Expression of Cyclooxygenase-2 and Matrix Metalloproteinase-9 in Gastric Carcinoma and its Correlation with Angiogenesis

Wei Hao Sun1, Yun Liang Sun1, Ren Nian Fang2, Yun Shao1, Hai Chen Xu1, Qi Ping Xue1, Guo Xian Ding1 and Yun Lin Cheng1

1Department of Geriatrics, First Affiliated Hospital of Nanjing Medical University, Nanjing and 2Department of Pathology, Chaohu City Second People’s Hospital, Chaohu, Anhui province, China

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Objective: To investigate the expression of cyclooxygenase (COX)-2 and matrix metalloproteinase (MMP)-9 in gastric carcinomas, and to correlate this expression with clinicopathological parameters and angiogenesis.

Methods: Ninety-six resected tumor specimens from patients with gastric carcinoma were obtained, and 30 corresponding paracancerous normal tissues were randomly selected as a control. Immunohistochemical staining was used for detecting the expression of COX-2 and MMP-9. Monoclonal antibody against CD34 was used for displaying vascular endothelial cells, and microvascular density (MVD) was calculated by counting of CD34-positive vascular endothelial cells.

Results: The positive expression rates of COX-2, MMP-9 and MVD in the cancerous tissue were 80.2%, 74.0%, and 32.5 ± 8.3, respectively, which were significantly higher than those in the normal tissue (P < 0.01). COX-2, MMP-9 expression rates and MVD in the patients with stages III and IV were 91.4%, 84.5% and 34.9 ± 8.7, respectively, which were significantly higher than those in the patients with stages I and II (P < 0.01). In addition, the Spearman rank correlation test showed that tumor MVD was closely associated with COX-2 (r = 0.311, P < 0.01) and MMP-9 (r = 0.349, P < 0.01) expressions.

Conclusions: Overexpression of COX-2 and MMP-9 is related to tumor invasion and lymph node metastasis in the gastric carcinoma. These results provide evidence that COX-2 contribute to gastric cancer development by promoting MMP-9 expression and angiogenesis.

Key words: cyclooxygenase-2 – matrix metalloproteinase-9 – gastric cancer – angiogenesis – immunohistochemistry

INTRODUCTION

Cyclooxygenase (COX: EC 1.14.99.1) is a key enzyme that catalyses the formation of prostaglandin (PG) and other eicosanoids from arachidonic acid. Two isoforms of COX have been identified: constitutively expressed COX-1 and mitogen-inducible COX-2 (1,2). Increased expression of COX-2 has been linked to carcinogenesis especially in the gastrointestinal cancers (3,4). Gastric carcinoma is one of the most common malignant diseases and remains an important cause of mortality worldwide (5). Although recent studies have indicated that the overall survival in patients with gastric carcinoma has improved in part because of the high detection rate of early cancer and wider implementation of radical surgery (6,7), the prognosis of advanced cancer still remains unsatisfactory.

Because the formation of new blood vessels is essential for tumor growth and progression, tumor-associated angiogenesis plays a critical role in the development and spread of malignant tumors (8). Matrix metalloproteinases (MMPs), a family of closely related enzymes that degrade the extracellular matrix (ECM), are considered to be important in facilitating tumor invasion and spread. MMPs may be related to the invasion, lymph node metastasis and survival of gastric carcinoma (9,10). Recent studies have suggested a major role for MMP-9 in the digestion of basement membrane type IV collagen, as an important mechanism for vessel invasion and metastasis (11). In vitro studies suggest that COX-2 induces angiogenesis by stimulating angiogenic growth factors in cancer cell lines (12,13). However, relationship between the expression of COX-2, MMP-9 and angiogenesis in gastric carcinomas remains unknown. Therefore, the aim of this study was to investigate the expression of COX-2 and MMP-9 in gastric carcinomas, and correlate this expression with clinicopathological parameters and angiogenesis.
MATERIALS AND METHODS

PATIENTS AND SAMPLES

Ninety-six patients who had undergone gastrectomy for primary gastric adenocarcinoma between January 2000 and December 2002 were included in this study. None of studied patients had received the treatment of non-steroidal anti-inflammatory drugs (NSAIDs), chemotherapy or irradiation before operation. All patients were thoroughly informed about the study and gave written consent for the investigation in accordance with the ethical guidelines of Nanjing Medical University.

