Clinicopathological Features and Overexpression of Matrix Metalloproteinases in Intramucosal Gastric Carcinoma with Lymph Node Metastasis

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ABSTRACT
Endoscopic mucosal resection, which has been widely accepted for the treatment of intramucosal gastric carcinoma (IMGC) because of the minimal invasiveness of the procedure and the sustained quality of life it provides, can only be used on the premise that the carcinoma has no lymph node metastasis. We evaluated the clinicopathological and biological features of IMGC with lymph node metastases in relation to matrix metalloproteinase (MMP) expression. Fifteen cases of lymph node metastasis-positive [n(+)] IMGC and 59 cases of lymph node metastasis-negative [n(–)] IMGC were obtained. The expression of MMP-2 and MMP-9 was investigated with immunohistochemical methods. Clinicopathologically, n(+)-IMGCs were more likely to be of a larger size, to be of poorly differentiated adenocarcinoma, to have had lymphatic permeation [ly(+)], and to have ulcerations within the lesion compared to n(–)-IMGCs. The incidence of the positive expression of MMP-9 in n(+)-IMGCs (67%) or ly(+)IMGCs (86%) was significantly higher than that in n(–)-IMGCs (32%) or ly(–)IMGCs (34%). Even in IMGCs, carcinoma cells may produce MMPs that can degrade the basement membrane, allowing them to permeate the lymph capillary. Ulcerations within the lesion may also facilitate the interchange of lymphatic flow between the mucosa and the submucosa, promoting the development of lymph node metastases.

INTRODUCTION
The incidence of early gastric carcinoma, designated as a lesion confined to the mucosa or submucosa, regardless of the presence or absence of lymph node metastasis, has been increasing steadily. The present rate comprises approximately 20–60% of all resected cases of gastric carcinoma (1–3). Recently, EMR has been widely accepted for the treatment of IMGC because of the minimal invasiveness of the procedure and the sustained quality of life it provides (1, 4). The prognosis of early gastric carcinoma is generally excellent (2). In early gastric carcinoma, the most important prognostic factor seems to be the presence or absence of lymph node metastasis, because hepatic metastasis or peritoneal dissemination is extremely rare in such patients (2, 5–7). Most cases of lymph node metastasis in early gastric carcinoma are those in which the carcinoma has invaded the submucosa, although metastasis is occasionally seen even in IMGCs (8). The positive rate of lymph node metastasis in IMGCs has been reported as ranging from 1.9 to 3.4% (3, 8). In such cases, if EMR is used, there is the possibility of failing to remove positive lymph nodes from around the stomach, thereby reducing the chance of a cure (6). It is, therefore, essential to select patients who have no lymph node metastasis for application of this technique. The staging by both macroscopic and histological examinations to exclude IMGCs with lymph node metastasis is considered to be essential (9).

MMPs, a family of closely related enzymes that degrade the extracellular matrix, are considered to be important in facilitating tumor invasion and spread (10). MMPs may be related to the invasion, lymph node metastasis, and survival of gastric carcinoma (11–15). Recent studies have suggested a major role for MMP-2 (72-kDa gelatinase, type IV collagenase) and MMP-9 (92-kDa gelatinase, type IV collagenase) in the digestion of basement membrane type IV collagen, as an important mechanism for vessel invasion and metastasis (16, 17).

In this study, our aim was to clarify the clinicopathological and biological features of IMGCs with lymph node metastasis and to determine whether there was a correlation between lymphogenous metastasis and MMP expression.

MATERIALS AND METHODS
Patients. Among our files of gastric carcinoma cases that were surgically resected at the Department of Surgery II, Kyushu University Hospital (Fukuoka, Japan), and the National Kyushu Cancer Center Hospital (Fukuoka, Japan), between 1975 and 1997, cases of IMGC were found and confirmed by serial step sections of 3–4 mm in width. Fifteen cases of IMGC revealed a n(+) condition. In addition, 59 n(–)-IMGC cases were randomly selected as a group of comparative cases.

The macroscopic and histological findings were evaluated based on the classification established by the Japanese Research Society for Gastric Cancer (18). The macroscopic classifications were divided into five types based on their histological permeation; n(+), positive for lymph node metastasis; n(–), negative for lymph node metastasis.
The predominant shape: type I (protruded), type IIa (elevated), type IIb (flat), type IIc (depressed), and type III (excavated). The histological grade was also divided into three categories based on the predominant features, consisting of well differentiated type (including papillary adenocarcinoma), moderately differentiated type, and poorly differentiated type (including signet-ring cell carcinoma). In addition, several discrete histological parameters were examined, including the presence or absence of an open ulcer or ulcer scar (including submucosal fibrosis) within the lesion, invasion of the muscularis mucosae, ly, and venous permeation.

