness of p53 and Ki-67 Immunohistochemical Analysis for Preoperative Diagnosis of Extremely Well-Differentiated Gastric Adenocarcinoma

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Key Words: Extremely well-differentiated gastric adenocarcinoma; p53 Immunohistochemistry; Ki-67 immunohistochemistry; Mucin histochemistry

Abstract

Of 987 cases of gastric adenocarcinoma seen at Nagoya University School of Medicine, we found 6 rare, extremely well-differentiated advanced gastric adenocarcinomas that could not be diagnosed as malignant tumors with only H&E staining, even with repeated biopsies under preoperative endoscopy. The aim of this study was to determine whether an immunohistochemical method using p53 and Ki-67 antibody would be helpful for preoperative pathologic diagnosis. The cancer control cases were 16 cases of ordinary well-differentiated advanced gastric adenocarcinoma, while the gastritis control cases were 22 cases of Helicobacter pylori–positive chronic gastritis. The p53 labeling index and the localization of Ki-67+ cells showed that the special adenocarcinomas in biopsy specimens were distinct from the surrounding normal mucosa and chronic gastritis, but not from the cancer control cases. These methods are useful markers for preoperative pathologic diagnosis of extremely well-differentiated gastric adenocarcinoma, which sometimes is confused with regenerative atypical glands before operation.

Diagnosis of gastric adenocarcinoma has not been difficult owing to technical advances in radiography, endoscopy, endoscopic biopsy, and endoscopic ultrasonography. However, pathologic diagnosis of endoscopic biopsy specimens with H&E staining sometimes does not agree with the clinical diagnosis, even though retrospectively, the biopsy specimens are found to contain adenocarcinoma. One of these types of gastric adenocarcinoma, which is histologically too well-differentiated to discriminate from inflamed or regenerative gastric epithelium, is called extremely well-differentiated gastric adenocarcinoma. Well-differentiated adenocarcinoma according to the Japanese Research Society for Gastric Cancer on the basis of the degree of glandular formation corresponds roughly to the intestinal type as defined by Lauren. The extremely well-differentiated gastric adenocarcinomas were intestinal-type carcinomas that mimicked well the complete type of intestinal metaplasia and showed only low-grade cytologic atypia but considerable structural atypia.

The p53 gene has a critical role in cell cycle regulation and tumor suppression as guardian of the genome. Mutations of the p53 tumor suppressor gene are one of the most frequently detectable genetic alterations in various human cancers. In gastric adenocarcinoma, intestinal-type carcinomas express p53 protein at a high frequency. Expression of p53 protein is an early event in tumorigenesis as observed in minute intramucosal gastric adenocarcinoma.

The monoclonal antibody Ki-67 reacts with a nuclear antigen present throughout the cell cycle (late G1, S, G2, and M phases) of proliferating cells, but is absent in quiescent (G0) cells. In intramucosal gastric adenocarcinoma, proliferating cancer cells were located most densely in the region corresponding to the proliferative zone of the nonneoplastic...
mucosa. This localization pattern of proliferating cells, however, tended to be absent when the carcinoma tissue invaded below the muscularis mucosa.17

We report 6 cases of extremely well-differentiated, advanced-type gastric adenocarcinomas that could not be diagnosed as malignant tumors with only H&E staining, even with repeated biopsies under preoperative endoscopy. We determined the usefulness of the immunohistochemical analyses of p53 oncoprotein, Ki-67 protein, and mucin phenotypes for pathologic diagnosis in preoperative biopsy specimens.

