Lymph node micrometastasis and its correlation with MMP-2 expression in gastric carcinoma

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Abstract

AIM: To examine matrix metalloproteinase-2 (MMP-2) expression in gastric cancer tissues and to evaluate its relationship with lymph node micrometastasis.

METHODS: The authors studied 850 lymph nodes resected from 30 patients with gastric carcinoma who underwent gastrectomy with lymphadenectomy using reverse transcription polymerase chain reaction (RT-PCR) assay in addition to H-E staining. MMP-2 expression of the tumor tissues was detected by immunohistochemical technique (EliVision™ plus).

RESULTS: MMP-2 expression was positive in 21 (70%) cases and negative in 9 (30%) cases. No significant correlations were found between MMP-2 expression and other variables such as age, gender, tumor location, tumor diameter, Lauren classification and lymphatic invasion. In contrast, MMP-2 expression correlated significantly with depth of tumor infiltration (P = 0.022), lymph node metastasis (P = 0.030) and tumor differentiation (P = 0.043). Lymph node micrometastases were detected in 77 (12.5%) lymph nodes of 14 (46.7%) gastric carcinoma patients. MMP-2 expression was positive in 12 (85.7%) of the 14 patients with lymph node micrometastases, and in 9 (56.3%) of the 16 patients without lymph node micrometastasis (P = 0.118).

CONCLUSION: Our results demonstrate that MMP-2 expression has significant correlation with tumor invasion, tumor differentiation and lymph node metastases. MMP-2 expression may be an important biological characteristics and significant prognostic parameter of gastric carcinoma. We also conclude that MMP-2 may participate in the development of lymph node micrometastasis of gastric carcinoma. Further investigations are needed to draw a conclusion.

INTRODUCTION

Lymph node metastasis is the most important prognostic factor of gastric carcinoma. Many clinical studies demonstrate that the existence of lymph node micrometastasis is often overlooked by conventional histopathological examination (H-E staining) [1-10]. However, the mechanisms of lymph node micrometastasis remain unclear heretofore. MMP-2 has been reported to be associated with tumor invasion and lymph node metastasis of gastric carcinoma by mediating the degradation of extracellular matrix components [11-14]. However, the relationships between MMP-2 expression in cancer tissues and lymph node micrometastasis have not yet been explored. Therefore, the aim of this study was to investigate correlations between MMP-2 expression and clinicopathologic characteristics of gastric carcinoma and possible relationships between lymph node micrometastasis and MMP-2 expression.

MATERIALS AND METHODS

Patients and specimens
A total of 850 lymph nodes resected from 30 patients with gastric carcinoma who underwent gastrectomy at the Department of Gastrointestinal and Pancreatic Surgery at Sun Yat-sen University of Medical Sciences were studied. There were 17 men and 13 women, ranging in age from 26 to 82 years, with a mean age of 56.8 years. None of these patients had received preoperative chemotherapy or radiotherapy. Total gastrectomy was performed in...
16 patients, distal subtotal gastrectomy in 13 patients, and proximal subtotal gastrectomy in 1 patient. One patient underwent D1 lymphadenectomy, 22 patients underwent D2 lymphadenectomy, 4 patients underwent D3 lymphadenectomy, and 3 patients underwent palliative resection. According to the Lauren’ criteria, 19 tumors were classified as diffusive type carcinomas, and 11 tumors were intestinal type carcinomas. Depth of tumor invasion and extent of lymph node metastasis were classified according to UICC TNM classification.

Half of each resected lymph node was fixed in 10% formalin and embedded in paraffin for routine histopathological examination. The other half was stored in 1ml RNA later (Sigma, USA) at 4 ℃ overnight, then transferred to a clean freezing tube and stored at -70 ℃ for RNA extraction. The resected primary tumors were also fixed in 10% formaldehyde and embedded in paraffin. Consecutive 4 μm sections were cut and stained with hematoxylin and eosin (H-E) staining and immunohistochemistry with anti-MMP-2 antibody.