**Immunohistochemistry of MMPs.** To evaluate the biological characteristics of the n(+)-IMGCs, the expression of MMP-2 and MMP-9 was investigated with immunohistochemical staining using monoclonal antibodies against MMP-2 (Fuji Industry, Toyama, Japan) and MMP-9 (Fuji Industry). Normal mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA) was used as an isotype control antibody. The sections (4 μm thick) were deparaffinized in xylene, hydrated through a graded series of ethanol, and immersed in 3% hydrogen peroxide in 100% methanol for 30 min to inhibit endogenous peroxidase activity. After being rinsed in PBS and incubated with normal rabbit serum for 30 min, the sections were then incubated in humid chambers with the primary antibody, MMP-2, MMP-9, or normal mouse IgG at 1:100 diluted solution overnight at 4, followed by three washes with PBS. The streptavidin-biotin method and Histofine SAB-PO (mouse) kits (Nichirei Corp., Tokyo, Japan) were used. The sections were then incubated with biotinylated rabbit antimouse immunoglobulins G, A, and M (Nichirei Corp.) for 20 min, followed by three washes with PBS. The slides were treated with peroxidase-conjugated streptavidin for 20 min. After being washed in PBS, the slides were developed by immersion into 0.01% H2O2 and 0.05% diaminobenzidine tetrahydrochloride for 1.5 min. A light counterstaining with Meyer’s hematoxylin was carried out.

**Statistical Analyses.** The BMDP Statistical Package program (BMDP, Los Angeles, CA) for the IBM (Armonk, NY) 4381 mainframe computer was used for all analyses. The relationship between the presence or absence of lymph node metastasis and clinico-pathological factors and the relationship between the presence or absence of lymph node metastasis and the expression of MMP-2 or MMP-9 were examined by Fisher’s exact probability test, the χ2 test, and Student’s test. The level of significance was P < 0.05.

**RESULTS**

**Clinicopathological Features.** The clinicopathological data of 15 n(+)-IMGCs and 59 n(−)-IMGCs are shown in Table 1. The median size of the overall of n(+)-IMGCs (5.02 ± 3.14 cm) was significantly larger than that of the n(−)-IMGCs (2.15 ± 1.15 cm). The incidence of poorly differentiated adenocarcinoma in n(+)-IMGCs (73%) was significantly higher than that in n(−)-IMGCs (37%). Seven of 74 cases were ly(−). Six of the seven ly(+) IMGCs revealed lymph node metastasis. The incidence of ly(+) in n(+)-IMGCs (40%) was significantly higher than that in n(−)-IMGCs (17%). Ulceration within the lesion was more frequently seen in n(+) IMGCs (80%) than in n(−)-IMGCs (32%).

**Immunohistochemical Analyses of MMP-2 and MMP-9.** Fig. 1 shows positive expressions for MMP-2 or MMP-9. These were all expressed within the cytoplasm of the carcinoma cells. The cells positive to MMP-2 or MMP-9 were checked to make sure that they were not stained with normal mouse IgG. The expression of MMP-2 or MMP-9 was defined as positive when more than 10% of the carcinoma cells showed positive cytoplasmic staining. In addition, we investigated the expression of MMP-2 and MMP-9 in normal gastric epithelium around carcinomas. The incidence of a positive expression of MMP-2 and MMP-9 in normal gastric epithelium was 7 and 23%, respectively, and there was no significant difference between n(−)-IMGCs and n(+)-IMGCs. Table 2 shows the relationship between lymph node metastasis and the expression of MMP-2 or MMP-9. The incidence of a positive expression of MMP-9 in n(+)-IMGCs (67%) was significantly higher than that in the n(−)-IMGCs (32%; P = 0.0146). As for MMP-2 expression, there was no significant difference between n(−)-IMGCs and n(+)-IMGCs. Table 3 shows the relationship between lymphatic permeation and the expression of MMP-2 or MMP-9. The incidence of a positive expression of MMP-9 in ly(+) IMGCs (86%) was significantly higher than that in the ly(−) IMGCs (34%; P = 0.0080).

**DISCUSSION**

The curative EMR for IMGCs should be based on excluding IMGCs with lymph node metastasis (6). However, it is difficult to diagnose whether there is an absence or presence of lymph node
metastasis preoperatively. According to previous studies (19–23), the criteria for curable IMGC using EMR have been considered to be: (a) intramucosal cancer; (b) a macroscopically protruding type and tumor size of less than 2 cm in diameter; (c) a macroscopically depressed type and tumor size of less than 1 cm in diameter; (d) a lack of mucosal ulceration within the lesion; or (e) a histologically well differentiated type. The clinicopathological results obtained in the current study, showing that n(+)-IMGCs were significantly larger than n(−)-IMGCs, that the average size of n(+)-IMGCs was larger than in n(−)-IMGCs, were compatible with the above criteria.

