Expression of Metallothionein II in Intestinal Metaplasia, Dysplasia, and Gastric Cancer

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ABSTRACT

Differential display is a valuable tool for the identification of differentially expressed genes in human carcinogenesis and development. The search for differentially expressed genes in gastric cancer and its premalignant lesions may help to define molecular alterations in the gastric mucosa that may precede the development of gastric cancer. Using the differential display technique, we identified a cDNA fragment, encoding metallothionein (MT) IIa mRNA. We performed immunohistochemical analysis using a monoclonal antibody directed against human MT and tissues obtained from 34 patients with gastric cancer and 20 healthy individuals to determine the expression and localization of MT in gastric cancer and its associated premalignant lesions and to correlate our findings with histomorphological features and Helicobacter pylori status. In addition, MT expression was assessed in gastric tissues obtained from patients with gastric cancer and first-degree relatives of patients with gastric cancers and healthy individuals using reverse transcription-PCR analysis. Northern blot analysis confirmed the overexpression of MT IIa in gastric cancer. In the normal gastric tissues, no MT immunoreactivity was observed at the superficial gastric epithelium toward the top of the gastric glands. However, MT immunoreactivity was detected at the foveolar neck of the gastric glands. Immunohistochemical analysis revealed an intense MT immunoreactivity in gastric cancer cells, independent of tumor stage, grade of differentiation, or tumor type. Furthermore, areas of dysplasia and intestinal metaplasia also exhibited intense MT immunoreactivity. Reverse transcription-PCR analysis of gastric biopsies obtained from first-degree relatives of patients with gastric cancer revealed the frequent expression of MT IIa in this high-risk group as compared with healthy subjects (P < 0.01). The overexpression of MT in gastric cancer and the expression of MT in intestinal metaplasia and dysplasia, as well as the expression of MT in the gastric mucosa of first-degree relatives of patients with gastric cancer, point to a role for MT in the early process of malignant transformation of the gastric mucosa.

INTRODUCTION

Gastric cancer remains a great challenge for clinicians and scientists. It is one of the most frequent cancers worldwide, and it is the second most common cause of cancer-related deaths (1–3). Because the clinical management is largely dependent on the stage of the tumor and the best response is achieved in early gastric cancers, understanding the biology of the tumor, identifying premalignant lesions, and detecting early cancers are the most viable options for improving the overall poor prognosis in these patients. Recently, many groups have analyzed molecular and genetic alterations underlying the pathogenesis of gastric cancer. Besides the activation of oncogenes, such as c-met and K-sam, as well as the inactivation of tumor suppressor genes, such as p53 and APC, the alteration of the adhesion molecule E-cadherin has been reported in gastric cancer (2, 3). Clearly, further molecular analysis is needed to identify other alterations that may contribute to gastric carcinogenesis and that may underlie the formation of premalignant lesions of gastric cancer and, thus, may function as markers for an increased risk of developing gastric cancer.

Differential display presents a novel method for the identification of aberrantly expressed genes in various biological states, such as carcinogenesis or development (4, 5). Generally, this method has proven to be highly effective for the identification of differentially expressed genes in the process of malignant transformation (4, 5). Furthermore, compared with other cloning methods, such as subtraction hybridization, this method is advantageous because of its high reproducibility and the identification of mRNAs with a low copy-number per cell. We used this method to search for differentially expressed genes in gastric cancer.

MTs constitute a family of several intracellular, low-molecular-weight MTs with a high affinity to various heavy metals, such as zinc and copper. Four different sets of MT genes, named MT I-IV, clustered on chromosome 16, have been described. MT I genes encode seven different transcripts, named MT IA, MT IB, MT IE, MT IF, MT IG, MT III, and MT IX (6, 7). The MT II gene encodes only one transcript, MT IIa, similar to MT III, also known as growth-inhibiting factor (7). These proteins are thought to contribute to the detoxification and metabolism of compounds such as heavy metal ions, and further interest in these proteins has been raised after demonstrating their potential role in the generation of cisplatin resistance in patients with malignancies undergoing cisplatin chemotherapy (8–10). Furthermore, the overexpression of MTs in brain tumors and in gastric and breast cancers is associated with poor survival (8, 9, 11, 12). Other groups have also demonstrated the expression of MTs in proliferating normal cells and regenerating cells apart from cancer cells (13).

