Transforming growth factor \( \beta 1 \) (TGF-\( \beta 1 \)) is a preoperative prognostic indicator in advanced gastric carcinoma

M Nakamura\(^1\), M Katano\(^1\), A Kuwahara\(^2\), K Fujimoto\(^2\), K Miyazaki\(^3\), T Morisaki\(^3\) and M Mori\(^4\)

Departments of Surgery, Saga Medical School, Saga, Japan; Department of Internal Medicine, Saga Medical School, Saga, Japan; Department of First Surgery, Faculty of Medicine, Kyushu University, Fukuoka, Japan; Department of Community Health Science, Saga Medical School, Saga, Japan

Summary
It has been generally accepted that transforming growth factor \( \beta 1 \) (TGF-\( \beta 1 \)) has both negative and positive effects on tumour growth and progression. This study analysed the prognostic value of TGF-\( \beta 1 \) mRNA in advanced gastric carcinoma. A reverse transcriptase–polymerase chain reaction analysis (RT-PCR) was used for TGF-\( \beta 1 \) in endoscopic biopsy specimens from 42 advanced gastric carcinomas. Thirty specimens expressed TGF-\( \beta 1 \) mRNA while 12 specimens did not. The follow-up duration ranged from 4 to 37 months (mean 22.6 months). TGF-\( \beta 1 \)-positive group demonstrated a shorter overall survival compared with the TGF-\( \beta 1 \)-negative group (\( P = 0.0014 \)). A significant correlation was also found in the 32 patients who underwent curative resection (\( P = 0.0048 \)). Significant correlations were found between TGF-\( \beta 1 \) mRNA expression and both stage (\( P = 0.0015 \)) and nodal involvement (\( P = 0.0060 \)). Multivariate analysis demonstrated that only TGF-\( \beta 1 \) mRNA expression (\( P = 0.0306 \)) was an independent prognostic factor. All of ten patients who underwent non-curative resection expressed TGF-\( \beta 1 \) mRNA. Expression of TGF-\( \beta 1 \) mRNA in gastric biopsy specimens may be an important preoperative prognostic variable for advanced gastric carcinoma.

Keywords: biopsy specimens; reverse transcriptase–polymerase chain reaction; high-risk group

It has been suggested that advanced gastric carcinomas may be divided into poor and good prognostic groups. Recent studies of various types of tumours have strongly suggested that the malignant potential of tumours can be correlated with gene expression (Tahara et al. 1986). Here, we focused on the preoperative evaluation of the malignant potential of gastric carcinoma related to mRNA expression of tumour growth-related factors at the tumour site (Nakamura et al. 1997). In advanced cases, mRNA expression for transforming growth factor-\( \beta 1 \) (TGF-\( \beta 1 \)) showed a significant positive correlation with nodal involvement (Nakamura et al. 1997). Traditional clinicopathological studies have shown that lymph node involvement is an important risk factor for predicting overall survival (Maruyama et al. 1989; Jatzko et al. 1995). These observations suggest that TGF-\( \beta 1 \) mRNA expression in biopsy specimens may identify a subgroup of gastric carcinoma patients with very aggressive disease.

TGF-\( \beta \) has a dimeric structure with a molecular weight of 25 kDa (Assion et al. 1983). It has been demonstrated that there are three forms in humans: TGF-\( \beta 1 \), TGF-\( \beta 2 \) and TGF-\( \beta 3 \), with TGF-\( \beta 1 \) being the most prevalent (Miyazono et al. 1988). TGF-\( \beta 1 \) is a potent inhibitor of epithelial cell growth (Masui et al. 1986; Shipley et al. 1986; Coffey et al. 1988; Moses et al. 1990). However, carcinoma cells, unlike normal cells, can escape from negative regulation by TGF-\( \beta 1 \) at the post-transcriptional level (Fowlis et al. 1992; Cui et al. 1994), receptor (Kimichi et al. 1988), or post-receptor level (Braun et al. 1990; Laiho et al. 1990; Pietenpol et al. 1990; Ito et al. 1992). Cui et al. (1994) have suggested that highly malignant carcinomas, especially advanced cases, may not be inhibited by TGF-\( \beta 1 \), and post-transcriptional down-regulation of TGF-\( \beta 1 \) production may enhance tumour growth. They have also suggested that once tumour cells are refractory to growth regulation, TGF-\( \beta 1 \) expression may confer a selective advantage to the tumour by enhancing angiogenesis or modulating stromal characteristics or the immune response to tumour growth, thus leading to increased invasion and metastasis (Torre-Amione et al. 1990; Welch et al. 1990). On the basis of these previous data, we hypothesized that TGF-\( \beta 1 \) expression at the site of carcinoma, especially advanced tumours, may contribute to highly malignant behaviour.

