Abstract. Gastric cancer is the second most common cause of cancer-related death worldwide and the highest incidence of this cancer has been reported in Asia, especially in China. Identification of early stage lesions is vital in achieving high survival rate. However, due to the lack of reliable biomarkers, the majority of gastric cancer is presented at an advanced stage. Recently, it has been reported that Id-1, a helix-loop-helix protein, may be a valuable diagnostic marker in many types of human cancer. In this study, we evaluated Id-1 protein expression in gastric cancer specimens and compared it with non-malignant tissues. In addition, to investigate whether Id-1 expression levels were associated with the aggressiveness of this disease as implicated in other cancer types, we also assessed Id-1 expression levels in primary tumours and their lymph node metastasized lesions. Our results indicate that up-regulation of Id-1 is a frequent event in gastric cancer but its expression levels are not associated with tumour metastasis. Our evidence provides a possible novel marker for the diagnosis of gastric cancer.

Gastric cancer is the second most common cause of cancer-related death worldwide. The highest incidence of gastric cancer has been reported in Asia, especially in Japan and China, while a much lower incidence has been observed in Western Europe and the United States (1). Although the incidence of this cancer has fallen significantly in developed countries, gastric cancer rates remain high in developing countries such as China (2). If detected early, for instance when the tumour is confined to the mucosa, the 5-year survival rate can reach 90% (3). However, due to the fact that the majority of gastric cancer is detected at an advanced stage, the 5-year survival rate is very low, with overall rates of less than 25% (4). Therefore, early detection of this disease is vital in improving long-term survival.

Unlike other common cancers, such as breast and prostate cancers, research on gastric cancer, especially on the diagnostic and prognostic factors, is limited. Several biological markers that are frequently detected in gastric cancer have been reported, however, due to either small sample sizes or limited number of studies, few definitive conclusions can be drawn from the data available. For example, activation of the Her-2/neu oncogene, which shares significant homology with the epidermal growth factor receptor, has been found in gastric cancer cells and increased Her2/neu levels were associated with an unfavourable outcome (5, 6), indicating its significance in prognosis. Expression of the anti-apoptotic factor Bcl-2 is found to be higher in metastatic cancers than in the primary lesions (7), suggesting its association with invasion potential. In addition, frequent alterations of tumour suppressor genes such as p53 and PTEN are related to poor diagnosis and survival of gastric cancer patients (7-9). Recently, down-regulation of E-Cadherin, a homophilic cell-to-cell adhesion molecule which plays a crucial role in establishing the structural integrity of epithelial tissues, has been found in more than 50% of the diffused gastric cancers (10,11). Although these results provide potential new prognostic factors for gastric cancer, research on the identification of novel molecular markers with high specificity for gastric cancer is still needed.

Recently, we have identified an up-regulation of a helix-loop-helix protein, Id-1 (inhibitor of differentiation/DNA synthesis), in the development of prostate cancer (12). We and others also found that Id-1 expression was unique in many types of human cancer such as breast (13), prostate (14) and nasopharyngeal (15) cancers and that increased Id-1 expression was associated with aggressiveness of these cancers. Recently, over-expression of Id-1 has been

Key Words: Id-1, gastric cancer, up-regulation.
Histologic type
Location

882

collected from the archival tissue bank of the Department of Gastric tissue samples. A total of 65 formalin-fixed and paraffin-

Materials and Methods

suggest as an independent marker for unfavourable prognosis in early stage cervical cancer (16). These lines of evidence suggest that Id-1 expression may be a specific biomarker for both diagnosis and prognosis of human cancer. Id proteins are a group of HLH transcription factors that lack the DNA binding domain. Therefore, they act mainly as inhibitors of basic HLH proteins which positively regulate cell differentiation, resulting in inhibition of cell differentiation (17,18). Recently, the oncogenic role of Id-1 has been suggested to be regulated through inactivation of tumour suppressor pathways such as the p16INK4a/RB pathway (19, 20), and activation of pathways that lead to cell proliferation and protection of apoptosis such as the Raf/MEK and NFκB pathways (21, 22). These lines of evidence strongly indicate that Id-1 may be a common oncopGene that plays an important part in the development and progression of human cancer. Since inactivation of the p16/ RB pathway (23, 24) and activation of the MAPK and NFκB pathways (25-27) are common events in gastric cancers, in this study we investigated the expression of Id-1 protein in gastric cancer specimens and studied its association with the aggressiveness of this cancer. Using 15 pairs of malignant and adjacent non-malignant tissues as well as 50 pairs of the primary and lymph node metastasized tumour samples, we studied, by immunohistochemistry, whether up-regulation of Id-1 was a frequent event in gastric cancer and its association with the progression of gastric cancer.

