Rate of Thymidylate Synthetase Inhibition in Gastric Cancer Tissue: Prognostic Indicators in Advanced Gastric Cancer Patients with Preoperative Chemotherapy

Toshiyuki HATAKEYAMA, Hiroshi NAKANO, Kimio NAMATAME, Masahiko YAMAGUCHI, Gaku KIGAWA, Kozo YOSHIDA, Hiroshi NEMOTO, Yasuo YOSHIZAWA, Minoru TAKEDA* and Kaoru KUMADA

Abstract: Fifty-one resected advanced gastric cancer patients who had preoperative chemotherapies, consisting of oral administration of 5-fluorouracil (5-FU) and 5-FU/cisplatin intravenous administration, were included in the present study. The rate of thymidylate synthetase inhibition (TSIR) was measured in resected tissues. Proliferating cell nuclear antigen expression (PCNA-S) and the rate of terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate-biotin nick end labeling expression (TUNEL-I) were also investigated in gastric cancer tissues obtained before and after chemotherapy. The aim of the present study was to examine whether these parameters were reliable prognostic indicators in these patients. Five-year survival analysis showed that a higher value of TSIR significantly predicted a better prognosis. A multiple regression analysis showed that TSIR and the conclusive stage were significant predictive indicators for prognosis in these patients. In curatively resected patients, TSIR was the only significant prognostic indicator. Therefore, TSIR in gastric cancer tissue is a useful indicator for predicting the efficacy of preoperative chemotherapy.

Key words: preoperative chemotherapy, advanced gastric cancer, proliferating cell nuclear antigen, thymidylate synthetase inhibition, TdT-mediated dUTP-biotin nick end labeling

Introduction

The concept of preoperative chemotherapy for advanced gastric cancer patients with severe locoregional spread has come to increasing in recent years since surgery alone has not been fully effective for these patients1). Preoperative chemotherapeutic regimens that include 5-fluorouracil (5-FU) have been shown to be suitable for potentially resectable advanced gastric cancer patients2, 3). A Japanese Classification of Gastric Carcinoma includes an evaluation of the histopathological as well as clinical response to preoperative chemotherapy4). However, other indicators that evaluate the response to preoperative chemotherapy-
apy are required, and indicators assessing the prognosis after preoperative chemotherapy are also needed, when preoperative chemotherapy is performed on potentially resectable patients and is performed just before surgery.

The present study examined three potential indicators which may assess the chemotherapeutic response or the prognosis after preoperative chemotherapy. The first is the rate of thymidylate synthetase inhibition (TSIR) in cancer tissue obtained after preoperative chemotherapy, which indicates the degree of inhibition of DNA synthesis by 5-FU. The TSIR has been shown to be a useful biochemical parameter for the evaluation of 5-FU chemotherapeutic response. The second is the suppression of proliferating cell nuclear antigen expression (PCNA-S), which is measured in cancer tissue specimens obtained before and after preoperative chemotherapy. PCNA is detected at the proliferating phase in the cell cycle, and could be another quantitative parameter for the evaluation of preoperative chemotherapeutic response in gastric cancer tissues. The third is an index of cancer cell apoptosis induced by preoperative chemotherapy, which is measured by terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate (dUTP)-biotin nick end labeling (TUNEL-I). There is a growing body of evidence to show that the efficacy of various antitumor agents is related to the susceptibility of the target tumor cells to apoptosis. The detection of DNA fragmentation by the TUNEL method has been shown to be a more sensitive method for assessing apoptosis than conventional morphological techniques.

Since it is still uncertain whether these three parameters are effective for predicting the prognosis of advanced gastric cancer patients who receive preoperative chemotherapy, the aim of the present study was to examine whether a comprehensive evaluation using the TSIR, PCNA-S and TUNEL-I in gastric cancer tissues could be effective for predicting prognosis when preoperative chemotherapy including 5-FU is performed.