MATERIALS AND METHODS
Patients and biopsy samples
Tumour biopsy specimens were obtained during preoperative endoscopy of 77 patients with gastric carcinoma. As none of the 35 early-stage patients died during the follow-up period, these were excluded from this study. Forty-two patients with advanced-stage gastric carcinomas underwent resection at the Department of Surgery, Saga Medical School, between 1993 and 1995. All 42 primary gastric carcinoma surgical specimens were classified histologically using Japanese Classification of Gastric Carcinoma (Japanese Research Society for Gastric Cancer. 1995). According to this classification, t1, t2, t3 and t4 correspond to tumour invasion of the mucosa or submucosa, muscularis propria or subserosa, the serosa without invasion of adjacent structures and serosa with adjacent structures respectively. Advanced gastric carcinomas consisted of t2, t3 and t4 specimens.
Reverse transcriptase–polymerase chain reaction (RT-PCR) and gel electrophoresis

Total RNA from each biopsy specimen was isolated by single-step, guanidine thiocyanate–phenol–chloroform extraction (Chromczynski and Sacchi. 1987). Biopsy specimens were minced on ice and homogenized manually in lysis buffer. The RNA fraction was suspended in diethyl pyrocarbonate-treated water and quantitated by absorbance at 260 nm. RT-PCR was carried out according to the Perkin-Elmer/Cetus protocol for reverse transcription of RNA and amplification of cDNA. The RT reaction was carried out with 0.5 μg of RNA per sample. cDNA amplification for β-actin was performed for 30 cycles with annealing temperature of 58°C. cDNA amplification for TGF-β1 was performed for 28 cycles with annealing temperatures of 65°C. Primer sequences were as follows. β-actin: 5'-GTG GGC CCC AGG CAC CA-3', 5'-CTC CTT AAT GTC ACG CAC GAT TTC-3' (Albino et al. 1991); TGF-β1: 5'-AAG TGG ATC CAC GAG CCC AA-3', 5'-GCT GCA CTT GCA GGA GGC CA-3' (Derynck et al. 1985). Aliquots of the PCR products (7.5 μl) were separated and visualized with ethidium bromide staining after electrophoresis on a 1.5% agarose gel in Tris acetate EDTA buffer at 100 V for 20 min. RT-PCR was performed immediately after sample collection by the same investigator, without knowledge of the corresponding clinical data. Identification of the PCR product specific for TGF-β1 mRNA (positivity) was performed by two investigators.

PCR product verification by Southern blot

PCR products were transferred to nylon membranes and probed with a radiolabelled oligonucleotide complementary to sequences within the region flanked by the primers. The blots were hybridized at 50°C with probes labelled on their 5' end with γ-32P (γ-32P)ATP: 7000 Ci mm⁻¹: ICN Pharmaceuticals, Costa Mesa, CA, USA) and T4 polynucleotide kinase (Pharmacia, Uppsala, Sweden) for 18 h. The membranes were washed for 10 min with 2 × standard saline citrate (SSC) and 0.1% sodium dodecyl sulphate (SDS), followed by 0.2 × SSC and 0.1% SDS at ambient temperature, then subjected to autoradiography.