Materials and Methods

Patients

Of 987 cases of gastric adenocarcinoma seen at Nagoya University School of Medicine, Nagoya, Japan, from June 1986 to May 2001, we found 6 intriguing cases of extremely well-differentiated advanced adenocarcinoma of the stomach that could not be diagnosed by H&E staining of endoscopic biopsy specimens taken at least 3 different times before operation. All preoperative biopsy specimens were diagnosed by 3 pathologists (N.N, T.N., T.H.) as regenerating epithelium without cytologic or structural atypia (group I, according to the histologic classification of the Gastric Cancer Study of the Japanese Research Society6), although retrospectively they eventually comprised extremely well-differentiated adenocarcinoma in operative specimens. These lesions were clinically diagnosed as advanced-type gastric adenocarcinomas. Therefore, gastrectomy was performed with the informed consent of the patients. Their clinical profiles are shown in Table I. We examined endoscopic biopsy specimens and operative specimens from the 6 cases. Case 3 was a prospective case. We diagnosed extremely well-differentiated adenocarcinoma in endoscopic biopsy specimens by immunohistochemical methods using p53 and Ki-67 antibody before operation.

For the cancer control cases, we examined biopsy specimens and resected samples from 16 unselected cases of well-differentiated, intestinal-type, advanced adenocarcinoma diagnosed preoperatively by H&E staining of endoscopic biopsy specimens (ordinary well-differentiated adenocarcinoma). For the gastritis control cases, we examined biopsy specimens from 22 cases of Helicobacter pylori–positive chronic gastritis with regenerative epithelium.

Immunohistochemical and Mucin Histochemical Analysis

Sections from 2 representative paraffin blocks of each case were immunostained with p53 (mouse monoclonal antibody, clone DO-1, Immunotech, Marseille, France); Ki-67 (mouse monoclonal antibody, clone MIB1, Immunotech); human gastric mucin (HGM) (mouse monoclonal antibody, clone 45M1, Novocastra, Newcastle upon Tyne, England); a marker for gastric foveolar mucin,18 MUC2 (mouse monoclonal antibody, clone Ccp58, Novocastra); an intestinal apomucin that is expressed predominantly in intestinal goblet cells19-21; and CD10 (mouse monoclonal antibody, clone 56C6, Novocastra), which detects the brush border of absorptive cells.22 Sections also were stained with galactose oxidase–Schiff, a marker for gastric foveolar mucin23-26; paradoxical concanavalin A class III, a marker for gastric gland mucin (mucous neck cells and pyloric gland cells)27-29; and high-iron diamine–alcian blue, pH 2.5, a marker for the detection of sialomucin and sulfomucin.30

Three-micrometer sections were mounted on silanized slides, deparaffinized, and rehydrated through graded alcohol to water. Next, slides were microwaved at 95°C for 6 cycles of 5 minutes each in a 10-mmol/L concentration of sodium citrate buffer (pH 6.0) for Ki-67 or for 7 cycles of 5 minutes each for p53. Then the slides were allowed to cool for approximately 1 hour at room temperature to enhance antigen retrieval. No pretreatment was performed for HGM, MUC2, and CD10. Endogenous peroxidase activity was blocked by incubation with 0.3% hydrogen peroxide for 20 minutes. Then specimens were treated with 10% normal rabbit serum for 10 minutes at room temperature in a cover plate.

Table I

<table>
<thead>
<tr>
<th>Case No./ Sex/Age (y)</th>
<th>Depth of Invasion</th>
<th>Tumor Type (Borrmann)</th>
<th>Location</th>
<th>TNM Classification</th>
<th>Frequency of Preoperative Biopsies</th>
<th>Chief Complaint</th>
<th>Plasma CEA (ng/mL)*</th>
<th>Chemotherapy Course</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/M/58</td>
<td>SE</td>
<td>70</td>
<td>IV</td>
<td>U/Less</td>
<td>T3 N0 M0</td>
<td>3</td>
<td>None</td>
<td>1.6</td>
</tr>
<tr>
<td>2/F/59</td>
<td>SI</td>
<td>47</td>
<td>III</td>
<td>U/Post</td>
<td>T4 N0 M0</td>
<td>3</td>
<td>None</td>
<td>7.8</td>
</tr>
<tr>
<td>3/F/78</td>
<td>SE</td>
<td>43</td>
<td>III</td>
<td>U/Post</td>
<td>T3 N0 M0</td>
<td>3</td>
<td>Fatigue</td>
<td>2.6</td>
</tr>
<tr>
<td>4/M/62</td>
<td>SS</td>
<td>48</td>
<td>II</td>
<td>M/Ant</td>
<td>T2 N0 M0</td>
<td>4</td>
<td>None</td>
<td>ND</td>
</tr>
<tr>
<td>5/M/69</td>
<td>SS</td>
<td>35</td>
<td>III</td>
<td>L/Less</td>
<td>T2 N1 M1</td>
<td>5</td>
<td>Epigastralgia</td>
<td>1.0</td>
</tr>
<tr>
<td>6/M/49</td>
<td>SS</td>
<td>32</td>
<td>III</td>
<td>L/Ant</td>
<td>T2 N0 M0</td>
<td>3</td>
<td>None</td>
<td>1.8</td>
</tr>
</tbody>
</table>