In our aim to assess the role of MT in the multistage process of gastric carcinogenesis, we used Northern blot analysis, immunohistochemical analysis, and RT-PCR analysis to explore the cellular expression and localization of this gene in the normal gastric mucosa, in premalignant lesions, and in gastric cancer. Furthermore, we assessed the expression of MT in the histologically normal gastric mucosa of individuals with an increased risk of developing gastric cancer.

MATERIALS AND METHODS

Tissues Samples. Tissue samples for molecular analysis were collected from patients undergoing gastric cancer surgery or endoscopy for diagnosis of gastric disease.

Gastric cancer tissues for differential display analysis were obtained from two male patients, ages 61 and 69 years, who were undergoing gastric resection for gastric adenocarcinoma of the intestinal type. In addition, a liver metastasis was resected in the older patient.
For immunohistochemical analysis, tumor tissue was obtained from 34 patients who underwent gastric cancer surgery or endoscopy for the diagnosis of gastric carcinoma. The characteristics and histomorphological data for these patients are given in Table 1. In addition, we collected gastric biopsies from eight patients (five male, three female) without any gastric disease. The median age of this group of patients was 51 years (range, 32–81 years). Another group, 12 patients with IM in the stomach, was studied. Apart from the IM, all of the gastric biopsies from this group of patients revealed gastritis (Table 2).

For RT-PCR analysis, tissue specimens were obtained by surgical resection or endoscopy from the tumor and from a tumor-free location of 19 patients with gastric cancer. Ten males and 9 females with a mean age of 62 years (range, 35–73 years) were enrolled in this study. Biopsies were also obtained from the corpus and antrum of 18 first-degree relatives (4 males) of gastric cancer patients; the mean age was 47 years (range, 27–65 years). Tissues from nine subjects with dyspepsia and histologically normal gastric mucosa, but without gastric cancer family history and without a history of peptic ulcers, were used as normal controls.

The individuals in all of the groups gave informed consent to participate in this study. Immediately after removal, all of the tissues for molecular analysis were put in liquid nitrogen and stored at −80°C until use. This study was approved by the Ethics Committee of the University of Magdeburg, Germany.

**Histology.** Formalin-fixed tissues were processed as previously described (14), and sections were stained with H&E for histological evaluation and with Warthin-Starry stain to detect *Helicobacter pylori* colonization. For the histological typing, we used Lauren’s classification; tumor stage was determined according to the TNM classification (15, 16). There were 31 carcinomas of the intestinal type, 19 carcinomas of the diffuse type, and 3 carcinomas of the mixed type. Tumor stage and grade of differentiation are given in Table 1. Gastritis was histologically classified according to the updated Sydney system (17). Subtyping of IM was performed with the use of Gomori’s aldehyde fuchsin (GAF)-alcian blue (AB) staining (18, 19), and three types of gastritis were identified as previously described by Jass and Felipe (20).

**Table 2 Characteristics and histological diagnosis of patients without gastric cancer**

<table>
<thead>
<tr>
<th>No.</th>
<th>Age</th>
<th>Sex</th>
<th>H. pylori</th>
<th>Grade</th>
<th>Activity</th>
<th>IM</th>
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</table>
| 9 | 64 | M | 2 | 2 | 1 | I
| 10 | 70 | F | 2 | 2 | 1 | II
| 11 | 51 | M | 2 | 3 | 1 | |
| 12 | 77 | F | 3 | 3 | 1 | II
| 13 | 70 | M | 1 | 2 | 1 | |
| 14 | 58 | F | 2 | 3 | 1 | |
| 15 | 45 | M | 1 | 2 | 1 | |
| 16 | 28 | M | 3 | 1 | 0 | X
| 17 | 26 | M | 2 | 2 | 1 | I
| 18 | 22 | F | 3 | 2 | 1 | III
| 19 | 11 | M | 2 | 2 | 1 | |
| 20 | 19 | F | 3 | 1 | 0 | X
| 21 | 11 | M | 1 | 1 | 0 | X
| 22 | 18 | F | 2 | 2 | 1 | |
| 23 | 10 | M | 1 | 2 | 1 | |
| 24 | 22 | F | 1 | 1 | 0 | |
| 25 | 14 | M | 1 | 2 | 1 | |
| 26 | 13 | F | 2 | 2 | 1 | |
| 27 | 8 | M | 1 | 1 | 0 | X
| 28 | 6 | F | 1 | 1 | 0 | |
| 29 | 3 | F | 2 | 2 | 1 | |
| 30 | 11 | F | 2 | 2 | 1 | |
| 31 | 11 | M | 1 | 1 | 0 | |
| 32 | 12 | F | 1 | 1 | 0 | |
| 33 | 8 | F | 1 | 1 | 0 | |
| 34 | 7 | F | 1 | 1 | 0 | |

* Dyspl., dysplasia; X, not determined; –, negative; +, mild immunoreactivity; ++, moderate immunoreactivity; ++++, strong immunoreactivity.