**RNA extraction**

Lymph node samples were homogenized in 1 mL of Trizol Reagent (Invitrogen) per 50-100 mg of tissue using a power homogenizer. RNA extraction was carried out according to the protocol recommended by the manufacturer. Total RNA was dissolved in diethylpyrocarbonate-treated water and the volume and quality of the RNA then assessed by the ultraviolet spectrophotometer.

**RT-PCR**

Complementary DNA (cDNA) was synthesized and amplified from total RNA using the Access RT-PCR system (Promega). The primer sequences used for CK-20 detection were 5'-gggctgctactcaggttacta-3' (sense) and 5' -ctctcagcagccagtttagcattatc-3' (anti-sense) [15]. cDNA synthesis was monitored by beta-actin RT-PCR using the following primers: 5'-caaatgcttctaggcggact -3' (sense) and 5' -cctcagcagccagtttagcattatc-3' (anti-sense). The RT-PCR was performed in a 25 μL reaction mixture containing 11 μL nuclease-free water, 5 μL 5 × Reacton Buffer, 0.5 μL each of beta-actin primers (20 μmol/L), 1.25 μL each of CK-20 primers (10 μmol/L), 1 μL MgSO4 (25 mmol/L), 0.5 μL AMV reverse transcriptase (5 u/μL), 0.5 μL Tli DNA polymerase (5 U/μL), and 3 μL RNA sample. The Access RT-PCR condition was set up as follows: 1 cycle at 48 ℃ for 45 min (reverse transcription), 1 cycle at 94 ℃ for 2 min (AMV RT inactivation), followed by 40 cycles at 94 ℃ for 30 s (denaturation) and at 62 ℃ for 1 min (annealing) and at 68 ℃ for 1.5 min (extension), followed by a final extension at 68 ℃ for 7 min. The resultant cDNA products of CK-20 and beta-actin were 121 base pairs and 381 base pairs, respectively. The RT-PCR products were analyzed by electrophoresis on 2% agarose gels stained with ethidium bromide.

In this report, “micrometastasis” in the regional lymph nodes was defined as metastasis that was detected only by the RT-PCR assay but not by routine H-E staining (Figure 1).

**Immunohistochemistry**

MMP-2 immunohistochemical staining was performed using EliVision™ plus IHC Kit (Maixin Biological, Fuzhou, China). After the sections were deparaffinized with xylene and dehydrated with ethanol, they were placed in 10 mmol/L citrate buffer and heated in a microwave at 700 W for 5 min for the retrieval of antigens in the specimens. Endogenous peroxidase activity was blocked by incubation of the slides in 3% hydrogen peroxide in absolute methanol at room temperature for 10 min. The sections were then incubated sequentially with 50 μL of mouse monoclonal antibody against MMP-2 (Maixin Biological, Fuzhou, China) overnight at 4 ℃, with 50 μL of polymer enhancer for 20 min, and 50 μL of polymerized HRP-anti mouse IgG for 30 min. The reaction products were visualized with diaminobenzidine (DAB Kit, Maixin Biological, Fuzhou, China), and sections were counterstained with hematoxylin, dehydrated, and evaluated under light microscope. Tris-buffered saline (TBS) solution was used instead of the primary antibody for negative controls.

For the purpose of data analysis, MMP-2 expression was graded according to the proportion of positive tumor cells. If more than 25% of the tumor cells were positively stained for MMP-2, the tumor was classified as positive MMP-2 expression. In contrast, if 25% or less of the tumor cells were positively stained, the tumor was classified as negative MMP-2 expression (Figure 2). The stained sections were observed independently by two pathologists who had no knowledge of the clinicopathological data.

**Statistical analysis**

Statistical analysis was performed by the Fisher’s exact test to examine the association of MMP-2 expression of tumor tissues with the clinicopathologic characteristics of gastric carcinoma, and the relationship between MMP-2 expression of tumor tissues and lymph node micrometastasis was evaluated. Statistical significance was defined as P < 0.05.