Cancer tissue was excised from each surgical specimen and adjacent normal tissue (at least 5 cm from the tumor) was randomly selected from 30 patients as a control. The tissue specimens were fixed in 10% formalin, embedded in paraffin, sectioned and stained with hematoxylin and eosin. The tumor specimens were subjected to detailed pathological examination, which identified depth of invasion, nodal status, marginal involvement and histological type of the tumors. Tumors were staged according to the TNM staging system of the International Union against Cancer (UICC) (14).

IMMUNOHISTOCHEMISTRY

Four micrometer thick sections cut from paraffin-embedded tissues were deparaffinized and rehydrated. Sections were then microwaved in citrate buffer, pH 6.1, at 95°C for 10 min for antigen retrieval. Endogenous peroxidase activity was quenched by incubation in 0.3% hydrogen peroxide in methanol for 30 min. Non-specific binding was blocked with 3% normal rabbit serum, or mouse monoclonal antibody against human COX-2 (M-19; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; 1:200 dilution in 1.5% normal rabbit serum), or mouse monoclonal antibody against human MMP-9 (IIA5; NeoMarkers, Inc., Fremont, CA, USA; 1:100 dilution in 1.5% normal goat serum), and CD34 (QBEnd/10; NeoMarkers, Inc.; 1:100 dilution in 1.5% normal goat serum) overnight at 4°C in a humidity chamber. The sections were incubated with primary goat polyclonal antibody against human COX-2 (M-19; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA; 1:200 dilution in 1.5% normal rabbit serum), or mouse monoclonal antibody against human MMP-9 (IIA5; NeoMarkers, Inc., Fremont, CA, USA; 1:100 dilution in 1.5% normal goat serum), and CD34 (QBEnd/10; NeoMarkers, Inc.; 1:100 dilution in 1.5% normal goat serum) overnight at 4°C in a humidity chamber. The sections were stained according to the avidin–biotin complex method using a commercial kit (Vectastain kit; Vector Laboratories, Burlingame, CA, USA) and visualized using 3,3′-diaminobenzidine (DAB) (Vectastain DAB kit; Vector Laboratories). The specimens were subsequently counterstained with hematoxylin (Merck, Darmstadt, Germany). To evaluate the specificity of the COX-2 and MMP-9 antibody, additional blocking experiments with the COX-2-blocking peptide (Santa Cruz Biotechnology, Inc.) or MMP-9-blocking peptide (Lab Vision Co.) were performed as reported previously (15).

EVALUATION OF COX-2 AND MMP-9 IMMUNOSTAINING

Semiquantitative analysis of the immunostainings for COX-2 and MMP-9 was performed for each case. The three most
The median age was 62 with a range from 36 to 76 years. Thirty tumors were located in the cardia, 26 in the corpus and 40 in the antrum.

**EXPRESSION OF COX-2 IN GASTRIC CARCINOMAS**

COX-2 expression was predominantly localized in the perinuclear and cytoplasm of carcinoma cells (Fig. 1A). Expression of COX-2 was also observed in vascular endothelial cells, fibroblasts and inflammatory mononuclear cells in the tumor stroma. Additionally, carcinoma cells permeated into the lymphoid plexus and venous vessel were also strongly immunoreactive to COX-2 protein (Fig. 1B). However, only minimal or negligible staining for COX-2 was detected in paracancerous normal tissue (Fig. 1C). When the antibody was preincubated with the blocking COX-2 peptide and applied to the sections, no immunoreactive signals appeared (data not shown).