Ant, anterior wall; CEA, carcinoembryonic antigen; L, lower third; Less, lesser curvature wall; M, middle third; ND, not done; Post, posterior wall; SE, exposed beyond the serosa; SI, invaded beyond serosa; SS, subserosa; U, upper third.

The normal level is <5 ng/mL. Systeme International units are given in micrograms per liter; the conversion factor is 1.0.
Primary antibodies were incubated with tissue sections for 18 hours at 4°C. After washing with a 0.01-mol/L concentration of phosphate-buffered saline, they were incubated with biotin-conjugated antimouse immunoglobulin for 10 minutes at room temperature and then incubated with peroxidase-conjugated streptavidin for 5 minutes at room temperature using a Histofine kit (Nichirei, Tokyo, Japan). Demonstration of binding sites with the peroxidase reaction was achieved with 3,3'-diaminobenzidine tetrahydrochloride (0.25 mg dissolved in 1 mL of 0.02% hydrogen peroxide). Faint nuclear staining, sufficient to aid in orientation but not enough to influence the judgment of positivity, was performed with Mayer hematoxylin solution.

The p53 labeling index (LI) was determined by calculating the number of positive nuclei per 500 gastric epithelial cells and cancer cells in 1 representative section. The count was performed under low magnification (×100) using a double-headed light microscope (BH-2, Olympus Optical, Tokyo, Japan).

The Ki-67 LI was determined as follows: the Net Micrometer (an ocular lens with grid marks; Net Micrometer Disk 7 mm/49, Olympus Optical) was used to count the numbers of both positive and negative nuclei under low-power magnification (×100). Next, we totaled the numbers of both positive/positive and negative nuclei of gastric epithelial cells and cancer cells in each horizontal row (of 7 squares each) and calculated the percentages. We designated Ki-67D as the dispersion of the percentage of positive cells (positive nuclei/total nuclei) that formed a vertical line.

Classification of Mucin Phenotypes

Galactose oxidase–Schiff, paradoxical concanavalin A class III, and HGM were defined as G-type markers. High-iron diamine–alcan blue, pH 2.5, MUC2, and CD10 were defined as I-type markers. Positive staining of these phenotypes in mucin was graded as diffuse (2+, >30% of cells), sporadic (+, <30% of cells), or negative (−, no positive cells).5 The criteria for the classification of mucin phenotypes were as follows: G type, positive with at least 1 G-type marker and negative with all I-type markers; I type, positive with at least 1 I-type marker and negative for all G-type markers; M type, positive with both G-type and I-type markers; and null type (N type), negative with all G-type and I-type markers.31

Data Analysis

The Wilcoxon signed-rank test was used to analyze the scores of the LIs and Ki-67D. Differences between confidence intervals were more than 95% (P < .05). Data are given as mean ± SE unless stated otherwise.

Results

Clinical History

Clinical profiles of the 6 cases of extremely well-differentiated gastric adenocarcinomas are summarized in Table 1. Cases without chief complaints were detected by physical checkup. All of these carcinomas were at the advanced stage and ulcerated. The carcinoma of case 5 metastasized to the liver. Carcinomas were located in every area of the stomach. We clinically diagnosed them as gastric adenocarcinomas by radiography and endoscopy. Image 1A and Image 1B show the double-contrast barium meal radiograph and endoscopic photograph of case 3. Ulcerated carcinoma (Borrmann type III) is localized in the gastric cardia to the upper body. The
irregular ulcer margin blends partly into the adjacent stomach mucosa, and the uneven base is amorphous.

