Multiparametric in situ mRNA Hybridization Analysis of Gastric Biopsies Predicts Lymph Node Metastasis in Patients with Gastric Carcinoma

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We examined the expression level of several genes that regulate different steps in metastasis formation in formalin-fixed, paraffin-embedded biopsies of 189 primary human gastric carcinomas prior to surgical resection in patients in whom lymph node metastasis was not evident by endoscopic ultrasonography or computed tomography (CT) scan. The expressions of epidermal growth factor receptor (EGF-R), vascular endothelial growth factor (VEGF), matrix metalloproteinase (MMP)-2 and E-cadherin were examined by a colorimetric in situ mRNA hybridization technique. The integrity of the mRNAs was verified, leaving 161 (85.2%) patients for study. After gastrectomy, 82 patients had positive lymph nodes and 79 patients had negative lymph nodes. The concurrent expression levels of MMP-2 and E-cadherin mRNAs were significantly higher and lower, respectively, in the metastatic tumors than the non-metastatic tumors. Expression of EGF-R and VEGF was not different between the metastatic and non-metastatic tumors. However, when only the intestinal-type of gastric cancer was evaluated, the level of VEGF mRNA was significantly higher in tumors associated with lymph node metastasis than in those without metastasis. However, a high MMP-2:E-cadherin ratio significantly correlated with lymph node metastasis in both types of gastric cancer. These results suggest that multiparametric in situ hybridization analysis for several metastasis-related genes may allow the preoperative prediction of lymph node metastasis from gastric cancer.

Key words: Metastasis-related genes — Gastric carcinoma — Biopsy specimen — Lymph node metastasis — in situ mRNA hybridization

The prognosis and choice of therapy for gastric cancer patients are based on the stage of the disease as well as its metastatic potential. However, routine histopathological examination of primary gastric cancer specimens cannot always identify patients with metastasis, especially lymph node metastasis. Recent advances in molecular biology and in our understanding of the pathogenesis of metastasis have provided new tools with which to predict the metastatic potential of human cancers.

To produce a metastasis, tumor cells must complete all the steps in the metastatic cascade, including transformation, growth, angiogenesis, invasion, survival in the circulation, adhesion, extravasation, proliferation, and again angiogenesis at the site of metastasis. Numerous reports have demonstrated that in different tumor systems the metastatic potential of cells directly correlates with the expression of different genes that encode for epidermal growth factor receptor (EGF-R), basic fibroblast growth factor (bFGF), vascular endothelial growth factor (VEGF), interleukin-8, matrix metalloproteinase (MMP)-2, multidrug resistance (mdr-1) and carcinoembryonic antigen (CEA). Most of these correlative studies reached the inevitable conclusion that the expression of a given gene is necessary, but insufficient, to produce clinically relevant metastasis. Because each individual step in the pathogenesis of metastasis is regulated by one or more independent factors, the identification of cells with metastatic potential in heterogeneous primary human tumors requires multi-parametric analyses of gene expression.

We previously reported the development of a rapid technique for detecting the activity of genes involved in the fundamental processes of metastasis, i.e., vascularization, invasion, adhesion, and proliferation. This colorimetric in situ hybridization (ISH) technique can be used to detect specific mRNA transcripts in cultured cells, frozen tissues and formalin-fixed, paraffin-embedded specimens. We have used ISH to study the expression level of several genes that regulate the different steps in the metastatic cascade and concluded that the expression level of metastasis-related genes correlates with the metastatic potential of human colon cancer, prostate carcinoma, pancreatic carcinoma, and non-small cell lung carcinoma. We examined whether ISH analysis of endoscopic biopsies of these genes can be used to predict the presence or absence of lymph node metastasis not otherwise noted on pre-operative imaging studies.
MATERIALS AND METHODS

Endoscopic biopsy specimens One hundred and eighty-nine formalin-fixed, paraffin-embedded endoscopic biopsy specimens of invasive primary gastric cancer from which lymph node metastases were not evident by endoscopic ultrasound or computed tomography (CT) scan in patients treated at the Cancer Research Institute, Kanazawa University from 1990 to 1997 were chosen at random. The patients ranged in age from 33 to 79 years with a mean of 61.8 years; 111 patients were men and 78 were women. The pathological N stages of the specimens were 88 patients with N0, 59 patients with N1, 31 patients with N2 and 11 patients with N3.

