Abstract. The clinical usefulness of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) chemosensitivity test (MTT assay; MTTA) in the selection of anticancer drugs against advanced gastric cancer (AGC) was evaluated. MTTA is widely used to predict patient responses to particular drugs, allowing for the selection of appropriate chemotherapeutic drugs and the avoidance of ineffective chemotherapeutic drugs, thereby improving patient survival. Since 1989, we have accumulated MTTA efficacy data from AGC patients. In this study, the present clinical roles of MTTA and the data from 202 patients with stage III or IV gastric cancer analyzed for survival outcome following surgery, with or without postoperative chemotherapy, evaluated by MTTA, are discussed. The patients were divided into 3 groups; an adapted group found to be sensitive to chemotherapy by MTTA, a non-adapted group found to be insensitive to chemotherapy by MTTA and a group that received no chemotherapy. For stage III gastric cancer patients, the adapted group had a statistically better survival rate compared to the other groups, while for stage IV patients, there was no difference in survival rate between any of the groups. However, further classification of stage IV patients as to the presence or absence of peritoneal dissemination (P) showed that the adapted group with P showed better prognoses than the other groups with P. The analysis of data collected since 2000 revealed that the 11 patients in the taxane-adapted group, who received chemotherapeutic regimens that included taxanes and were found to be sensitive to taxanes by MTTA, demonstrated better survival than the taxane non-adapted group (n=11) (p=0.045). In conclusion, MTTA results predicted patient prognoses, based on the selection of appropriate chemotherapy. Although new anticancer drugs have made significant gains in the treatment of advanced gastric cancer (AGC), AGC remains a serious disease without a completely effective therapy. Conventional anticancer drugs, such as 5-Fluorouracil (5-FU), doxorubicin (DXR) and cisplatin (CDDP), have shown comparatively low efficacy against AGC and a standard protocol for adjuvant chemotherapy in AGC remains to be determined (1, 2). However, the recent development of new drugs such as S-1, CPT-11 and the taxanes may allow the better control of AGC, with improved efficacy rate and patient survival (3).

Generally, patients who respond to treatment in terms of decreased tumor volume after chemotherapy demonstrate better prognostic outcomes than non-responders that show no improvement in AGC. Thus, evaluation of the effectiveness of an anticancer drug prior to its use in treatment is a critical issue for the AGC patient as it may predict prognostic outcome. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) chemosensitivity test (MTT assay; MTTA) is a rapid and quantitative method for the determination of cell viability as described by Mosmann (4). Numerous studies from a variety of clinics have shown MTTA to be a useful method to predict chemotherapeutic effectiveness, such that particular drugs can be selected on the basis of this assay. Positive MTTA results would be expected to lead to improved survival in AGC patients (5-11). We have performed MTTA at our institute since 1989, and in 2000 we started to evaluate the efficacy of the taxanes (docetaxel or paclitaxel) using MTTA. Based on the accumulated data from 1989 to 2003, we attempted to clarify the present
clinical role of MTTA in the selection of anticancer drugs, to improve patient prognosis based on the use of appropriate chemotherapies and the elimination of ineffective chemotherapies against AGC following surgery.

Patients and Methods

Patients. A total of 202 patients with pathological stage III or IV primary gastric cancer, but not residual gastric cancer which accompanied cancer at another site, who underwent resection of the stomach and successful evaluation by MTTA without preoperative chemotherapy between January 1989 and December 2003, were enrolled in the study. The results of the chemosensitivity test on surgical specimens and patient survival were compared, the patients having been divided into 3 groups; an adapted group treated with at least one effective drug as determined by MTTA, a non-adapted group treated with ineffective drugs as determined by MTTA and a group that received no chemotherapy. All the patients in the adapted and non-adapted groups were retrospectively identified based on the equivalent intensity and duration of chemotherapeutic treatment following surgery. An inhibition rate (IR) of 30% was set as the cut-off IR for MTTA such that an IR of more than 30% was determined to be effective. Detailed clinicopathological backgrounds of the patients are shown in Tables I and II. No statistically significant differences were observed between the 3 groups in terms of background factors.