Materials and Methods

Pathology, the third affiliated hospital, Harbin Medical University (Harbin, China). This included 15 adenocarcinomas and adjacent non-malignant tissues, 50 pairs of primary tumour tissues and their lymph node metastasis. All of the tissues were obtained by surgical resection. For the 15 pairs of malignant and non-malignant tissues, the age distribution was between 40 and 76 years old and the male and female ratio was 2.75:1. The 50 pairs of primary and metastasized cancer tissues were collected from patients aged from 20 to 70 years and the male to female ratio was 7.3:1. The histopathology of the specimens was first examined and classified by pathologists in the Department of Pathology in the same hospital. Table I summarizes the detailed clinicopathological features of all cancer specimens used in this study. Three normal gastric tissues were collected from patients with duodenal ulcer who underwent surgical resection.

**Table I. Clinicopathological parameters of gastric cancer specimens.**

<table>
<thead>
<tr>
<th>Clinicopathological parameter</th>
<th>Number of cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gender</td>
<td>Male 55, Female 10</td>
</tr>
<tr>
<td>Location</td>
<td>Cardia 8, Body 48, Antrum 9</td>
</tr>
<tr>
<td>Histologic type</td>
<td>Moderately-differentiated 18, Moderately-poorly-differentiated 11, Poorly-differentiated 27, Poor-signet ring 1, Signet ring cell 5, Mucinous 3</td>
</tr>
</tbody>
</table>

Gender, parameter number of cases: 20 to 70 years and male to female ratio was 7.3:1. The age distribution was between 40 and 76 years old and the male to female ratio was 2.75:1. The 50 pairs of primary and metastasized cancer tissues were collected from patients aged from 20 to 70 years and the male to female ratio was 7.3:1. The histopathology of the specimens was first examined and classified by pathologists in the Department of Pathology in the same hospital. Table I summarizes the detailed clinicopathological features of all cancer specimens used in this study. Three normal gastric tissues were collected from patients with duodenal ulcer who underwent surgical resection.

Immunohistochemistry. Detailed experimental procedures were described in our previous studies (14). Briefly, tissues were fixed in 10% buffered formalin and embedded in paraffin. Five-micrometer-thick sections were deparaffinized in xylene and rehydrated in graded alcohols and distilled water. After antigen retrieval, endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 30 min, followed by rehydration in PBS and incubation with 5% rabbit serum for 30 min to bind nonspecific antigens. The slides were then incubated overnight at 4°C with polyclonal antibodies against Id-1 (Santa Cruz Biotechnology, CA, USA) in TBS containing 2% rabbit serum and 1% bovine serum albumin. This was followed by incubation with biotinylated anti-rabbit IgG at a dilution of 1:200 for 30 min at room temperature, followed by peroxidase-conjugated avidin-biotin complexes and 3,3'-diaminobenzidine (DAKO, Glostrup, Denmark). The sections were then counterstained with Mayer's haematoxylin and analyzed by standard light microscopy. Sections were incubated with TBS containing 2% rabbit serum and 1% bovine serum albumin without primary antibody as negative controls.

Evaluation of Id-1 immunostaining intensity. The staining intensity for Id-1 protein was scored on a scale from “+” to “++++” where the highest staining intensity was defined as “++++”. For each experiment, both non-malignant and malignant specimens were included so that the relative staining intensity between these two groups could be justified and compared. All sections were evaluated by three investigators (Q.W., X.W, and W.X) in a blinded manner without any information relating to clinical pathological characteristics. When there was a disagreement among the investigators regarding the scores, final results were generated after mutual agreement.