Patients and Methods

Patients and chemotherapeutic regimens: During the period from 1989 to 1994, 51 advanced gastric cancer patients (Group Ib-IVb) underwent gastrectomy after two regimens of preoperative chemotherapy as described below at the Showa University Fujigaoka Hospital. The clinical stages of these patients were stage Ib, II, IIIa, IIIb, IVa and IVb according to the Japanese Classification of Gastric Carcinoma. Forty-three patients (Group Ib-IVa) of those were classified into conclusive stage Ib to IVa. During the same period, 88 advanced gastric cancer patients underwent gastrectomy without preoperative chemotherapy. Their stage, age, and sex did not significantly differ from those of the 51 patients with preoperative chemotherapy.

5-FU was orally administered to 30 of the Group Ib-IVb patients. Three hundred mg/day of 5-FU was administered for 14 days, and 100 mg was orally administered on the day of surgery. The other 21 patients received 5-FU and cisplatin (CDDP) intravenously (5-FU/CDDP). Five days' serial administration of 5-FU just before surgery was performed (500 mg/m²). CDDP was administered on day 3 (50 mg/m²). Five hundred mg/m² of 5-FU was intravenously administered on the day of surgery. In the cases of oral 5-FU administration, 5-FU was administered up to the morning of the operation. In the cases of 5-FU/CDDP therapy, 5-FU was administered by drip infusion right up to the beginning of surgery.
Informed consent was obtained from all patients and approval was obtained from the
designated review board of the institution. There were no operative deaths for 30 days after
the surgery and there were no deaths due to other causes in the 51 patients.

Histopathological response to preoperative chemotherapy was assessed according to the
Japanese Classification of Gastric Carcinoma.4

Surgical Procedures and Methods of Follow Up: Surgical operations were standardized and
performed by a single surgical team. Histopathologically curative resection including
curative lymph node dissection was performed on the stage Ib-IVa 43 patients. Eight
patients with stage IVb disease underwent palliative gastric resections. No adjuvant
chemotherapies after the surgery were performed for the 43 patients undergoing curative
resection. When recurrence was diagnosed, 5-FU/CDDP therapy was performed every 4
weeks. In the 8 patients undergoing palliative resection, 5-FU/CDDP therapy was
administered every 4 weeks after the surgery.

A retrospective review of all available inpatient and outpatient records including operative
and surgical pathology reports was performed in the present study. Patient follow-up and
outcome were documented by clinical visits, telephone interview or correspondence.

PCNA-S: The PCNA labeling index (PCNA-LI) was measured by an immunocytochemical
method described previously.9, 10 In brief, approximately 7.0 × 7.0 × 7.0 mm of fresh cancer
tissues immediately obtained from the resected cancer tissues were used. In addition, 4
small biopsied specimens with cancer tissues, which were endoscopically obtained 2 or 3
days before the beginning of preoperative chemotherapy, were used. PCNA staining was
carried out by the avidine-biotin complex method (Dakopatts, Copenhagen, Denmark),
using anti-PCNA monoclonal antibody (PC10; Novocastra, Newcastle, UK) as a primary
antibody. All labeled nuclei were regarded as positive. The PCNA-LI was given by the
number of PCNA-positive cells per 1000 cancer cells. The PCNA-LI was measured using
several photographs of high magnitude light microscopy (×400), which were obtained from
different parts of the specimens. Suppression of PCNA expression (PCNA-S) was
 calculated with both the PCNA-LI in endoscopically biopsied tissues and the PCNA-LI in
the resected tissue. The PCNA-S was calculated as :

\[ \frac{(PCNA-LI \text{ in the endoscopically biopsied specimens obtained before preoperative chemotherapy}) - (PCNA-LI \text{ in the resected tissues})}{(PCNA-LI \text{ in the endoscopically biopsied specimens obtained before preoperative chemotherapy}) \times 100.} \]