Drugs. Mitomycin C (MMC), doxorubicin (DOX) and 5-Fluorouracil (5-FU) were purchased from Kyowa Hakko Kogyo Co. Ltd., Tokyo, Japan. Cisplatin (CDDP) was purchased from Nippon Kayaku Co. Ltd., Tokyo, Japan. Docetaxel (DOC) and Paclitaxel (PAC) were kindly provided by Aventis Pharma, Japan (Tokyo, Japan), and Bristol Myer, Japan (Tokyo, Japan), respectively.

MTTA chemosensitivity test. The in vitro chemosensitivity of fresh surgical specimens of gastric cancer was evaluated using the tetrazolium salt MTT as reported by Mossman, with some modifications (4). The tissue specimens were obtained during surgery from patients who had given written informed consent. Resected specimens were stored in Hank's balanced salt solution (Gibco, Gaithersburg, MD, USA) that contained 100 IU penicillin (Gibco), 100 µg streptomycin (Gibco) and 0.25 µg amphotericin B (Gibco) per ml and were brought directly to our laboratory. Single-cell suspensions were prepared enzymatically by incubating the specimens for 30 min in 0.5 mg/ml pronase (Boehringer Mannheim GmbH, Mannheim, Germany), 0.2 mg/ml collagenase type I (Sigma) and 0.2 mg/ml DNase (Sigma). After 2 centrifugations, the tumor cells were suspended in RPMI 1640 (Gibco) medium supplemented with 10% fetal bovine serum (FBS; CSL Ltd., Australia), diluted to 1x10^5 cells/ml and 100-µl aliquots were plated into 96-well microplates (Gibco) per ml and were brought directly to our laboratory. Single-cell suspensions were prepared enzymatically by incubating the specimens for 30 min in 0.5 mg/ml pronase (Boehringer Mannheim GmbH, Mannheim, Germany), 0.2 mg/ml collagenase type I (Sigma) and 0.2 mg/ml DNase (Sigma). After 2 centrifugations, the tumor cells were suspended in RPMI 1640 (Gibco) medium supplemented with 10% fetal bovine serum (FBS; CSL Ltd., Australia), diluted to 1x10^5 cells/ml and 100-µl aliquots were plated into 96-well microplates (Gibco) to give approximately 10^4 cells per well. The drug solutions were dissolved in RPMI 1640 and 100-µl aliquots were added to each well to give final concentrations of 10 µg/ml MMC, 50 µg/ml 5-FU, 25 µg/ml CDDP, 30 µg/ml DOC, or 100 µg/ml PAC. The control wells contained 100 µl of the cell suspension and 100 µl RPMI 1640 containing 10% FBS, while 200 µl RPMI with 10% FBS was used as a blank. The plates were incubated for 48 h at 37°C in a humidified atmosphere.

Table I. Clinicopathological factors of stage III patients with gastric cancer enrolled in this study.

<table>
<thead>
<tr>
<th></th>
<th>Adapted (n=38)</th>
<th>Non-adapted (n=20)</th>
<th>Surgery alone (n=31)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (range)</td>
<td>64.7(36-81)</td>
<td>60.4(41-73)</td>
<td>68.2(33-84)</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>28/10</td>
<td>13/7</td>
<td>24/7</td>
</tr>
<tr>
<td>Macroscopic type (Type 1/2/3/4/5)</td>
<td>1/7/25/3/3</td>
<td>0/5/9/5/1</td>
<td>1/8/12/7/3</td>
</tr>
<tr>
<td>Serosal invasion (mp/ss/se/si)</td>
<td>2/13/22/1</td>
<td>2/2/14/2</td>
<td>1/1/17/2</td>
</tr>
<tr>
<td>Lymph node metastasis (N0/N1/N2/N3)</td>
<td>0/7/30/1</td>
<td>0/6/14/0</td>
<td></td>
</tr>
<tr>
<td>Histology (pap/tub/por/sig/muc)</td>
<td>0/15/22/0/1</td>
<td>0/5/13/1/1</td>
<td>1/1/15/3/1</td>
</tr>
</tbody>
</table>

No statistically significant differences between the groups of stage III gastric cancer were observed.

Table II. Clinicopathological factors of stage IV patients with gastric cancer enrolled in this study.