Statistical analysis. The staining intensity of each slide was scored as “−”, “+”, “++”, “+++” or “++++”, representing none, mild, moderate, or heavy staining, respectively. Specimens were then grouped according to gender of the patients, location, growth pattern, depth of invasion and histological types of the tumour. The difference between Id-1 expression intensity was analysed using the SPSS statistical software (SPSS Inc, Chicago, IL, USA). The analysis of the association between Id-1 expression and the clinical subgroups was performed by Chi-square (χ²) test for categorical variables and by Spearman correlation test for continuous variables. A level of p < 0.05 was considered as statistically significant.

Results

Differential Id-1 expression between malignant and non-malignant gastric tissues. To investigate if there was an upregulation of Id-1 protein in gastric cancer cells, we studied Id-1 expression on 15 primary gastric cancer specimens and their adjacent non-malignant tissues. As shown in Figure 1, while there was no evidence of Id-1 protein expression in the non-malignant gastric epithelial cells (A and B), the adenocarcinoma cells showed strong positive cytoplasmic staining for the Id-1 protein (C and D), which agrees with our previous findings that Id-1 protein was detected in the cytoplasm of prostate and nasopharyngeal carcinoma cells but absent in non-malignant cells (14, 15). Thirteen out of 15 (86.7%) of the cancer tissues showed positive staining for Id-1, while it was undetectable in all of the non-malignant tissues.

Figure 1. Immunostaining of Id-1 protein in gastric cancer and adjacent non-malignant tissues. A-B: Negative Id-1 staining is found in non-malignant gastric epithelial cells. C-D: Positive cytoplasmic Id-1 staining was observed in a case of intestinal-type gastric cancer. E-F: A case of non-malignant gastric tissue used as a negative control with omission of primary antibody. Photos were taken under 200x (A, C, E) and 400x (B, D, F) magnifications. Note that up-regulation of Id-1 is shown in malignant gastric cancer cells but absent in the non-malignant cells.
To further confirm the results on the non-malignant tissues, 3 normal gastric tissues from non-cancer patients were also studied under the same conditions and no Id-1 expression was detected. Among the Id-1-positive malignant tissues, over 50% showed high staining intensity ("++" to "+++"") (Table II). Statistical analysis indicated that Id-1 expression was significantly higher in malignant gastric cancer tissues compared to the non-malignant tissues (Table II, \( p < 0.0001 \)).

**Table II. Summary of Id-1 expression in primary tumour and adjacent normal tissues.**

<table>
<thead>
<tr>
<th>No. of samples</th>
<th>Staining intensity</th>
<th>Fisher’s exact test (Two-tailed)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary tumour (%)</td>
<td>Adjacent normal tissue (%)</td>
<td></td>
</tr>
<tr>
<td>5 (33.3%)</td>
<td>0</td>
<td>++++</td>
</tr>
<tr>
<td>3 (20%)</td>
<td>0</td>
<td>+++</td>
</tr>
<tr>
<td>2 (13.3%)</td>
<td>0</td>
<td>++</td>
</tr>
<tr>
<td>2 (13.3%)</td>
<td>0</td>
<td>+</td>
</tr>
<tr>
<td>3 (20%)</td>
<td>18 (100%)*</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>18</td>
</tr>
</tbody>
</table>

Differential Id-1 expression between primary and lymph node metastasized gastric cancers. To further investigate the significance of Id-1 expression in gastric cancer, we next increased the sample size and studied 50 cases of primary tumour samples and their lymph node metastasis using non-malignant tissues as controls. Similar to the results observed previously (Figure 1 and Table II), the majority of the primary tumour samples (96%) stained positive for Id-1, while a high percentage (66%) had relatively high expression ("+++" to "++++") (Figure 2 and Table III). Id-1 expression in lymph node metastasized tumours was lower (Figure 2E and F), with 34% showing negative staining, compared to the primary tumours. However, over 50% of the tumour samples, regardless of being primary or metastasized, showed high staining intensity ("+++" to "++++") as observed previously (Table II). These results indicate that up-regulation of Id-1 protein is a frequent event in gastric cancer but its expression levels are not associated with metastasis.