Pathologic Findings

Image 2A and Image 2B show the H&E staining of the resected sample of case 3. The little neoplastic and well-defined glandular structures with tubules were spread sparsely without any change in atypia in an expanding growth pattern. Although they showed tortuous, branching, anastomosing, and plexiform structures, they lacked overt back-to-back glands and solid areas. Although there were irregular glandular structures, the histologic structural atypia was very slight. The surface of the mucosa had regenerated epithelium. Inflammatory cell infiltration was somewhat pronounced, and connective tissue proliferation was evident. There was chronic atrophic gastritis surrounding the carcinoma. The tumor cells were relatively mature, with hyperchromatic, well-rounded nuclei containing an even distribution of chromatin and few prominent nucleoli. Carcinoma cells were normal in size and contained reduced amounts of cytoplasm and had an increased nuclear/cytoplasmic ratio, but nuclear polarity and few mitoses. There was little cellular atypia in these adenocarcinomas. Image 2C and Image 2D show H&E staining of the endoscopic biopsy specimen. They showed a nearly normal order of maturation and differentiation of gastric proper glands. In the deep mucosal layer, the atypical glands described were observed but barely exhibited any structural atypia, which was too difficult to diagnose as adenocarcinoma only by biopsies.

Immunohistochemical Analysis

$p53$ Expression

Immunohistochemically, overexpression of the p53 oncoprotein was observed in all cases of extremely well-differentiated
adenocarcinoma, both in resected samples and biopsy specimens. Figure 1A demonstrates that the p53 LI of extremely well-differentiated adenocarcinoma (0.37 ± 0.16) was significantly higher than that of the corresponding nonneoplastic mucosa surrounding the cancer (0.02 ± 0.01) in resected samples. Ten cases (62%) of ordinary well-differentiated adeno-
Adenocarcinoma were p53+, and their p53 LI was 0.57 ± 0.15 in resected samples. The p53 LI in resected samples of total cases of ordinary well-differentiated adenocarcinoma (0.32 ± 0.18) was significantly higher than that of gastritis control cases. Twelve gastritis control cases (55%) were p53+, and their p53 LI was 0.02 ± 0.02 in biopsy specimens.

Ki-67 Expression

In Ki-67-immunostained sections, 15 (94%) of 16 cases of ordinary well-differentiated adenocarcinoma had positive cells, as did all cases of extremely well-differentiated adenocarcinoma and *H pylori*-positive chronic gastritis. Positive cells appeared in the lower third of the mucosa, suggesting a proliferative zone of chronic gastritis tissues and normal nonneoplastic surrounding adenocarcinomas, but were randomly localized throughout adenocarcinoma tissue as shown in Image 4. Figure 2A demonstrates that the Ki-67D of extremely well-differentiated adenocarcinoma (46 ± 23) was significantly lower than that of corresponding normal mucosa surrounding the cancer (532 ± 247) in resected samples. The Ki-67D of ordinary well-differentiated adenocarcinoma (41 ± 44) also was significantly lower than that of the corresponding nonneoplastic mucosa surrounding adenocarcinoma (469 ± 236) in resected samples. Figure 2B demonstrates that the Ki-67D of extremely well-differentiated adenocarcinomas (46 ± 70) was significantly lower than that of gastritis control cases in biopsy specimens (876 ± 288).

Mucin Phenotypes

Mucin phenotypes of the 6 cases of extremely well-differentiated adenocarcinoma and surrounding nonneoplastic mucosa are summarized in Table 2. There were all kinds of mucin phenotypes, (ie, no characteristic mucin phenotype) in these adenocarcinomas. The phenotypes of adenocarcinoma were not so distinct from those of the surrounding nonneoplastic mucosa.