COX-2 expression in cancerous tissue was significantly higher than that in the normal gastric tissue ($P < 0.001$, Table 2). Among the 96 patients with gastric carcinoma examined, COX-2 overexpression was found in 53 (55.2%) cases. There was no significant correlation between COX-2 expression and clinicopathological parameters with respect to age, gender, tumor site, histological type and the degree of tumor cell differentiation. However, significant difference was noted with respect to the depth of invasion and lymph node metastasis. Both the expression rate and expression level for COX-2 were significantly higher in the patients with stages III and IV or lymph node metastasis than that in those with stages I and II or without lymph node metastasis ($P < 0.005$ or $P < 0.05$, Table 1).

**EXPRESSION OF MMP-9 IN GASTRIC CARCINOMAS**

Cytoplasmic and cell membranous staining for MMP-9 was mainly seen in the tumor stromal cells including fibroblasts, vascular endothelial cells and inflammatory mononuclear cells (Fig. 1D). The distribution of MMP-9 positive carcinoma cells

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**Table 1. Correlation of clinicopathological parameters with COX-2 and MMP-9 expression**

<table>
<thead>
<tr>
<th>Clinicopathological parameters</th>
<th>$n$</th>
<th>COX-2 expression</th>
<th>MMP-9 expression</th>
<th>MVD</th>
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<tbody>
<tr>
<td></td>
<td></td>
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<tr>
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<td></td>
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<tr>
<td>≥60</td>
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<td>15</td>
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<td>7</td>
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<td>7</td>
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<td>26</td>
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<td>6</td>
<td>9</td>
</tr>
<tr>
<td>Antrum</td>
<td>40</td>
<td>5</td>
<td>11</td>
<td>17</td>
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<td></td>
</tr>
<tr>
<td>I + II</td>
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<td>14</td>
<td>9</td>
<td>10</td>
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<tr>
<td>III + IV</td>
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<td>15</td>
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<td>Well and moderately</td>
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<td>17</td>
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<td>Poorly</td>
<td>31</td>
<td>8</td>
<td>7</td>
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<td>Absent</td>
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<td>12</td>
<td>11</td>
<td>9</td>
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</table>

*Result from grade comparisons of COX-2 (or MMP-9) expression among the patients with different clinicopathological characteristics using Wilcoxon test or Kruskal–Wallis non-parametric test.

**Result from percentage comparisons of COX-2 (or MMP-9) expression among the patients with different clinicopathological characteristics using $\chi^2$-test.

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The median age was 62 with a range from 36 to 76 years. Thirty tumors were located in the cardia, 26 in the corpus and 40 in the antrum.
was frequently located in deeply invading tumor cell nests, especially at the invasive margin (Fig. 1E). However, MMP-9 was only weakly expressed in the normal gastric tissue (data not shown). When the antibody was preincubated with the blocking MMP-9 peptide and applied to the sections, no immunoreactive signals appeared (data not shown). The positive expression rate of MMP-9 was 74.0% (71/96) in the carcinoma tissue, which was significantly higher than those in the normal tissue ($P < 0.001$, Table 2).

The correlation between MMP-9 expression and clinicopathological characteristics of patients was similar to COX-2 expression (Table 1). Additionally, MMP-9 expression level progressively rises from patients with low COX-2 expression to intermediate and high COX-2 expression. There was a significant correlation between COX-2 and MMP-9 expression ($r = 0.24$, $P < 0.05$; Fig. 2) in human gastric carcinoma.

**CD34 EXPRESSION AND MVD**

The positive expression of CD34 was mainly presented as brownish yellow or brownish granules in the cytoplasm of vascular endothelial cells. Although new microvessels in the cancerous lesions had no regular contour and were not evenly distributed, they were always found to be most numerous at the periphery of the tumor (hot spot; Fig. 1F). The mean of MVD in the 96 gastric carcinomas was $32.5 \pm 8.3$ (range $13–62$), which was significantly higher than that in the normal mucosa ($13.1 \pm 2.4$, $P < 0.001$). High MVD was significantly associated with the depth of tumor invasion and lymph node metastasis. The relationship between MVD and clinicopathological parameters was shown in Table 1.