Paraffin blocks were cut into 4-µm sections and processed for ISH analysis. Adjacent sections were stained with hematoxylin and eosin for histopathological evaluation. Only specimens with intact mRNA, determined in terms of poly d(T) signal, were further evaluated for expression of metastasis-related genes.

Oligonucleotide probes Specific antisense oligonucleotide DNA probes were designed to be complementary to the mRNA transcripts of five metastasis-related genes (Table I) based on published reports of the cDNA sequences. The specificity of the oligonucleotide sequences was initially determined by a GenEMNL database search using the Genetics Computer Group sequence analysis program (GCG, Madison, WI) based on the FastA algorithm, which showed 100% homology with the target gene and minimal homology with nonspecific mammalian gene sequences. The specificity of each sequence was also confirmed by northern blot analysis. A d(T)20 oligonucleotide was used to verify the integrity of the mRNA in each sample. All DNA probes were synthesized with six biotin molecules (hyperbiotinylated) at the 3'-end via direct coupling using standard phosphorimidine chemistry (Research Genetics, Huntsville, AL). The lyophilized probes were reconstituted to a stock solution at 1 µg/µl in 10 mM Tris (pH 7.6) and 1 mM EDTA. The stock solution was diluted with Probe Diluent (Research Genetics) immediately before use. The working dilutions of each probe are shown in Table I.

ISH ISH was performed as described previously. ISH was carried out according to the Microprobe manual staining system (Fischer Scientific, Pittsburgh, PA). Tissue sections (4 µm thick) of formalin-fixed, paraffin-embedded specimens were mounted on silane-treated ProbeOn slides (Fisher Scientific). The slides were placed in the Microprobe slide holder, dewaxed, and dehydrated with Autodewaxer and Autoalcohol (Research Genetics), followed by enzymatic digestion with pepsin. Hybridization of the probe was carried out for 45 min at 45°C, and the samples were then washed three times with 2× standard saline citrate for 2 min at 45°C. The samples were incubated with alkaline-phosphatase-labeled avidin for 30 min at 45°C, briefly rinsed in 50 mM Tris buffer (pH 7.6), rinsed with alkaline phosphatase enhancer (Biomedia Corp., Foster City, CA) for 1 min, and finally incubated with the chromogen substrate Fast Red (Research Genetics) for 30 min at 45°C. A positive reaction in this assay stains red. Controls for endogenous alkaline phosphatase included treatment of the samples in the absence of the biotinylated probe and use of chromogen in the absence of any oligonucleotide probes.

To check the specificity of the hybridization signal, the following controls were used: 1) RNase pretreatment of tissue sections, 2) substitution of the antisense probe with a biotin-labeled sense probe, and 3) competition assay with unlabeled antisense probes. A markedly decreased or absent signal was obtained after all of these treatments.

Image analysis to quantify intensity of color reaction Stained sections were examined in a Zeiss photomicroscope (Carl Zeiss, Thornwood, NY) equipped with a three-chip charge-coupled device (CCD) color camera (model DXC-960 MD, Sony Corp., Tokyo). The images were analyzed using the Optimas image analysis software (version 4.10, Bothell, WA). The slides were prescreened by one of the investigators to determine the range of staining intensity of the slides to be analyzed. Images covering the range of staining intensities were captured electronically, a color bar was created, and a threshold value was set in the red, green and blue modes of the color camera. All subsequent images were quantified based on this threshold. The integrated optical density of the selected fields was deter-