TUNEL-I: To measure the difference in the TUNEL labeling index (TUNEL-LI) between
prechemotherapy and postchemotherapy (i.e., TUNEL-I), half of each fresh cancer tissue
specimen was fixed for 24 hours in 20% phosphate-buffered formalin and embedded in
paraffin, and the endoscopically obtained tissues were also used. The TUNEL
immunocytochemical expression was performed by the immunocytochemical method
described previously.14 A newly developed kit, the Mebstain apoptosis kit (code no. 8440;
Medical and Biological Laboratories Co., Nagoya, Japan) was used. The TUNEL labeling
index in cancer tissue was given by the number of TUNEL-positive cells per 1000 cancer
cells, and TUNEL-I was calculated as :

\[ \frac{(TUNEL-LI \text{ in the resected tissues})}{(TUNEL-LI \text{ in the endoscopically biopsied specimens})} \]

TSIR: In each case, 500 mg or more of fresh cancer tissue obtained from resected tissue
was prepared for the measurement of the TSIR. Each tissue was immediately stored at
−80°C for later analysis. The concentration of isolated thymidylate synthetase (TS-free),
which does not combine with 5-FU, was measured by the protein-bound radiochemical assay described by Spears et al.\textsuperscript{6,7} with slight modifications by our laboratory\textsuperscript{9,10}. Simultaneously, the concentrations of total thymidylate synthetase (TS-total), which are TS-free and TS combined with FdUMP (TS-bound), were also measured by the above-mentioned method. The results of TSIR were calculated as:

$$\text{TSIR (\%)} = \frac{(\text{TS-total} - \text{TS-tree})}{\text{TS-total} \times 100}.$$  

FdUMP was measured by the competitive ligand-binding assay described by Spears et al.\textsuperscript{6,7} with slight modifications by our laboratory\textsuperscript{9,10}.

DNA ploidy: Approximately $4.0 \times 4.0 \times 4.0$ mm of fresh cancer tissue was obtained and frozen ($-80^\circ\text{C}$) until measurement of DNA ploidy pattern. These tissues were minced with scissors and a single cell suspension was generated by the addition of 5 ml of 0.1% RNase (Sigma Chemical Co., St. Louis, MO, USA) and Triton X-100. Then the samples were agitated for 5 minutes at room temperature. The solvent was then filtered through a 40-$\mu$m mesh, and centrifuged at 1000 rpm for 10 minutes. The supernatant was discarded. The sediment was washed twice with phosphate-buffered saline. It was finally treated with a fluorescence dye containing 50 $\mu$g/ml of propidium iodide. Flow cytometric analysis was performed with a FACScan flow cytometer (Becton Dickinson, Mountain View, CA, USA). A cell population was considered DNA-diploid when the cells exhibited a single G0G1 peak and a DNA index (DI) of 1.0 to 1.1. Cell populations showing 2 or more discrete G0G1 peaks were considered DNA-aneuploid. Human blood lymphocytes were used as an internal standard to identify the diploid population. The DI was calculated by comparing the channel ratio of G0/G1 populations among human lymphocytes with that among tumor or gastric remnant cells. The histogram was prepared with a minimum of 20000 cells.

The following clinical and pathologic risk factors were examined for prognostic influence: age, the conclusive stage distribution according to the General Rules for Gastric Cancer Study, the grade of peritoneal dissemination, the grade of lymph node metastasis, the grade of the depth of invasion, the type of chemotherapeutic regimens, the grade of the histopathological response to preoperative chemotherapy, histological types, the grade of curability, the grade of lymph node dissection, TSIR, PCNA-S and TUNEL-I.

Unpaired two-group comparisons were made by the Mann-Whitney U nonparametric test. Survival time was calculated from the date of gastrectomy until death. The cumulative 5-year survival rate was estimated by the method of Kaplan and Meier. The log rank test was used to assess the significance. A univariate Cox's proportional hazard model was used to calculate the relative risk of mortality and 95% confidence intervals. A multivariate stepwise Cox's regression analysis (backward elimination method) was performed to identify factors that were independently associated with mortality. All statistical analyses were performed using the software package Statview IV (Abacus Concepts, Berkley, CA, USA). The survival analysis was made by the Statview IV equipped with Survival Tools (Abacus Concepts). When the p-value in any analysis was less than 0.05, the difference was considered to be significant.