**CORRELATION BETWEEN MVD AND COX-2, MMP-9 EXPRESSION**

MVD in the COX-2 positive expression tumors was $33.6 \pm 3.4$, and significantly higher than that in COX-2 negative expression tumors ($28.1 \pm 7.3$, $P = 0.01$). MVD in the MMP-9 positive expression tumors was $33.7 \pm 8.6$, and significantly higher than that in MMP-9 negative expression tumors ($29.1 \pm 7.2$, $P < 0.05$). MVD was significantly

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**Figure 1.** Representative examples of immunohistochemistry for COX-2 (A–C), MMP-9 (D and E) and CD34 (F). Strong COX-2 immunoreactivity was mainly detected in the perinuclear and cytoplasm of cancer cells within the malignant gland (A). COX-2 protein was also expressed in the carcinoma cells permeated into the lymphoid plexus and venous vessel (B), but minimal or negligible in paracancerous normal tissue (C). MMP-9 immunostaining was mainly seen in the tumor stromal cells (D) and invading tumor cells especially at the invasive margin (E). Detection of microvessels in cancerous tissues was highlighted by immunostaining against CD34 (F). Original magnification, ×100.

**Figure 2.** The scatter plot of MMP-9 expressions in gastric carcinomas with different levels of COX-2 expression (grades 0–3) ($r = 0.24$, $P < 0.05$).

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**Table 2.** Expression of COX-2 and MMP-9 in the cancerous and paracancerous normal tissue

<table>
<thead>
<tr>
<th>Groups</th>
<th>$n$</th>
<th>COX-2 expression</th>
<th>MMP-9 expression</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Cancerous tissue</td>
<td>96</td>
<td>19</td>
<td>24</td>
</tr>
<tr>
<td>Normal tissue</td>
<td>30</td>
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<td>4</td>
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</tbody>
</table>

*Result from grade comparisons of COX-2 (or MMP-9) expression between two groups using Wilcoxon test or Kruskal–Wallis non-parametric test.

**Result from percentage comparisons of COX-2 (or MMP-9) expression between two groups using $\chi^2$-test.
associated with COX-2 \( (r = 0.311, \ P < 0.01; \text{Fig. 3A}) \) and MMP-9 \( (r = 0.349, \ P < 0.01; \text{Fig. 3B}) \) expression in the tumors.

**DISCUSSION**

Epidemiological studies have shown that NSAIDs can reduce the incidence rate and mortality of digestive tract carcinomas, including esophageal, gastric and colorectal cancers. The PG synthetic enzyme COX is a target for NSAIDs therapy. Recent studies have demonstrated that COX-2 is overexpressed in many malignant tumors including gastric carcinomas \((4,18,19)\). The present study also demonstrated that COX-2 expression was significantly higher in cancer tissue compared with normal tissue. Furthermore, we analysed the relationship between COX-2 expression and clinicopathological characteristics in patients with gastric carcinoma. It was clearly shown that COX-2 expression was closely correlated with tumor staging and lymph node metastasis, indicating COX-2 may contribute to invasive growth in gastric carcinoma.

These results are in accordance with previous reports that overexpression of COX-2 is significantly associated with lymph node metastasis \((20,21)\) and depth of invasion \((22)\).

It is well known that tumor invasion and metastasis are the major causes of death for cancer patients. However, the exact mechanisms responsible for the formation of metastases are not fully understood. Tumor invasion is considered to be a dynamic, complex, multi-step process, which involves detachment of malignant cells from their point of origin, traversal of ECM and basement membranes, and invasion into lymphoid vascular channels. The metastatic process involves intravasation and extravasation of tumor cells, followed by reimplantation of tumor cells, formation of a new tumor stroma and neoangiogenesis to consolidate a secondary tumor at a distant site \((23)\). Degradation of ECM and basement membranes by the tumor cells is a critical step in the processes of tumor invasion and metastasis. MMPs, a family of nine or more highly homologous zinc-dependent endopeptidases that degrade ECM, are considered to be important in facilitating tumor invasion and spread. Among human MMPs reported previously, MMP-2 (gelatinase \( \text{A/MT}_{72,000} \) type IV collagenase) and MMP-9 (gelatinase \( \text{B/MT}_{92,000} \) type IV collagenase) are thought to be key enzymes for degrading type IV collagen, which is a major component of the basement membrane. Contributions of both enzymes to invasion and metastasis have been documented in numerous reports \((9–11,24–26)\). The present study demonstrated that MMP-9 expression in gastric carcinoma was related to tumor staging as well as to lymph node metastasis. This finding supports previous study that activated MMP-9 correlated well with tumor invasion and lymphatic permeation in human gastric carcinomas \((27)\).