Cells were grown to 80–90% confluence and subcultured, the cells were harvested after passages 6–8.

**mRNA Isolation.** Total cellular RNA was isolated from 100–300 mg of tissue and cells by using the guanidinium thiocyanate method and ultracentrifugation. The chromosomal DNA was removed by the incubation of 50–100 µg of RNA for 15 min at 37°C with 10 units DNase I (Promega, Mannheim, Germany) and 20 units RNasin (Promega) in 40 mM Tris-HCl (pH 8.3), 6 mM MgCl₂, 10 mM NaCl, 2 mM spermidine, and 10 mM DTT. After phenol/chloroform extraction, the RNA was precipitated and dissolved in diethyl pyrocarbonate and water (21).

**RT-PCR Analysis.** One µg of total RNA was reverse transcribed using dNTPs (1 mM), DTT (5 mM), reverse transcription buffer [50 mM Tris-HCl (pH 8.3), 2.5 mM KCl, 3 mM MgCl₂, and 1 mM DTT], and 40 units of RNasin; 6 µl of Superscript plus were added to a total volume of 20 µl. After incubation at 37°C for 1 h, the reaction was terminated by incubating the mixture at 95°C for 10 min. Three different pairs of primers were designed to amplify the coding region of MT Ia, MT IIa and MT III mRNA. Primer sequences were as reported by Blauweggers et al. (22) and were chosen outside the coding regions to avoid amplification of pseudogenes as follows: (a) MT Ia sense: 5′-TTCCACGTGCAGCTTATAGCC-3′; (b) MT Ia antisense: 5′-ATGGGTCAGGGTTGTATGGAA-3′; (c) MT IIa sense: 5′-AAACCTTGTC- CGACTCTTACGGC-3′; (d) MT IIa antisense: 5′-TACTAGCAAACGGT- CACGGTCA-3′; (e) MT III sense: 5′-TGGGAAGAAGCCGGCCTTAC-3′; and (f) MT III antisense: 5′-TGGGATTATGTCATTCCCTC-3′. The 30 µl of reaction mixture was incubated for 4 min at 95°C. The samples were denatured at 95°C for 30 s, annealed at 59°C for 60 s, and then extended at 72°C for 2 min. After 35 cycles, there was a final elongation for 5 min at 72°C, and the samples were stored at 4°C until further use. Eight µl of the PCR product was then electrophoresed on a 1.25% agarose gel together with size markers. The integrity of the cDNAs was confirmed by RT-PCR analysis using primers specific for β-actin, as previously described (21, 23).