**Results**

The distribution of the conclusive stage in the 51 patients is shown in Tables 1. In the 43 patients undergoing curative surgery, 15 patients died of recurrent diseases. The sites of recurrence which were first diagnosed were peritoneal dissemination in 9 patients, distant
Thymidylate Synthetase Inhibition for Prognosticators

Table 1 Distribution of conclusive stage of the 51 advanced gastric cancer patients

<table>
<thead>
<tr>
<th>Conclusive stage*</th>
<th>stage Ib</th>
<th>stage II</th>
<th>stage IIIa</th>
<th>stage IIIb</th>
<th>stage IVa</th>
<th>stage IVb</th>
</tr>
</thead>
<tbody>
<tr>
<td>All patients (n=51)</td>
<td>10 (19.6%)</td>
<td>10 (19.6%)</td>
<td>14 (27.5%)</td>
<td>4 (7.8%)</td>
<td>5 (9.8%)</td>
<td>8 (15.7%)</td>
</tr>
<tr>
<td>Patients with 5-FU/ora administration (n=30)</td>
<td>7 (23.3%)</td>
<td>7 (23.3%)</td>
<td>9 (30%)</td>
<td>3 (10%)</td>
<td>1 (3.3%)</td>
<td>3 (10%)</td>
</tr>
<tr>
<td>Patients with 5-FU/CDDP intravenous administration (n=21)</td>
<td>3 (14.3%)</td>
<td>3 (14.3%)</td>
<td>5 (23.8%)</td>
<td>1 (4.8%)</td>
<td>4 (19.0%)</td>
<td>5 (23.8%)</td>
</tr>
</tbody>
</table>

* The conclusive stage analysis was determined in accordance with the Japanese Classification of Gastric Carcinoma.

In the stage Ib-IVb patients, the 5-year survival rates were 56.7% in the PCNA-S ≥ 50 group, 28.3% in the 0 ≤ PCNA-S < 50 group and 31.2% in the PCNA-S < 0 group. There were no significant differences among these groups. The 5-year survival rates were 41.7% in the TUNEL-I ≥ 5 group, 38.1% in the 2 ≤ TUNEL-I < 5 group and 41.5% in the TUNEL < 2 group. There were also no significant differences among the three groups. The 5-year survival rates were 79.5% in the TSIR ≥ 60% group, 38.3% in the TSIR 30–60% group and 12.5% in the TSIR < 30% group (Fig. 1). The higher TSIR significantly predicted better survival (TSIR ≥ 60% vs. TSIR 30–60%, p < 0.05; TSIR ≥ 60% vs. TSIR < 30%, p < 0.0001). There was no significant association between TSIR and conclusive stage (Table 2).

In the stage Ib-IVa curatively resected patients, the 5-year survival rates were 90.5% in the TSIR ≥ 60% group, 44.8% in the TSIR 30–60% group and 15.6% in the TSIR < 30% group. The higher TSIR also significantly predicted better survival in the patients with curative resection (TSIR ≥ 60% vs. TSIR 30–60%, p < 0.05; TSIR ≥ 60% vs. TSIR < 30%, p < 0.01). Neither PCNA-S nor TUNEL-I significantly predicted the 5-year survival rates.

lymph node metastasis in 3, liver metastasis in 2, and bone metastasis in one patients.

In the stage Ib-IVb patients, the 5-year survival rates were 56.7% in the PCNA-S ≥ 50 group, 28.3% in the 0 ≤ PCNA-S < 50 group and 31.2% in the PCNA-S < 0 group. There were no significant differences among these groups. The 5-year survival rates were 41.7% in the TUNEL-I ≥ 5 group, 38.1% in the 2 ≤ TUNEL-I < 5 group and 41.5% in the TUNEL < 2 group. There were also no significant differences among the three groups. The 5-year survival rates were 79.5% in the TSIR ≥ 60% group, 38.3% in the TSIR 30–60% group and 12.5% in the TSIR < 30% group (Fig. 1). The higher TSIR significantly predicted better survival (TSIR ≥ 60% vs. TSIR 30–60%, p < 0.05; TSIR ≥ 60% vs. TSIR < 30%, p < 0.0001). There was no significant association between TSIR and conclusive stage (Table 2).