**RESULTS**

**Correlations between MMP-2 expression and clinicopathologic characteristics of gastric carcinoma**

MMP-2 expression was positive in 21 (70%) cases and negative in 9 (30%) cases. No significant correlations were found between MMP-2 expression and other variables such as age, gender, tumor location, tumor diameter, Lauren classification and lymphatic invasion. In contrast, MMP-2 expression correlated significantly with depth of tumor infiltration (P = 0.022), lymph node metastasis (P = 0.03) and tumor differentiation (P = 0.043) (Table 1).
Figure 2 Expression of MMP-2 in gastric carcinoma. A: poorly-differentiated gastric carcinoma (× 400); B: moderately-differentiated gastric carcinoma (× 400); C: well-differentiated gastric carcinoma (× 400).

Table 1 Correlation between MMP-2 expression and clinicopathologic characteristics of gastric carcinoma

| Variable               | Patients (n) | MMP-2 expression | P value |
|------------------------|--------------|-------------------|---------|
|                        |              | Negative (%)      | Positive (%) |       |
| Gender                 |              |                   |           |       |
| Male                   | 17           | 6 (35.3)          | 11 (64.7) |       |
| Female                 | 13           | 3 (23.1)          | 10 (76.9) | 0.691  |
| Age                    |              |                   |           |       |
| < 50 yr                | 10           | 1 (10)            | 9 (90)    |       |
| ≥ 50 yr                | 20           | 8 (40)            | 12 (60)   | 0.204  |
| Superficial diameter   |              |                   |           |       |
| < 5 cm                 | 17           | 7 (41.2)          | 10 (58.8) |       |
| ≥ 5 cm                 | 13           | 7 (53.8)          | 6 (46.2)  |       |
| Tumor location         |              |                   |           |       |
| Upper/Middle third     | 17           | 5 (29.4)          | 12 (70.6) |       |
| Lower third            | 13           | 4 (30.8)          | 9 (69.2)  | 1.000  |
| Histologic type        |              |                   |           |       |
| Intestinal             | 11           | 5 (45.5)          | 6 (54.5)  |       |
| Diffuse                | 19           | 4 (21.1)          | 15 (78.9) | 0.225  |
| Depth of invasion      |              |                   |           |       |
| T1                     | 5            | 4 (80)            | 1 (20)    |       |
| T2                     | 16           | 4 (25)            | 12 (75)   |       |
| T3/T4                  | 9            | 1 (11.1)          | 8 (88.9)  | 0.022  |
| Histologic differentiation |          |                   |           |       |
| Well                   | 7            | 4 (57.1)          | 3 (42.9)  |       |
| Moderate               | 6            | 3 (50)            | 3 (50)    |       |
| Poorly                 | 17           | 2 (11.8)          | 15 (88.2) | 0.043  |
| Lymph node metastasis  |              |                   |           |       |
| Positive               | 20           | 3 (15)            | 17 (85)   |       |
| Negative               | 10           | 6 (60)            | 4 (40)    | 0.030  |
| Lymphatic invasion     |              |                   |           |       |
| Positive               | 18           | 3 (16.7)          | 15 (83.3) |       |
| Negative               | 12           | 6 (50)            | 6 (50)    | 0.102  |

Table 2 Correlation between MMP-2 expression of tumor tissues and lymph node micrometastasis

| Micrometastasis | Patients (n) | MMP-2 expression | P value |
|-----------------|--------------|-------------------|---------|
|                |              | Negative (%)      | Positive (%) |       |
| Positive        | 4            | 2 (43.3)          | 12 (56.7) | 0.118  |
| Negative        | 16           | 7 (43.7)          | 9 (56.3)  |       |

Relationships between lymph node micrometastasis and MMP-2 expression

Lymph node micrometastases were detected in 77 (12.5%) lymph nodes of 14 (46.7%) gastric carcinoma patients. MMP-2 expression was positive in 12 (85.7%) of the 14 patients with lymph node micrometastasis, and in 9 (56.3%) of the 16 patients without lymph node micrometastasis. However, the difference between these two groups was not statistically significant (P = 0.118) (Table 2).