Figure 1 Expression of TGF-β1 in 42 advanced gastric carcinoma specimens

Table 1 Relationship between the expression of TGF-β1 mRNA and the pathological parameters in 42 patients with advanced gastric carcinoma

|                      | TGF-β1 (-) | TGF-β1 (+) | P-value |
|----------------------|------------|------------|---------|
| Age                  | 61.6 ± 16.5 | 61.6 ± 14.2 | 0.9957  |
| Sex                  |            |            |         |
| Male                 | 9          | 20         | 0.7225  |
| Female               | 3          | 10         |         |
| Stage                |            |            |         |
| I                    | 5          | 0          | < 0.001 |
| II                   | 4          | 5          |         |
| III                  | 3          | 15         |         |
| IV                   | 0          | 10         |         |
| Depth of invasion    |            |            |         |
| T2                   | 6          | 8          | 0.1016  |
| T3                   | 6          | 19         |         |
| T4                   | 0          | 3          |         |
| Nodal status         |            |            |         |
| Nodal negative       | 8          | 5          | 0.0030  |
| Nodal positive       | 4          | 25         |         |
| Histological type    |            |            |         |
| Well differentiated   | 2          | 2          |         |
| Moderately differentiated | 5   | 8          |         |
| Poorly differentiated | 4          | 16         | 0.5451  |
| Signet ring cell     | 0          | 2          |         |
| Mucinous             | 1          | 2          |         |
Table 2  Risk factors affecting survival rate by multivariate analysis in the 42 patients with advanced gastric carcinoma

| Parameter                        | Hazards ratio | P-value |
|----------------------------------|---------------|---------|
| Serosal invasion                 | 3.766         | 0.0476  |
| Lymph node metastasis            | 1.533         | 0.5545  |
| Lymphatic invasion               | 2.137         | 0.4010  |
| Venous invasion                  | 0.773         | 0.6155  |
| Histological type                | 0.545         | 0.2258  |
| Expression of TGF-β1 mRNA        | 12.598        | 0.0186  |

Intestinal type: well-differentiated, moderately differentiated and mucinous adenocarcinoma. Diffuse type: poorly differentiated adenocarcinoma and signet ring cell carcinoma.

Statistics

Chi-square test and Mann–Whitney’s U-test were used for statistical analyses between mRNA expression for TGF-β1 and traditional clinical and pathological parameters. Survival curves were calculated using the Kaplan–Meier method and analysed using the log-rank test. The influence of each variable on overall survival was assessed by Cox’s proportional hazard model. Calculations were carried out using Stat View (Abacus Concepts, Berkeley, CA, USA). A P-value < 0.05 was considered to be significant.

RESULTS

Of the 42 patients examined, 12 (TGF-β1-negative cases) had no visible PCR products specific for TGF-β1 mRNA, while 30 (TGF-β1-positive cases) had a sharply visible PCR product (Figure 1). The correlations between expression of TGF-β1 mRNA and clinicopathological parameters are shown in Table 1. Significant correlations were found between TGF-β1 mRNA expression and tumour stage (P < 0.0001), as well as nodal involvement (P = 0.0030). The prognosis of the TGF-β1-positive group was worse than that of the TGF-β1-negative group (P = 0.0014) (Figure 2). Multivariate analysis indicated that TGF-β1 mRNA expression (P = 0.0186) was a significant independent prognostic factor (Table 2). The prognosis of the 32 advanced carcinoma patients who underwent curative resection was studied. Overall survival of the 20 TGF-β1-positive patients was worse than that of the 12 TGF-β1-negative patients (P = 0.0048) (Figure 3). Significant correlations were found between TGF-β1 mRNA expression and both stage (P = 0.0015) and nodal involvement (P = 0.0060) (Table 3). Multivariate analysis also demonstrated that only TGF-β1 mRNA expression (P = 0.0306) was an independent prognostic factor (Table 4).

Ten patients underwent non-curative resection because of peritoneal dissemination (four cases), hepatic metastasis (five cases) or ovarian metastasis (one case) (Table 5). All of these carcinoma specimens expressed TGF-β1 mRNA.
We have demonstrated that TGF-β1 mRNA expression in biopsied carcinoma specimens is a potent preoperative prognostic indicator independent of clinicopathological parameters in advanced-stage gastric carcinomas. Current therapeutic strategies for individual patients with gastric carcinoma are generally determined by the stage of disease at postoperative pathological examination, which is especially affected by the presence and grade of involved regional lymph nodes. Therapeutic strategies determined by preoperative assessment of nodal involvement may improve the survival rate. We have demonstrated previously that TGF-β1 mRNA expression showed a positive correlation with nodal involvement in advanced carcinomas (Nakamura et al. 1997). In the present study, TGF-β1 mRNA expression similarly showed a positive correlation with lymph node disease. In addition, the TGF-β1-positive patient had a shorter overall survival compared with TGF-β1-negative patients who underwent gastrectomy or curative resection. Although we hypothesized that poor prognosis in TGF-β1 positive cases resulted from lymph node involvement, multivariate analysis indicated that transcription of TGF-β1, not nodal status, was a prognostic factor. This finding may explain why tumours recur in some patients without nodal involvement. One possibility is that TGF-β1 mRNA levels in carcinoma specimens may predict lymph node metastasis, even when conventional histological examination is negative (Hayashi et al. 1995; Maehara et al. 1996). Another is that the determination of TGF-β1 mRNA may be independent of the traditional pathological characteristics predicting survival.