**Differentiation Display.** For mRNA differential display, 8 µl of total RNA (3 µg) of each sample was denatured for 10 min at 65°C and then reverse transcribed for 1 h at 37°C in a 20-µl reaction volume using 2.5 mM MgCl₂, 25 µM each dNTP (TaKaRa, Otsu, Japan), 2.5 µM primer (5′-AGGTGACCGGT-3′), 2.5 µM primer (5′-GGTGCACCGGT-3′), 2.5 µM primer (5′-AGGTGACCGGT-3′), and 2.5 µM primer (5′-GGTGCACCGGT-3′). The resulting cDNA, after a 1:37 dilution, was used as template in a 20-µl PCR reaction that contained 0.5 µM arbitrary 10mer primer (5′-AGGTGACCGGT-3′), 2.5 µM primer (5′-GGTGCACCGGT-3′), 2.5 µM primer (5′-AGGTGACCGGT-3′), and 2.5 µM primer (5′-GGTGCACCGGT-3′). After incubation at 15°C for 10 min and then at 95°C for 5 min, the samples were stored at 4°C until further use. Eight µl of the PCR product was then electrophoresed on a 1.25% agarose gel together with size markers. The integrity of the cDNAs was confirmed by RT-PCR analysis using primers specific for β-actin, as previously described (21, 23).
94°C, 1 min at 42°C, and 30 s at 72°C. After completion of the PCR reaction, 14 μl of sequencing stop solution (95% formamide, 20 mM Na₂EDTA, 0.05% w/v bromphenol blue, and 0.05% w/v xylene cyanol) were added to each resulting PCR product. The samples (8 μl) were then loaded on a horizontal 6% polyacrylamide gel and were visualized by silver staining (24). The band of interest (Fig. 1A, arrowhead) was cut from the gel and then transferred into a PCR tube with 100 μl of sterile double distilled H₂O. The gel slice was minced with a fine sterile needle and then boiled for 15 min. After centrifugation, the supernatant was transferred in a new tube to precipitate the cDNA with 3 μl of 3 M sodium acetate (pH 6.0) and 450 μl of ethanol at −80°C for at least 30 min. The cDNA was pelleted by centrifugation and dissolved in 10 μl of sterile double distilled H₂O. For re-amplification, 2 μl of the dissolved cDNA fragment was included as template for a further PCR reaction using the initial primer set and the same conditions described above, with two changes: 6 units of Stoffel fragment of AmpliTaq DNA polymerase (Perkin-Elmer, Langen, Germany) and a 2.5-μM concentration of both primers were used for successful reamplification (24, 25).

**Sequencing.** The reamplified PCR product was cloned using the Original TA Cloning Kit (Invitrogen, Leek, the Netherlands) and was sequenced on a Model 373A-DNA-Sequencer (Applied Biosystems, Langen, Germany) using the DNA-Sequencing-Kit, Ready-Reaction (Perkin-Elmer). Sequencing of the clones revealed a 99-bp fragment corresponding to the 3’ end of the human MT IIa mRNA (7).

**Northern Blot Analysis.** For Northern blot analysis, 20 μg of total RNA from each sample was denatured with glyoxal and DMSO at 50°C for 1 h and fractionated on a 1.2% agarose/4-morpholinepropanesulfonic acid gel. After running at 90 V for 3 h, the RNA was transferred onto a nylon membrane (Hybond-N+, Amersham, Braunschweig, Germany) using 20× SSC. The 99-bp PCR fragment of MT II was purified with QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and labeled with [α-32P]dCTP using the Oligolabeling Kit (Pharmacia, Freiburg, Germany). The hybridization of the probe was performed at 63°C for 16 h as described previously (21, 23). After hybridization, the filter was washed in 2× SSC/0.1% SDS and was evaluated after autoradiography. To verify mRNA integrity and equal loading, a 1.1-kb EcoRI β-actin cDNA fragment (ATCC) was hybridized to the same filters after removal of the first probe by boiling in 0.1× SSC/0.1% SDS. The blot was exposed at −80°C to Kodak XAR-5 film with intensifying screens, and the intensity of the radiographic bands was quantified by laser densitometry (Ultrascan XL, Pharmacia, Uppsala, Sweden).

**Immunohistochemistry.** The presence of MT was assessed using formalin-fixed, paraffin-embedded gastric tissue sections. Sections (4-μm) were deparaffinized in graded alcohol (21, 23). After rinsing with Tris buffer, the sections were incubated with anti-MT antibody. Highly specific monoclonal antibodies for MT were used at a dilution of 1:50 and incubated at 37°C for 60 min. The MT antibody E9 (DAKO, Carpinteria, CA) is a monoclonal mouse antibody derived from the ascites of immunized mice. Horse self-polymerized MT-1 and MT-2 were used as immunogen. Further analysis revealed the specific inhibition of the antibody by glutaraldehyde-polymerized human, horse, sheep, and rat MT-1 and MT-2, pointing to a single and highly conserved epitope (12, 26). Detection of the bound primary antibody was performed using the avidin-biotin-complex method (Vectastain ABC-AP KIT, Vector Laboratories, Burlingame, CA). The sections were incubated with diluted biotinylated secondary antibody (1:200 in RPMI) at 22°C for 30 min, then rinsed with Tris buffer for 5 min, and finally incubated with Vectastain ABC-AP reagent (1:100 in RPMI) for 30 min. The sections were rinsed with Tris buffer again and incubated with Fast Red Chromogen System (Immunotech, Marseille, France). Finally, the slides were counterstained and mounted with aqueous mounting medium (21, 23). All of the immunohistochemical studies were performed using a number of negative and positive controls. As positive controls, breast cancer samples that had previously been shown to express abundant MT by immunohistochemistry were used in our immunohistochemical analysis (12, 26). Furthermore, to ensure the specificity of immunostaining, we performed immunohistochemistry using consecutive sections in the absence of the primary antibody and with preimmune serum. In all of these cases, no immunostaining was detected (not shown). Immunohistochemical data were reviewed by an experienced pathologist (T. G.) who was blinded to the clinical data of the patients. The number of positive cells was counted, and the immunoreactivity was graded as absent, low (<5%), moderate (5–20%), or strong.