DISCUSSION

The protein MMP-2 (type IV collagenase) belongs to the family of metalloproteinases. Its function is related to cellular matrix degradation including basement membrane type IV collagen. MMP-2 has been reported to play an important role in the metastatic process and tumor invasion of gastric carcinoma[16-19].

Monig et al[13] investigated correlations between MMP-2 immunoreactivity and currently used classification systems and possible relationships between lymph node metastasis and MMP-2 expression in a prospective study. MMP-2 expression correlated significantly with depth of tumor infiltration, lymph node metastasis, distant metastasis, and UICC stage. In contrast, no significant correlations were found between MMP-2 expression and other variables such as age, tumor differentiation, WHO, Lauren, Goseki, and Ming classifications. Sundblad et al[12] also reported that MMP-2 expression showed significant correlations with parietal depth of infiltration (P = 0.03) and with metastases in regional lymph nodes (P < 0.05). However, Ko et al[20] reported that MMP-2 expression was not correlated significantly with lymph node micrometastasis or depth of invasion (P > 0.05). They also reported that TIMP-2 expression (MMP-2 inhibitor) showed a strong relationship with lymph node micrometastasis and depth of tumor infiltration (P < 0.05).

In the present study, MMP-2 expression was observed in 70% (21 of 30 cases) of gastric carcinoma. MMP-2 expression increased in accordance with the depth of tumor invasion. MMP-2 expression was more frequent in T3/T4 tumors (8 of 9 cases, 88.9%) and T2 tumors (12 of 16 cases, 75%), as compared with T1 tumors (1 of 5 cases, 20%). The difference was statistically significant (P = 0.022). In 20 gastric carcinomas with lymph node metastasis 17 (85%) showed positive MMP-2 expression, while in 10 gastric carcinomas without lymph node metastasis only 4 showed positive MMP-2 expression (P = 0.030). In addition, we also found that there was a significant correlation between MMP-2 expression and tumor differentiation. 88.2% (15 of 17 cases) poorly differentiated gastric carcinomas showed MMP-2 expression, while positive MMP-2 expres-
sion of moderately- and well-differentiated gastric carcinomas were only 50% (3 of 6 cases) and 42.9% (3 of 7 cases) respectively. The difference was also statistically significant ($P = 0.043$).

Our results demonstrate that MMP-2 expression has significant correlation with tumor invasion, tumor differentiation and lymph node metastasis. For gastric carcinoma, MMP-2 expression may be an important biological characteristic and a significant prognostic parameter.

Recent advances in immunohistochemistry and molecular biology techniques have made it possible to detect lymph node micrometastasis not evidenced by routine H-E staining. 28%-68.1% of patients with gastric carcinoma were reportedly identified micrometastases in regional lymph nodes.[21-23] However, the mechanism of lymph node micrometastasis is still not completely known now. Therefore, the main objective of this study was to examine MMP-2 expression in the tumor tissues and explore its relationship with lymph node micrometastasis. Totally, 77 (12.5%) lymph nodes of 14 (46.7%) patients with gastric carcinoma were detected lymph node micrometastases. In 14 gastric carcinoma patients with lymph node micrometastases 12 (85.7%) showed positive MMP-2 expression, while in 16 gastric carcinoma patients without lymph node micrometastases only 9 (56.3%) were examined MMP-2 expression. The results indicate that MMP-2 may participate in the development of lymph node micrometastasis of gastric carcinoma, however, the difference was not statistically significant ($P = 0.118$). This may be explained by the fact that the cases in our study were comparatively few. To draw a further conclusion, a large-scale investigation is needed.

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