TGF-β is a prototype of multifunctional growth factors that either inhibit or stimulate cell proliferation (Mackay et al. 1995). In vitro studies using cell lines have demonstrated that TGF-β inhibits the growth of most epithelial cells (Masui et al. 1986; Shipley et al. 1986; Coffey et al. 1988; Moses et al. 1990), including colon carcinoma (Hoosein et al. 1987) and gastric carcinoma (Ito et al. 1992). Little is known, however, about the association of TGF-β with progression of malignant diseases in vivo. Recent observations have demonstrated that TGF-β may facilitate tumour growth through immunosuppression, angiogenesis or changes in the extracellular matrix (Pepper et al. 1990; Kehrl et al. 1991; Tada et al. 1991; Ueki et al. 1992). Several studies have demonstrated high levels of TGF-β1 mRNA in gastric carcinoma (Tahara. 1990; Hirayama et al. 1992), especially the scirrhous type (Yoshida et al. 1989). We have previously examined TGF-β1 mRNA in surgically resected primary gastric carcinomas (Morisaki et al. 1996). We have reported that TGF-β1 mRNA was frequently expressed in poorly differentiated adenocarcinomas and in tumours with an advanced stage or lymph node spread (Morisaki et al. 1996). An association between TGF-β1 mRNA expression with metastatic spread to axillary lymph nodes has been shown in breast cancer (Walker and Dearing. 1992). Tsushima and colleagues (1996) have reported that TGF-β1 expression may be associated with colorectal adenocarcinoma invasiveness (Tsushima et al. 1996). Friess and colleagues (1993) have demonstrated that human pancreatic cancers have increased levels of TGF-β isoforms and suggested that TGF-β may contribute to disease progression (Friess et al. 1993).

We suggest that investigation of TGF-β1 mRNA in carcinoma specimens may be a new tool for the preoperative evaluation of the aggressive potential of gastric carcinoma. The present study showed a significant correlation between expression of TGF-β1 mRNA in gastric carcinoma specimens and a poor prognosis. Similar results have been reported in other types of solid tumours. Takamani and colleagues (1994) have demonstrated that TGF-β1 was an independent prognostic indicator in patients with pulmonary adenocarcinoma (Takamani et al. 1994). Gorsch and colleagues (1992) described an association between the intensity of TGF-β1 immunoreactivity in breast carcinoma specimens and disease-free survival that was independent of established prognostic variables (Gorsch et al. 1992).

### Table 4
Risk factors affecting survival rate by multivariate analysis in the 32 patients with advanced gastric carcinoma who underwent curative resection

| Parameter                              | Hazards ratio | P-value |
|----------------------------------------|---------------|---------|
| Serosal invasion Negative vs positive  | 4.578         | 0.0770  |
| Lymph node metastasis Negative vs positive | 1.872       | 0.4931  |
| Lymphatic invasion Negative vs positive | 1.632       | 0.6916  |
| Venous invasion Negative vs positive   | 0.905         | 0.8820  |
| Histological type Intestinal vs diffuse| 0.331         | 0.1180  |
| Expression of TGF-β1 mRNA Negative vs positive | 11.043      | 0.0306  |

Intestinal type: well-differentiated, moderately differentiated and mucinous adenocarcinoma. Diffuse type: poorly differentiated adenocarcinoma and signet ring cell carcinoma.

### DISCUSSION

We have demonstrated that TGF-β1 mRNA expression in biopsied carcinoma specimens is a potent preoperative prognostic indicator independent of clinicopathological parameters in advanced-stage gastric carcinomas.