In the stage Ib-IVa curatively resected patients, the 5-year survival rates were 90.5% in the TSIR ≥ 60% group, 44.8% in the TSIR 30–60% group and 15.6% in the TSIR < 30% group. The higher TSIR also significantly predicted better survival in the patients with curative resection (TSIR ≥ 60% vs. TSIR 30–60%, p < 0.05; TSIR ≥ 60% vs. TSIR < 30%, p < 0.01). Neither PCNA-S nor TUNEL-I significantly predicted the 5-year survival rates.
Table 2 Relationship between TSIR and conclusive stage.

<table>
<thead>
<tr>
<th>TSIR</th>
<th>stage Ib</th>
<th>stage II</th>
<th>stage IIIa</th>
<th>stage IIIb</th>
<th>stage IVa</th>
<th>stage IVb</th>
</tr>
</thead>
<tbody>
<tr>
<td>TSIR &lt; 30% (n=15)</td>
<td>2</td>
<td>3</td>
<td>6</td>
<td>1</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>TSIR 30-60% (n=18)</td>
<td>4</td>
<td>3</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>3</td>
</tr>
<tr>
<td>TSIR ≥60% (n=18)</td>
<td>4</td>
<td>4</td>
<td>4</td>
<td>1</td>
<td>2</td>
<td>3</td>
</tr>
</tbody>
</table>

There was no significant relationship between TSIR and the conclusive stage.

(33.3% in the PCNA-S<0 group, 53.3% in the 0≤PCNA-S<50 group and 46.7% in the PCNA-S≥50 group; 46.7% in the TUNEL-I≥5 group, in the 2≤TUNEL-I<5 group and 54.9% in the TUNEL-I<2 group).

There was more stage Ib and II diseases in patients with 5-FU oral administration than in those with 5-FU/CDDP administration (Table 1). Therefore, 5-year survival rates depending on TSIR were compared to those of the 5-FU oral administration group or those of 5-FU/CDDP group. In the 5-FU oral administration group, the 5-year survival rates were 78.7% in the TSIR≥60% group, 58.3% in the TSIR 30-60% group and 12.5% in the TSIR<30% group (TSIR<30% vs. TSIR 30-60%, p<0.05; TSIR≥60% vs. TSIR<30%, p<0.01). In the 5-FU/CDDP group, the 5-year survival rates were 100% in the TSIR >60% group, 37.5% in the TSIR 30-60% group and 20.0% in the TSIR<30% group.

Multiple regression analysis of stage Ib-IVb patients showed that the conclusive stage and TSIR were significant prognostic indicators (Table 3; p=0.006 and p=0.035, respectively). In the Ib-IVa curatively resected patients, another multiple regression analysis showed that TSIR was the only significant prognostic indicator (Table 4; p=0.036).

The FdUMP concentration in the 5-FU oral administration group was 3.91±0.93 pmol/g tissue, and that in the 5-FU/CDDP group was 4.70±1.82. The differences between the chemotherapeutic regimens did not significantly affect the concentration of FdUMP in cancer tissue.

Five-year survival rate of the 5-FU oral administration group was 31.4%, and that in the 5-FU/CDDP group was 37.7% in the stage Ib-IVb patients. On the other hand, 5-year survival rate in patients without preoperative chemotherapy, but with adjuvant chemotherapy of 5-FU/CDDP every 4 weeks after the diagnosis of recurrence, was 25.4% (n=88; stage Ib-IVb). The preoperative chemotherapy increased survival rate, but not significantly.

Discussion

The present study showed that the two regimens of chemotherapy including 5-FU improved postoperative survival as compared to the surgical results without preoperative chemotherapy. The present results also showed that TSIR and conclusive stage were significant prognostic indicators in the stage Ib-IVb advanced gastric cancer patients who received preoperative chemotherapy including 5-FU. In addition, there were no significant relationships between TSIR and conclusive stage in the stage Ib-IVb patients. The result also indicates that TSIR is a reliable prognostic indicator in these patients. Furthermore, TSIR was shown to be the only significant prognostic indicator in the curatively resected patients with preoperative chemotherapy.