| Probe        | Sequence 5’-3’ (GC content) | Working dilution | Ref. |
|--------------|----------------------------|-----------------|-----|
| EGF-R        | GGA GCG CCC CGG CCG TCC CGG (87.5%) | 1:800           | 30, 36 |
| VEGF         | TGG TGA TGT TGG ACT CCT CAG TGG GC (55.6%) | 1:200           | 37  |
| MMP-2        | TGG GCG ACG GCG CGG CGG CGG CTG GGC (85.2%) | 1:500           | 38  |
| E-Cadherin   | TGG GCG ACG GCG CGG CGG CGG CGG CTG GGC (62.5%) | 1:200           | 39  |
| (mixture)    | GAC GCC GGC GCC CCC TTC ACA GTC (75.0%) | 1:1000          |     |

Table I. Sequences of Oligonucleotide Probes
specimens of human gastric carcinomas are shown in Table II. Calculated characteristics, patterns of metastasis and tumor stage (77 patients) and diffuse-type (84 patients) tumors. Clinical characteristics, patterns of metastasis and tumor stage (77 patients) and diffuse-type (84 patients) tumors. Clini-

cal characteristics, patterns of metastasis and tumor stage (77 patients) and diffuse-type (84 patients) tumors. Clinical characteristics, patterns of metastasis and tumor stage (77 patients) and diffuse-type (84 patients) tumors. Clinical characteristics, patterns of metastasis and tumor stage (77 patients) and diffuse-type (84 patients) tumors. Clinical characteristics, patterns of metastasis and tumor stage (77 patients) and diffuse-type (84 patients) tumors. Clinical characteristics, patterns of metastasis and tumor stage (77 patients) and diffuse-type (84 patients) tumors.

Table II. Characteristics of Patients

| Metastasis | No metastasis |
|------------|---------------|
| Intestinal type (n=77) | 38 | 39 |
| Diffuse type (n=84) | 44 | 40 |
| Total (161) | 82 | 79 |

We examined the expression levels of several genes that regulate different steps in the process of gastric cancer metastasis, growth (EGF-R), angiogenesis (VEGF), invasion (MMP-2) and cell adhesion/cohesion (E-cadherin) in

Table III. Median Level of Gene Expression by Metastasis

| Gene          | Metastatic tumors | Non-metastatic tumors | P value |
|---------------|-------------------|-----------------------|---------|
| EGF-R         | 55 (15–100)       | 52 (12–100)           | 0.413   |
| VEGF          | 61 (25–100)       | 54 (20–90)            | 0.101   |
| MMP-2         | 74 (28–100)       | 36 (0–70)             | <0.001  |
| E-Cadherin    | 21 (5–40)         | 43 (8–76)             | <0.001  |

There was a strong significant correlation between high MMP-2:E-cadherin ratios and lymph node metastasis in intestinal-type and diffuse-type gastric tumors. The levels of VEGF and MMP-2 mRNAs were significantly greater in the intestinal-type tumors with associated metastasis than in those without associated metastasis. On the other hand, significantly higher levels of MMP-2 and significantly lower levels of E-cadherin were observed in diffuse-type gastric tumors with associated metastasis than in those without associated metastasis (Table IV). The MMP-2:E-cadherin ratios predict lymph node metastasis. We also compared the expression levels of EGF-R in the biopsy specimens and resected primary tumors from the same 15 patients. There were only three patients with more than a 30% decrease or increase of EGF-R expression level. The mean levels of both tumors are 68.5 and 71.8, respectively, which are very similar (Fig. 2).