Current therapeutic strategies for individual patients with gastric carcinoma are generally determined by the stage of disease at postoperative pathological examination, which is especially affected by the presence and grade of involved regional lymph nodes. Therapeutic strategies determined by preoperative assessment of nodal involvement may improve the survival rate. We have demonstrated previously that TGF-β1 mRNA expression showed a positive correlation with nodal involvement in advanced carcinomas (Nakamura et al. 1997). In the present study, TGF-β1 mRNA expression similarly showed a positive correlation with lymph node disease. In addition, the TGF-β1-positive patient had a shorter overall survival compared with TGF-β1-negative patients who underwent gastrectomy or curative resection. Although we hypothesized that poor prognosis in TGF-β1 positive cases resulted from lymph node involvement, multivariate analysis indicated that transcription of TGF-β1, not nodal status, was a prognostic factor. This finding may explain why tumours recur in some patients without nodal involvement. One possibility is that TGF-β1 mRNA levels in carcinoma specimens may predict lymph node metastasis, even when conventional histological examination is negative (Hayashi et al. 1995; Maehara et al. 1996). Another is that the determination of TGF-β1 mRNA may be independent of the traditional pathological characteristics predicting survival.

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### Table 5
Ten patients with advanced gastric carcinoma who underwent non-curative resection

| Patient no. | Reasons for non-curative resection | Expression of TGF-β1 mRNA | Outcome |
|-------------|-----------------------------------|---------------------------|---------|
| 1           | Peritoneal dissemination           | Positive                  | 3 M Dead|
| 2           | Peritoneal dissemination           | Positive                  | 5 M Dead|
| 3           | Peritoneal dissemination           | Positive                  | 8 M Dead|
| 4           | Peritoneal dissemination           | Positive                  | 15 M Alive|
| 5           | Hepatic metastasis                | Positive                  | 1 M Dead|
| 6           | Hepatic metastasis                | Positive                  | 1 M Dead|
| 7           | Hepatic metastasis                | Positive                  | 5 M Dead|
| 8           | Hepatic metastasis                | Positive                  | 9 M Dead|
| 9           | Hepatic metastasis                | Positive                  | 25 M Alive|
| 10          | Ovarian metastasis                | Positive                  | 4 M Dead|
Because TGF-β1 mRNA may be elevated in normal tissue adjacent to tumour tissue (Anzano et al. 1985; Travers et al. 1988; Gomella et al. 1989), it remains unclear whether gastric carcinoma cells are the main source of TGF-β1 in our study. We have shown that TGF-β1 mRNA was expressed more frequently in carcinoma tissues than in adjacent normal cells (Morisaki et al. 1996). We confirmed that TGF-β1 protein was expressed in the tumour cells of five TGF-β1-positive carcinoma specimens determined by RTPCR (data not shown). We postulate that TGF-β1 produced by gastric carcinoma cells may act as a growth factor for the tumour through autocrine and paracrine loops.

TGF-β action is tightly regulated through transcriptional control (Kim et al. 1989a and b). It is possible that a highly malignant carcinoma lose the inhibitory response to TGF-β1 (Torre-amione et al. 1990; Welch et al. 1990; Gorsch et al. 1992; Eklöv et al. 1993; Glick et al. 1993; Jirtle et al. 1993; Cui et al. 1994). Some carcinoma cells may escape negative regulation by TGF-β through post-transcriptional down-regulation of TGF-β production (Fowlis et al. 1992; Cui et al. 1994). Other carcinomas may escape at the receptor (Kimchi et al. 1988) or post-receptor level (Braun et al. 1990; Laiho et al. 1990; Pietenpol et al. 1990; Ito et al. 1992). It may also be possible that autocrine or paracrine stimulation of carcinoma cell proliferation by TGF-β1 may directly influence progression of gastric carcinoma. Leof and colleagues (1986) have demonstrated a positive growth response to TGF-β1 (Leof et al. 1986).

TGF-β1 mRNA expression significantly correlated with the presence of involved lymph nodes. Extended gastrectomy including D3 or D4 lymph node dissection may be recommended for improved survival in TGF-β1-positive cases.

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