As for the pathway to lymph node metastasis in IMGC, it is undeniable that lymphatic permeation is the first step in lymph node metastasis and an important factor for lymph node metastasis. In the current study, the incidence of ly positivity in n(+)-IMGCs was significantly higher than that in n(−)-IMGCs. In the current study, all of the n(−)-IMGCs, with the exception of one lesion, were larger than 2.0 cm in diameter, and they were significantly larger than the n(−)-IMGCs. As the size of a carcinoma increases, the chance that carcinoma cells come into contact with lymphoid vessels within the lesion will increase (6). In addition, large lesions are not well suited for EMR because of various technical problems (6).

Sano et al. (24) have reported that no lymph node metastasis was recognized in IMGCs without mucosal ulceration. It has been widely reported that the incidence of ulcerations within the lesions in n(+)-IMGCs was significantly higher than that in the n(−)-IMGCs (8, 25–29). In the current study, ulcerations within the lesion were significantly more common in n(+)IMGCs than in n(−)-IMGCs. In such lesions, the basement membrane is destroyed, and inflammatory reaction or inflamed granulation tissue is enlarged in both the mucosa and the submucosa (30). Thus, large lymphatic vessels within the submucosa can be considered to easily intermingle with intramucosal lymphatic vessels, and the potential for carcinoma cells to invade the submucosal layer through the large lymphatic vessels should thus be estimated to be higher than in IMGCs without ulcers (8, 24, 26, 31).

Although the large size of carcinoma and ulceration within the lesion can contribute to the contact between carcinoma cells and the lymphoid vessels, specific enzymes are necessary for the degradation of the vessel wall and for invasion of the lymphoid vessel. The ability for carcinoma cells to degrade the vessel wall and invade the lymphoid vessel cannot be evaluated directly by only a common clinicopathological study with H&E staining. MMPs are considered to be important in facilitating tumor invasion and spread (10). Bando et al. (32) reported that MMP expression is associated with advanced-stage cancer and that it contributes to tumor progression, invasion and metastasis. Endo et al. (13) reported that the levels of serum and plasma MMP-2 and MMP-9 were important factors relating to metastasis and invasion in gastric cancer, although it has also been reported that MMP-2 and MMP-9 expression were recognized in early gastric carcinoma but were not correlated with lymph node metastasis or the degree of invasion depth (11, 14).

In the current study, it was proved that MMP-9 was significantly correlated with lymphatic permeation and lymph node me-

**Table 2** Relationship between lymph node metastasis and the expression of MMPs

<table>
<thead>
<tr>
<th>Lymph node metastasis</th>
<th>Negative (n = 59)</th>
<th>Positive (n = 15)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>54 (92%)</td>
<td>12 (80%)</td>
<td>NS*</td>
</tr>
<tr>
<td>Positive</td>
<td>5 (8%)</td>
<td>3 (20%)</td>
<td></td>
</tr>
<tr>
<td>MMP-9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>40 (68%)</td>
<td>5 (33%)</td>
<td>0.0146</td>
</tr>
<tr>
<td>Positive</td>
<td>19 (32%)</td>
<td>10 (67%)</td>
<td></td>
</tr>
</tbody>
</table>

* NS, not significant.

**Table 3** Relationship between lymphatic permeation and the expression of MMPs

<table>
<thead>
<tr>
<th>Lymphatic permeation</th>
<th>Negative (n = 67)</th>
<th>Positive (n = 7)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>60 (90%)</td>
<td>6 (86%)</td>
<td>NS*</td>
</tr>
<tr>
<td>Positive</td>
<td>7 (10%)</td>
<td>1 (14%)</td>
<td></td>
</tr>
<tr>
<td>MMP-9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>44 (66%)</td>
<td>1 (14%)</td>
<td>0.0080</td>
</tr>
<tr>
<td>Positive</td>
<td>23 (34%)</td>
<td>6 (86%)</td>
<td></td>
</tr>
</tbody>
</table>

* NS, not significant.

*Fig. 1* Carcinoma cells that are positive for MMP-2 or MMP-9. a and b, in carcinoma cells positive for both MMP-2 (a) and MMP-9 (b), the cytoplasm of the carcinoma cells was stained immunohistochemically (×100). c, the positive section for MMP-9 was not stained with normal mouse IgG (×100). d, section shows carcinoma cells permeating into the lymphoid vessel. The carcinoma cells were positive for MMP-9 (×100).
tastasis in IMGCs. However, MMP-2 did not reveal the results same as MMP-9. It has been reported that the matrix-degrading activity of MMP-9 was nearly 25 times higher than that of MMP-2 and that MMP-9 was more important for the metastatic potential of carcinoma than MMP-2 (33, 34).

In conclusion, this study correlated the clinicopathological features of size, differentiation, lymphatic permeation, and presence of ulcerations with the presence of lymph node metastases. Moreover, this study demonstrated a correlation between MMP-9 expression (but not MMP-2 expression) and lymph node positivity. These findings suggest the possibility that MMP-9 expression, which has been considered to be a marker of advanced carcinoma, may be important factor facilitating lymphatic invasion and metastases in early gastric carcinoma.

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REFERENCES

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