**Statistical Analysis.** Whenever indicated, the Fisher’s exact test and χ² test were used to determine statistical analysis, with P < 0.05 taken as the level of significance (27).

**RESULTS**

**MT IIA Is Overexpressed in Gastric Cancer.** By using differential display analysis and human gastric cancer tissues and the adjacent noncancerous gastric mucosa obtained from two patients with gastric adenocarcinoma, we identified a single band in both cancer tissues (Fig. 1A, arrowhead) and in the cell line. In the matched noncancerous gastric tissues of the cancer patients, this band was only faintly present. After an additional round of PCR using the primers as outlined in “Materials and Methods,” we obtained the identical picture, thus confirming the differential expression of this cDNA fragment (not shown). After the cloning of the PCR product, sequencing of the cDNA fragment revealed a cDNA fragment corresponding to the 3’ end of the human MT IIA mRNA. To confirm overexpression of MT IIA, we used this fragment for the generation of a randomly primed labeled cDNA probe for Northern blot analysis. The Northern blot analysis demonstrated enhanced expression of MT IIA mRNA in the tumor tissue (Fig. 1B, T) as compared with the normal counterpart (Fig. 1B, N). This case is identical to the first pair of gastric tissues that were used for the initial differential display analysis (Fig. 1A).

Thus, Northern blot analysis confirmed the overexpression of MT IIA in gastric cancer as compared with the noncancerous gastric mucosa.
Fig. 2. Immunohistochemical analysis of MT expression in gastric cancer. Strong MT immunoreactivity is present in the cytoplasm of gastric cancers of the intestinal (A, B) and diffuse type (C). The pattern of immunostaining was heterogeneous with areas of gastric cancer cells exhibiting MT immunoreactivity next to areas without MT immunoreactivity (A, B). A, ×100; B and C, ×400.
In Gastric Cancers, MT Expression Is Present in IM and Dysplasia and in the Cancer Cells. To determine the cellular site of MT expression in gastric cancers and to assess the expression of MT in preneoplastic lesions of the gastric mucosa, we performed immunohistochemical analysis using a panel of 34 paraffin-embedded gastric cancer tissues. MT immunoreactivity was observed in all 10 gastric cancers of the diffuse type, in 19 (90%) of the 21 intestinal type, and in all of the 3 mixed type (Table 1). Immunoreactivity was present in the cytoplasm and the surface of the cancer cells (Fig. 2). The degree of MT immunoreactivity in the gastric cancers was classified as absent or low in 19 cases, whereas moderate to intense immunoreactivity was observed in 15 cases (Table 1). However, neither the tumor stage, nor the grade of differentiation, nor the histological tumor type correlated with the degree of MT expression in the gastric carcinomas. In addition, we also observed MT immunostaining in dysplasia adjacent to the cancer cells in five cases of gastric cancer (Table 1). In addition, 16 of the 34 gastric cancers exhibited abundant MT immunostaining in areas of IM (Fig. 3).

In the Noncancerous Stomach, MT Expression Is Observed Only at the Foveolar Neck and in IM. To further study the role of MT expression in IM, we performed immunohistochemical analyses using gastric biopsies obtained from patients with IM but without cancer of the stomach and from patients without gastric disease (Table 2). Gastric biopsies of eight healthy patients without IM and without histological signs of gastritis that were negative for H. pylori exhibited MT immunoreactivity only at the foveolar neck of the gastric glands but not in the superficial gastric epithelium (Fig. 4). In all cases, goblet cells exhibited intense MT immunoreactivity.