We next determined whether the expression level of these metastasis-related genes correlated with metastasis in intestinal-type and diffuse-type gastric tumors. The levels of VEGF and MMP-2 mRNAs were significantly greater in the intestinal-type tumors with associated metastasis than in those without associated metastasis. On the other hand, significantly higher levels of MMP-2 and significantly lower levels of E-cadherin were observed in diffuse-type gastric tumors with associated metastasis than in those without associated metastasis (Table IV). The MMP-2:E-cadherin ratios predict lymph node metastasis. There was a strong significant correlation between high MMP-2:E-cadherin ratios and lymph node metastases in both intestinal- and diffuse-type gastric cancer (P<0.001). The sensitivity and specificity of high MMP-2:E-cadherin ratios (>3) in predicting lymph node metastasis were 73.7% and 74.4%, respectively, in intestinal type, and 82.6% and 84.2%, respectively, in diffuse type. The ratio of MMP-2:E-cadherin was significantly more predictive of lymph node metastasis than either factor alone (Table V).

DISCUSSION

We examined the expression levels of several genes that regulate different steps in the process of gastric cancer metastasis, growth (EGF-R), angiogenesis (VEGF), invasion (MMP-2) and cell adhesion/cohesion (E-cadherin) in
biopsy specimens of primary human gastric carcinomas. We have previously reported that the metastatic potential of human colon cancer cells and human colon cancer can be identified by multiparametric ISH analysis for the expression of EGF-R, bFGF, MMP-2 and E-cadherin.\textsuperscript{31, 32} We examined VEGF, as the most important angiogenic factor, in this study because we have previously reported that VEGF was more closely associated with angiogenesis of human gastric cancer than bFGF.\textsuperscript{43}

EGF-R is present on many normal and tumor cells. Increased levels and amplification of EGF-R have been found in many human tumors and cell lines, including

Fig. 1. ISH analysis of metastasis-related genes in gastric biopsy specimens from metastatic (A, C, E, F, G, and I) and non-metastatic tumors (B, D, F, H, and J). Hybridization with hyperbiotinylated poly d(T)\textsubscript{20} probe confirmed the integrity and lack of mRNA degradation. Positive reactions in this assay show as red. The expression intensity of the poly d(T)\textsubscript{20} probe (A, B) was assigned the value of 100. The expression intensity values for EGF-R (C, D), MMP-2 (E, F), VEGF (G, H) and E-cadherin (I, J) were 85, 80, 90, 42, 100, 85, 15, 40, respectively.
breast cancer, colon cancer, and gastric cancer. Our laboratory has shown that expression of EGF-R directly correlated with ability to produce hepatic metastasis in human colon cancer.

MMPs are members of a unique family of proteolytic enzymes that can degrade native collagens and other extracellular matrix components. Because their substrates are the major components of the extracellular matrix, which supports tissue structure, the increased expression of MMP by tumor cells is believed to play an important role in tumor cell invasion and metastasis. Enhanced expression of MMPs, especially MMP-2, has been reported in various human malignant tumors. Immunohistochemical studies demonstrated that the MMP-2 protein is present in gastric carcinoma cells and its expression levels at the invasive edge correlate well with tumor progression. Ohtani et al. have shown that MMP-2 mRNA is localized in stromal fibroblastic cells, but not in tumor cells by ISH using an RNA probe. They speculated that fibroblasts are the major source of synthesis of MMP-2, and carcinoma cells may not be important in the secretion of MMP-2, based on an immunoelectron microscopic study. Under our experimental conditions with the specific oligonucleotide probe, signals indicating MMP-2 expression were detected in the cytoplasm of the tumor cells. Although weak signals were also observed in stromal cells, no correlation was observed between intensity of MMP-2 in stromal fibroblastic cells and lymph node metastases of gastric carcinomas (data not shown).

E-Cadherin is a cell surface glycoprotein involved in calcium-dependent homotypic cell-to-cell adhesion. It is localized at the epithelial junction complex and is responsible for the organization, maintenance and morphogenesis of epithelial tissue. Reduced levels of E-cadherin are associated with a decrease in cellular/tissue differentiation and increased grade in different epithelial neoplasms. Transfection of E-cadherin-encoding cDNA into invasive cancer cells has been shown to inhibit their motility and invasiveness.