Although the efficacy of various antitumor agents appears to be related to apoptosis\textsuperscript{12,15}, using the sensitive TUNEL-method\textsuperscript{13,16} we found no prediction of postoperative survival...
PCNA is a cell cycle-dependent auxiliary protein for the enzyme activation of DNA polymerase $\delta^{17,18}$, which is detected during the proliferating phase from the G1 to S phase. Recent reports showed that the proliferating activity of cancer cells could be assessed by the grade of PCNA expression in cancer tissues $^{19,20}$. Our previous study demonstrated that the PCNA labeling index in resected gastric cancer tissues correlated with the histopathological chemotherapeutic response after preoperative chemotherapy $^{8,10}$. These findings indicated that the measurement of the PCNA expression in cancer tissue was effective for evaluating the proliferative activity of cancer cells after preoperative chemotherapy. However, PCNA-S did not correlate with postoperative survival in patients with preoperative chemotherapy.

Kubota et al. showed that the DNA ploidy pattern changed from aneuploid to diploid after chemotherapy, and that the decrease of the G1 phase fraction and increase of the S-phase fraction correlated with poor chemotherapeutic response of preoperative Futraful.
administration in patients with gastric and colorectal cancers\textsuperscript{21}). In the present study, however, the DNA ploidy pattern was not found to be a reliable indicator for prognosis.

Some clinical studies have shown that the maximum tolerated dose and dose-limiting toxicity of 5-FU depend upon the schedule of administration\textsuperscript{22,23}, and that the slopes of the dose-response curve to 5-FU are different depending on the schedule of drug administration\textsuperscript{23}). These reports have indicated that pulsed 5-FU administration increases incorporation of 5-FU into RNA, which is not mediated by TSIR. The continuous infusion of 5-FU also increases incorporation into DNA, which is mediated by the binding of FdUMP and TS\textsuperscript{24,25}). Therefore, the present protocol of 5-FU administration, oral or continuous venous administration of 5-FU, might increase incorporation of 5-FU into DNA. These results may explain why the measurement of the TSIR in cancer tissue significantly predicted postoperative prognosis in the patients with preoperative chemotherapy in the present study.

The present study examined two regimens of preoperative chemotherapy. The effectiveness of 5-FU oral administration in the treatment of gastrointestinal cancers may be questionable, since the absorption of 5-FU through the gastrointestinal tract is variable and inconsistent\textsuperscript{26}). However, Phillips et al. demonstrated the efficacy of 5-FU oral administration because of its high concentration in the portal blood\textsuperscript{27}). In the present study, the concentrations of FdUMP (an active form of 5-FU) in the cancer tissue were not different between the 5-FU oral administration group and the 5-FU/CDDP group. The finding suggests that the two regimens of preoperative chemotherapy provided equivalent delivery of 5-FU to cancer tissue. In addition, higher values of TSIR in both 5-FU oral administration and 5-FU/CDDP groups predicted better survival. These findings suggest the efficacy of both these regimens of preoperative chemotherapy.

In any case, it would be of much more clinical significance to identify molecular markers predicting the outcome of preoperative chemotherapy, so that patients who benefit from chemotherapy would be treated preoperatively and patients who do not benefit would directly undergo surgical resection. Measurement of TSIR in endoscopically biopsied sections may be a useful indicator of gastric cancer patients in whom preoperative chemotherapy including 5-FU may be beneficial.

Acknowledgments

This work was supported in part by grants from the Scientific Research Fund of the Ministry of Education and a Grant-in Aid for Cancer Research from the Ministry of Health and Welfare, Japan.

References

6) Spears CP, Gustavsson BG, Mitchell MS, Spicer D, Berne M, Bernstein L and Danenberg PV: Thymidylate synthetase inhibition in malignant tumors and normal liver of patients given intravenous 5-fluorouracil. Cancer
Thymidylate Synthetase Inhibition for Prognosticators