In the second group of individuals without gastric cancer undergoing endoscopy, H. pylori colonization was observed in 11 of 12 cases, and IM was detected in all of these patients. Eight cases demonstrated type I IM, whereas types II and III IM were present in one and three cases, respectively. All of these cases exhibited histological signs of gastritis of varying degrees (Table 2). In all of the cases, MT expression was found in areas of IM and at the foveolar neck of the gastric glands but, again, was not observed at the superficial gastric epithelium.

In summary, neither group of patients—who differed with respect to the presence of IM and H. pylori colonization of the gastric mucosa—expressed MT at the superficial gastric epithelium, but both groups exhibited MT expression at the foveolar neck of the gastric glands.

First-Degree Relatives of Patients with Gastric Cancer Express MT mRNA in the Gastric Mucosa. A total of 18 first-degree relatives were studied for the presence of MT expression in biopsies taken from the antrum and the corpus. Using specific primers for MT Ia, IIa, and III, we performed RT-PCR analyses. Whereas individuals with a documented history of gastric cancer in their family exhibited MT Ia and III mRNA in 16 and 5 of 18 cases, respectively, individuals without a familial history of gastric cancer exhibited MT Ia mRNA transcripts in only 3 cases (P < 0.01). MT III was not detected at all in the biopsies of healthy individuals. In gastric cancers, the expression of MT Ia and MT III was observed in 13 and 5 of the 19 cases, respectively (Table 3). MT IIa mRNA was observed in all of the cases; however, due to the limited amount of RNA available, quantification of mRNA levels was not performed (not shown).

DISCUSSION

The pathogenesis of gastric cancers remains largely unknown. From histomorphological analysis, a sequence of histological changes of the gastric mucosa, eventually reaching the final step of malignant transformation to gastric adenocarcinoma, has been described. Thus, Correa (28) has proposed the development of gastric cancers arising from chronic gastritis, to IM, to dysplasia, and, finally, to cancer. To date, multiple genetic and molecular alterations that are present in the gastric cancer cell and that may underlie the multistage process of gastric carcinogenesis have been reported. Thus, gastric cancers present with inactivated tumor suppressor genes, such as p53 or APC—
both inactivated by mutations—as well as with activated oncogenes, such as c-met and K-sam, which are frequently amplified and overexpressed in gastric cancers (1–3). Furthermore, new reports demonstrate microsatellite instability and alteration of adhesion molecule expression in these tumors, too (29). To identify molecular changes that are present in gastric cancers and that may also occur in premalignant lesions of gastric cancer, we used the differential display technique to analyze gastric cancer tissues. We compared the differential expression of cDNA fragments in human gastric cancers with a well-known gastric cancer cell line, to assure that differentially expressed bands in the tumor tissues were present in gastric cancer cells. Using this approach, we identified the overexpression of a member of the MT family in gastric adenocarcinoma. To evaluate whether the overexpression of MT might play a role in the multistage process of gastric carcinogenesis and thereby contribute to the pathogenesis of preneoplastic lesions in gastric cancer, we analyzed the expression of MT in histomorphological lesions that have been implicated in the process of gastric carcinogenesis, as well as in the histologically normal gastric mucosa of individuals who have a documented family history of gastric cancer and who have been shown to exhibit an increased risk of developing gastric cancer (30, 31).

Our immunohistochemical analysis revealed that MT is expressed in at least 90% of gastric cancers and is present in premalignant lesions such as dysplasia and IM. Although other groups have demonstrated MT expression in various cancers and found an association between the overexpression of MT and poor survival in astrocytic and pancreatic tumors (8, 9), we could not find an association of MT expression with tumor stage or grade of differentiation in gastric cancer. In addition, MT expression was independent of the type of gastric cancer. Although survival data were not available for these patients, an association seems highly unlikely, inasmuch as the above mentioned parameters seemed to be independent of MT expression. Whereas areas of dysplasia and IM exhibited abundant MT expression, the adjacent noncancerous gastric mucosa as well as the gastric mucosa of healthy individuals did not appear to exhibit MT expression except for cells of the foveolar neck, which is known to be a zone of high proliferative activity (32). When we compared our findings in the cancers with noncancerous gastric tissues, we found expression of MT in areas of IM in gastric cancer patients and in H. pylori-infected patients without gastric cancer. However, the superficial epithelium of the gastric mucosa in healthy individuals did not exhibit MT expression, no matter whether these patients were infected with H. pylori or not. The lack of association between MT expression and the stage of the tumor according to the TNM classification—together with the presence of MT in preneoplastic lesions of gastric cancer and the localized expression of MT in areas of high proliferative activity and regeneration in the normal gastric mucosa—support the hypothesis that the alteration of MT expression may be an early event in the process of gastric carcinogenesis.