However, we did not observe any statistically significant association between Id-1 expression levels and the gender of the patients, location, growth pattern, depth of invasion or histological type of the tumour, indicating that Id-1 expression levels may not play an essential role in the clinical characteristics of gastric cancer.

**Table III. Summary of Id-1 expression in primary tumours and their lymph node metastasis.**

<table>
<thead>
<tr>
<th>No. of samples</th>
<th>Staining intensity</th>
<th>Rank sums test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primary tumour (%)</td>
<td>Lymph node metastasis (%)</td>
<td></td>
</tr>
<tr>
<td>17 (34%)</td>
<td>14 (28%)</td>
<td>++++</td>
</tr>
<tr>
<td>16 (32%)</td>
<td>12 (24%)</td>
<td>+++</td>
</tr>
<tr>
<td>9 (18%)</td>
<td>5 (10%)</td>
<td>+</td>
</tr>
<tr>
<td>6 (12%)</td>
<td>2 (4%)</td>
<td>-</td>
</tr>
<tr>
<td>2 (4%)</td>
<td>17 (34%)</td>
<td>-</td>
</tr>
<tr>
<td>Total</td>
<td>50</td>
<td>50</td>
</tr>
</tbody>
</table>

**Discussion**

In this study, we report an up-regulation of a helix-loop-helix protein, Id-1, in gastric cancer using 65 clinical samples. Since Id-1 expression was found in the majority of gastric cancer tissues (over 80%), our results indicate that Id-1 may provide a novel marker for gastric cancer. The fact that Id-1 expression levels were not found to be significantly associated with the metastatic potential of gastric cancer suggests that it may not be a crucial factor in metastasis.

Like other Id family members, Id-1 prevents helix-loop-helix proteins from binding to DNA, thus inhibiting transcription of a number of genes responsible for differentiation and proliferation (18). A number of recent studies have also suggested that Id-1 may function as an oncogene since its overexpression is found in many types of human cancer and its activation is able to interfere with several cell signalling pathways involved in human carcinogenesis (12,14,15, 20-22).

In the present study, we provide the first evidence on the possible role of Id-1 in gastric cancer. Previously, it has been reported that inactivation of the p16/RB pathway is a common event in gastric cancer (23, 24). In addition, activation of the MAPK and NFkB pathways are associated with gastric cancer development and progression (25-27). Since these cell signalling pathways have been shown to be regulated by Id-1 in other cell systems (14, 15, 20-22), it is possible that the increased Id-1 expression in gastric cancer may also contribute to the alteration of these pathways frequently observed in this cancer. We are currently studying the direct role of Id-1 expression in gastric cancer cell proliferation and its effect on p16/RB, MAPK and NFkB pathways.

In addition to its role in cell proliferation, Id-1 has been indicated to promote the invasiveness of human cancer cells (12) and its expression levels were correlated with the
progression of several human cancers (14, 16, 28). However, using 50 primary gastric tumour samples and their lymph node metastasis, we did not observe any correlation between Id-1 expression levels and the metastatic ability of the tumours (Figure 2, Table III). In fact, the Id-1 expression levels seemed to be lower in the metastatic tumours than in the primary tumour cells (Table III). It is possible that, unlike observations in breast and ovarian cancers (28, 29), Id-1 expression may not be essential for the progression and metastasis of gastric cancer.

In summary, we have identified an up-regulation of a novel factor, Id-1, in gastric cancer. Our results further support the role of Id-1 as an oncogene in the development of human cancer. Since the molecular pathogenesis of human gastric cancer is largely unknown, our evidence implicates a possible novel factor in the tumourigenesis of gastric cancer.

Figure 2. Immunostaining of Id-1 protein in primary gastric cancer and the lymph node metastasis. A-B: Negative Id-1 staining is found in non-malignant gastric epithelial cells. C-F: Positive cytoplasmic Id-1 staining was observed in a case of diffused-type gastric cancer (C, D) and its lymph node metastasis (E-F). G-H: A case of malignant gastric tissue used as a negative control with omission of primary antibody. Photos were taken under 200x (A, C, E, G) and 400x (B, D, F, H) magnifications. Note that Id-1 protein expression is shown in both primary gastric cancer and its metastasized lesions.
References


Received August 29, 2003
Accepted December 12, 2003