Our results showed that higher expression of MMP-2 and lower expression of E-cadherin are significantly related with metastasis in diffuse-type gastric carcinomas, and higher expressions of MMP-2 and VEGF are correlated with metastasis in intestinal-type gastric carcinomas. The biological behavior of gastric cancer depends on the pathological tumor type. Intestinal-type tumors tend to be exophytic, metastasizing to the liver by hematogenous dissemination. In contrast, diffuse-type gastric cancers are more invasive with predominantly peritoneal dissemination. These results suggest that a reduced level of E-cadherin is very important in peritoneal dissemination, VEGF is essential for liver metastasis, and MMP-2 is necessary for both metastases. Our laboratory has reported that high MMP-2:E-cadherin ratios correlate well with metastatic potential in several carcinomas such as colon, stomach, prostate, pancreas, and lung. Therefore, we studied whether this ratio can be used to predict lymph node metastasis preoperatively. We found that a high ratio (>3) showed high sensitivity and specificity, especially in diffuse type.

![Graph showing the expression levels of EGF-R in biopsy specimens and resected primary tumors from the same 15 patients. Only three patients showed more than 30% decrease or increase of EGF-R expression level. The means at the two tumor sites are similar.](image-url)

**Table IV.** Median Levels of Gene Expression in Intestinal Type and Diffuse Type Gastric Cancers

| Gene    | Intestinal type Metastatic tumors Non-metastatic tumors | Diffuse type Metastatic tumors Non-metastatic tumors |
|---------|---------------------------------------------------------|---------------------------------------------------|
| EGF-R   | 57                                                      | 54                                                |
| VEGF    | 74                                                      | 53 P=0.006                                         |
| MMP-2   | 73                                                      | 43 P<0.001                                         |
| E-Cadherin | 30                                                      | 34                                                |

**Table V.** Correlation of MMP-2:E-cadherin Ratios and Lymph Node Metastasis in Both Types of Gastric Cancer

| MMP-2:E-cadherin ratio | Intestinal type Metastatic tumors Non-metastatic tumors | Diffuse type Metastatic tumors Non-metastatic tumors |
|------------------------|--------------------------------------------------------|---------------------------------------------------|
| >3                     | 28                                                      | 10                                                |
| ≤3                     | 10                                                     | 29                                                |

![Table IV. Median Levels of Gene Expression in Intestinal Type and Diffuse Type Gastric Cancers](image-url)

![Table V. Correlation of MMP-2:E-cadherin Ratios and Lymph Node Metastasis in Both Types of Gastric Cancer](image-url)
The ISH technique was used here to examine the concurrent expression of metastasis-related genes in formalin-fixed and paraffin-embedded biopsy specimens. The integrity of the mRNA was confirmed in 161 (85.2%) samples. This rate was very high compared to surgical specimens (60–70%), because biopsy specimens are generally small and immersed in formalin just after they are obtained. Controversy exists regarding the consistency between the expression level of a factor in a biopsy specimen vs. that in the resected surgical specimen. In this study, there was no significant difference in the expression level of EGF-R between biopsy specimens and the resected tumors.

In summary, this ISH technique for biopsy specimens of gastric cancer is an easy method for studying metastasis-related genes, and is expected to be useful to predict lymph node metastasis preoperatively, an issue which is difficult even with advanced imaging tools such as computed tomography (CT), magnetic resonance imagination (MRI), echo, and endoscopical ultrasonography. The list of metastasis-related genes used here is incomplete; additional ones should be added as probes become available. It will be necessary to study other genes in large prospective series before we can draw more definitive conclusions about the usefulness of this ISH technique for biopsy specimens as a predictor of lymph node metastasis for gastric cancer.

(Received June 20, 2002/Revised August 28, 2002/Accepted September 17, 2002)

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