In general, normal tissues usually do not exhibit MT expression. However, after treatment with cytokines, metal ions, or UV irradiation, expression of MT is induced (6, 7). In addition to its potential beneficial effect via detoxification of metal ions, MT may also exert an antiapoptotic and mitogenic effect. Abdel-Mageed and Agrawal (33) have demonstrated that the down-regulation of MT inhibited cell growth and initiated apoptosis in MCF-7 cells. This effect was associated with the induction of p53 and c-fos expression, whereas c-myc and bcl-2 transcripts were down-regulated. Additional evidence for an antiapoptotic role for MT was derived from studies demonstrating a specific interaction between MT and the p50 subunit of NF-κB in MCF-7 cells, which leads to the transactivation of this transcription factor by MT, thus supporting the hypothesis that NF-κB may mediate the antiapoptotic effects of MT (34). Together, these findings support

Table 3 Expression of MT Ia and MT III in gastric cancer patients, relatives, and healthy controls

<table>
<thead>
<tr>
<th></th>
<th>MT Ia</th>
<th>MT III</th>
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<tr>
<td>Normal controls</td>
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<td>0/9</td>
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<td>Gastric cancer</td>
<td>13/19</td>
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<sup>a</sup> P < 0.01 as determined by Fisher’s exact test.
<sup>b</sup> Relatives are individuals without clinical disease but with a first-degree relative suffering from gastric cancer.
the hypothesis that MT is a growth-promoting and antiapoptotic intracellular protein that may contribute to the pathogenesis of human malignancies.

Epidemiological analysis has revealed that individuals with a family history of gastric cancer have a 3-fold increased risk of developing gastric cancer as compared with the unaffected population (30, 31). Furthermore, it has recently been shown that cell proliferation in the gastric mucosa of these individuals increases independent of H. pylori infection (35). MT Ia mRNA transcripts were found in gastric biopsies obtained from first-degree relatives and cancer patients in 16 of 18 and 13 of 19 cases, respectively. In contrast, healthy controls exhibited MT Ia in only 3 cases. MT Ia mRNA was present in all of the tissue samples. However, we did not quantitate the mRNA levels of MT Ia in these specimens because the amount of RNA was very limited. Inasmuch as the expression of MT III (also known as growth-inhibiting factor; Ref. 7) was not significantly altered in the relatives of cancer patients nor in gastric cancer patients as compared with healthy controls, the fact that the growth-promoting factors MT Ia and MT Ila are aberrantly expressed in the gastric mucosa raises the hypothesis that these two factors may contribute to increased cell proliferation in the gastric mucosa.

The mechanisms leading to overexpression of MT in tumor tissues are largely unknown. It has been demonstrated by several groups that MT expression is induced by growth factors, cytokines, and UV irradiation (6, 7). Overexpression of MT resulting from MT gene amplification has been demonstrated in cell lines chronically exposed to cadmium. Furthermore, Ha-ras mutation may also lead to enhanced MT transcription (6, 7). Clonal overexpression of MT in the colon of mice treated with the mutagen dimethylhydrazine was linked to somatic mutations in the morphologically normal mucosa (36). In this model, it has been proposed that MT overexpression may be the consequence of cis-activating mutations of the MT gene or trans-activating mutations of regulatory genes (36). In our study, we found expression of MT in the normal mucosa of the stomach that was confined to the foveolar neck. Because the number of cases was limited, the presence of H. pylori and its role in the expression of MT in the areas of high proliferative activity of the gastric mucosa was not addressed in this study. Thus, additional studies are necessary to elucidate the molecular mechanisms that lead to the aberrant expression of MT in the normal-appearing gastric mucosa.

In conclusion, we have demonstrated the overexpression of MT Ila in gastric cancer and the expression of MT in intestinal metaplasia and dysplasia. Together with the presence of MT Ia isoforms in gastric biopsies of individuals with an increased risk of developing gastric cancer, our findings point to a role for MT in the early phases of gastric carcinogenesis.

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