Discussion

We report 6 cases of extremely well-differentiated, intestinal-type gastric adenocarcinoma. This type of gastric adenocarcinoma is very rare (6/987 [0.6%] in our study) and clinicopathologically difficult to discriminate from inflamed or regenerative changes of the gastric epithelium. We could not diagnose biopsy specimens from this type of adenocarcinoma as neoplasms with only H&E staining owing to the slight nucleic and gland structural atypia. The present study indicated that the p53 LI was helpful for diagnosing this type of gastric adenocarcinoma. Mutations of the p53 tumor suppressor gene are one of the most frequently detectable

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**Figure 1** p53 Labeling index (LI). Data are shown as mean ± SE (error bars). **A**, Resected samples. Bars represent the following: a, extremely well-differentiated adenocarcinoma (n = 6); b, surrounding nonneoplastic mucosa of extremely well-differentiated adenocarcinoma (n = 6); c, ordinary well-differentiated adenocarcinoma (n = 16); d, surrounding nonneoplastic mucosa of ordinary well-differentiated adenocarcinoma (n = 16). **B**, Biopsy specimens. Bars represent the following: a, extremely well-differentiated adenocarcinoma (n = 5); b, ordinary well-differentiated adenocarcinoma (n = 14); c, *Helicobacter pylori*-positive chronic gastritis (n = 22). NS, not significant.
The frequency of p53 protein expression in gastric cancers ranges from 42.5% to 57%. Many investigators have found higher frequencies of p53 positivity in intestinal-type gastric cancers (62%-70%) and advanced cancers (61%) than in the diffuse type (35%-52%) and early cancers (30%). Moreover, there is an
intratumoral heterogeneous distribution of p53+ cells. A p53 LI greater than 5% was observed in 70% of gastric tumors. On the other hand, p53 was overexpressed in 15% of *H. pylori*–positive chronic gastritis to 38% of metaplastic gastritis. However, the p53 LI was approximately 4% in the mucosa of *H. pylori*–positive gastric antrum and body specimens. In a previous study, all cases of extremely well-differentiated, intestinal-type gastric adenocarcinoma were p53+ and their LI was 37%, which suggests a similar pattern of p53 immunohistochemical overexpression with ordinary well-differentiated intestinal-type adenocarcinoma but a pattern significantly different from that of chronic gastritis. Thus, one can distinguish extremely well-differentiated adenocarcinoma from chronic gastritis even with biopsy specimens by means of immunohistochemical overexpression of p53 oncoprotein.

We found that the disturbed localization of Ki-67+ cells is a useful pathologic feature for diagnosing extremely well-differentiated, advanced-type adenocarcinoma. The frequency of Ki-67 protein expression in gastric cancers was 88%. In our cases, 97% of cancer cells were positive with Ki-67. Its expression is stable and has a high frequency, so it provides an adequate method for evaluating the proliferative zone of the gastric mucosa. In intramucosal gastric adenocarcinoma tissues showing organoid differentiation, proliferating cancer cells were located most densely in the region corresponding to the proliferative zone of the nonneoplastic mucosa. Actually, proliferating cancer cells in adenocarcinoma of the pyloric mucosal type appeared most densely in the middle layer of the mucosa, whereas the cells in adenocarcinoma of the intestinal metaplastic type appeared in the lower layer of the mucosa. This pattern of localization of proliferating cells, however, tended to be absent when the carcinoma tissue invaded below the muscularis mucosa, suggesting that the disturbance of organoid differentiation possibly triggers invasion below. Another study described that even in minute intramucosal gastric adenocarcinoma, less than 5 mm in longest dimension, only 23% of adenocarcinoma conserved a proliferative zone at the bottom of the carcinomatous tubules. The rate of p53 overexpression in these minute cancers without a proliferative zone was significantly higher than in those with the zone. On the other hand, proliferating epithelial cells were located most frequently in the neck region in the normal fundic and pyloric mucosa. While infected by *H. pylori*, proliferating epithelial cells extend beyond the neck region toward the surface epithelium. Eradication of *H. pylori* significantly decreased epithelial cell proliferation. In chronic gastritis with intestinal metaplasia, Ki-67 nuclear immunoreactivity was translocated to the lower part of the glands. Taken together, distribution of Ki-67–immunoreactive proliferating cells is disturbed in invasive gastric adenocarcinoma, whereas it is only enhanced in chronic gastritis infected by *H. pylori* compared with normal gastric mucosa. We applied this pathologic feature to the diagnosis of extremely well-differentiated, advanced-type gastric adenocarcinomas. The distribution pattern of Ki-67 immunoreactivity was reflected in the dispersion of its positive ratio in the microscopic matrix area of the mucosal layer (Ki-67D). Ki-67D was significantly lower in both resected samples and biopsy specimens obtained from extremely well-differentiated, advanced-type gastric adenocarcinoma than in the surrounding nonneoplastic mucosa (accompanying chronic