It is interesting that there was a positive association between COX-2 and MMP-9 expressions in human gastric carcinoma. Both COX-2 and MMP-9 immunoreactivity were strongly detected not only in gastric cancer cells but also in the tumor stromal cells including fibroblasts, vascular endothelial cells and inflammatory cells. These findings are compatible with our hypothesis that overexpression of COX-2 is directly involved in the induction of MMP-9 in human gastric carcinoma. The exact mechanism underlying COX-2 upregulation of MMP-9 remains unknown. The production of MMP-9 by endotoxin-stimulated monocytes/macrophages was significantly inhibited by indomethacin, a non-specific COX-2 inhibitor, indicating that PGs mediate this effect \((28)\). The induction of COX-2 in monocytes and the resulting production of PGE\(_2\) has been shown to be involved in the signal transduction pathway leading to MMP production by these cells \((29,30)\). Recently, there is evidence that aspirin inhibits the invasiveness of Epstein–Barr virus-associated tumors through suppression of MMP-9 expression \((31)\). Moreover, prostate cancer cells that received specific and non-specific COX-2 inhibitors showed a significant reduction in the levels of MMP-9 in the culture medium \((32)\). Taken together these in vivo and in vitro data, inducing MMP-9 expression may be one of the mechanisms that COX-2 promotes the development and metastasis in gastric carcinoma.
Angiogenesis is well recognized to be essential for the growth of solid tumors (8,33). It has been demonstrated that COX-2 expression correlates with tumor neovascularization and prognosis in human colorectal carcinoma patients (34). In a rat model of angiogenesis, corneal blood vessel formation is suppressed by a COX-2 inhibitor celecoxib, but not by a COX-1 inhibitor (35). MVD is a reliable index of tumor angiogenesis (36). We found that the MVD in COX-2 positive tumors was significantly higher than that in COX-2 negative tumors, a close correlation was present between MVD and COX-2 expression in human gastric carcinoma. COX-2 expression was also detected in the angiogenic vasculature present within the tumors and preexisting vasculature adjacent to cancer lesions, suggesting that COX-2 may induce newly formed blood vessels to sustain tumor cell viability and growth. In addition, MVD in the tumors with lymph node metastasis was significantly higher than that without and it was also correlated with the depth of tumor invasion. These results are in accordance with Li and co-workers (37) reported previously, and indicating that COX-2 or COX-2-derived PGs may play a major role in the development of cancer through neovascularization. Therefore, inducing tumor angiogenesis may be one of the mechanisms by which COX-2 promotes the development and metastasis in gastric carcinoma.

To produce new vasculature during angiogenesis, endothelial cells must migrate, divide and form tubes (38). Proteolysis of components of the ECM allows endothelial cells to migrate and releases stored angiogenic signaling molecules from the ECM. High levels of MMP-9 in tissues have been associated with active neovascularization (39,40). Studies in mice that were genetically modified to lack MMP-9 expression have shown that MMP-9 contributes to the angiogenic switch that occurs during carcinogenesis (39–42). Zhao et al. (43) recently demonstrated that MMP-9 promote the angiogenesis in the gastric cancers. A positive correlation between MVD and MMP-9 expression has also been confirmed in the present study. Therefore, host-derived MMP-9 expression, most likely induced by COX-2 overexpression, appears to play a critical role in angiogenesis and progressive growth in human gastric carcinoma. Both COX-2 and MMP-9 protein may be served as a marker for invasiveness and metastasis in gastric carcinoma.

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References