**Figure 2** Dispersion of Ki-67+ cells (Ki-67D). Data are shown as mean ± SE (error bars). A, Resected samples. Bars represent the following: a, extremely well-differentiated adenocarcinoma (n = 6); b, surrounding nonneoplastic mucosa of extremely well-differentiated adenocarcinoma (n = 6); c, ordinary well-differentiated adenocarcinoma (n = 16); d, surrounding nonneoplastic mucosa of ordinary well-differentiated adenocarcinoma (n = 16). B, Biopsy specimens. Bars represent the following: a, extremely well-differentiated adenocarcinoma (n = 5); b, ordinary well-differentiated adenocarcinoma (n = 14); c, *Helicobacter pylori*–positive chronic gastritis (n = 22). NS, not significant.
of extremely well-differentiated gastric adenocarcinoma cases.

Table 2
Mucin Phenotypes of Carcinoma and Surrounding Mucosa of Extremely Well-Differentiated Gastric Adenocarcinoma Cases

<table>
<thead>
<tr>
<th>Case No.</th>
<th>GOS</th>
<th>HGM</th>
<th>Con A III</th>
<th>HID AB pH2.5</th>
<th>MUC2</th>
<th>CD10</th>
<th>Phenotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2+/+</td>
<td>2+/+</td>
<td>2+/+</td>
<td>–/–</td>
<td>–/–</td>
<td>–/–</td>
<td>G/G</td>
</tr>
<tr>
<td>2</td>
<td>–/–</td>
<td>–/2+</td>
<td>2+/2+</td>
<td>–/–</td>
<td>–/+</td>
<td>–/+</td>
<td>G/M</td>
</tr>
<tr>
<td>3</td>
<td>–/2+</td>
<td>–/–</td>
<td>–/2+</td>
<td>–/2+</td>
<td>–/+</td>
<td>–/+</td>
<td>I/G</td>
</tr>
<tr>
<td>4</td>
<td>–/–</td>
<td>–/2+</td>
<td>+/2+</td>
<td>2+/2+</td>
<td>–/+</td>
<td>+/+</td>
<td>M/M</td>
</tr>
<tr>
<td>5</td>
<td>–/–</td>
<td>–/–</td>
<td>–/–</td>
<td>–/–</td>
<td>–/–</td>
<td>–/–</td>
<td>I/M</td>
</tr>
<tr>
<td>6</td>
<td>–/2+</td>
<td>–/2+</td>
<td>–/2+</td>
<td>–/–</td>
<td>–/–</td>
<td>–/–</td>
<td>N/M</td>
</tr>
</tbody>
</table>

Con A III, paradoxical concanavalin A class III; GOS, galactose oxidase–Schiff; HGM, human gastric mucin; HID-AB, high-iron diamine–alcian blue; 2+, diffuse, >30% of cells; +, sporadic, <30% of cells; –, negative, no positive cells.

The criteria for the classification of mucin phenotypes were as follows: G type, positive with at least 1 G-type marker and negative with all I-type markers; I type, positive with at least 1 I-type marker and negative for all G-type markers; M type, positive with both G-type and I-type markers; and null type (N type), negative with all G-type and I-type markers.16

The p53 LI and distribution pattern of Ki-67+ cells in preoperative biopsy specimens obtained from extremely well-differentiated gastric adenocarcinoma will be helpful markers for pathologic diagnosis.

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