<table>
<thead>
<tr>
<th></th>
<th>Adapted (n=57)</th>
<th>Non-adapted (n=24)</th>
<th>Surgery alone (n=32)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Median age (range)</td>
<td>63.4(33-84)</td>
<td>59.5(40-72)</td>
<td>66.5(44-90)</td>
</tr>
<tr>
<td>Gender (male/female)</td>
<td>38/19</td>
<td>20/4</td>
<td>15/17</td>
</tr>
<tr>
<td>Macroscopic type (Type 1/2/3/4/5)</td>
<td>0/12/29/15/1</td>
<td>1/1/8/14/0</td>
<td>1/5/15/9/2</td>
</tr>
<tr>
<td>Serosal invasion (mp/ss/se/si)</td>
<td>2/12/32/11</td>
<td>1/1/15/7</td>
<td>1/6/17/8</td>
</tr>
<tr>
<td>Lymph node metastasis (N0/N1/N2/N3/N4)</td>
<td>2/11/15/16/13</td>
<td>2/4/5/0/13</td>
<td>0/7/9/15/5</td>
</tr>
<tr>
<td>Histology (pap/tub/por/sig/muc)</td>
<td>0/12/39/3/3</td>
<td>0/2/19/1/2</td>
<td>0/10/21/0/1</td>
</tr>
<tr>
<td>Liver metastasis (+/-)</td>
<td>11/46</td>
<td>2/22</td>
<td>5/27</td>
</tr>
<tr>
<td>Peritonel dissemination</td>
<td>26/31</td>
<td>12/12</td>
<td>18/14</td>
</tr>
</tbody>
</table>

No statistically significant differences between the groups of stage IV gastric cancer were observed.
atmosphere containing 95% air and 5% CO₂. A mixture of 0.4% MTT (Sigma Chemical Co., St. Louis, MO, USA) and 0.1 M sodium succinate (Wako Pure Chemical Ind., Ltd., Osaka, Japan), each dissolved in 10 μl phosphate-buffered saline and filtered through a 0.45-μm membrane filter (Millipore, Bedford, MA, USA), was then added and the plates incubated for an additional 3 h at 37°C. After the final incubation, 150 μl dimethyl sulfoxide (Nacalai Tesque, Kyoto, Japan) were added to each well to dissolve the MTT-formazan salt and the plates were mechanically shaken for 10 min on a mixer (Model 250, Sonifier, Branson, MO, USA). The optical densities of each well were determined using a model EAR 340 easy reader (SLT-Labinstruments, Salzburg, Austria) at 540 nm and 630 nm. The inhibition rates (IR) were calculated using the formula: (1 - A/B) X 100%, where A and B represent the mean absorbances of drug-treated and control wells, respectively. The effects were regarded as positive when IR values were greater than or equal to 30%

Statistical analysis. The patient clinicopathological factors examined included age, gender, macroscopic tumor type, microscopic serosal invasion, microscopic lymph node metastasis and histological differentiation category based on the “Japanese Classification of Gastric Carcinoma” (12). Background factors were compared using the Mann-Whitney U-test, χ² test, or the Fisher’s direct probability test. The overall survival rate was calculated by the Kaplan and Meier method and the statistical significance was calculated by the log-rank test. P<0.05 was regarded as statistically significant (13).

Results

In the stage III cohort, the adapted group (n=38) showed improved survival compared to the non-adapted (n=20) and no chemotherapy (n=31) groups (adapted vs. non-adapted p=0.039, adapted vs. no chemotherapy p=0.035, by log-rank test). No statistically significant difference in survival outcome was observed between the non-adapted and no chemotherapy groups (Figure 1). In contrast, for the stage IV gastric cancer cohort, the adapted group showed no statistically significant survival benefit compared to the other 2 groups (Figure 2), with the 5-year survival rate of the adapted group (10.0%) only slightly higher than that of the non-adapted (5.8%) and no chemotherapy (7.7%) groups.

When stage IV gastric cancer patients were further categorized as to the presence or absence of peritoneal dissemination (P) or liver metastasis (H), only those stage IV gastric cancer patients with P showed statistically significant differences between the adapted and the non-adapted groups. The adapted group (n=26) showed significantly longer survival compared to the non-adapted (n=12) and no chemotherapy (n=18) groups (adapted group vs. non-adapted group p=0.040, adapted group vs. no chemotherapy group p=0.017, by the log-rank test) (Figure 3). The patient numbers were too small to allow the statistically evaluation of patients with H (Figure 4). No statistically significant differences between the adapted and the non-adapted groups were observed for any of the stage IV patients or stage IV patients without P or H (Figure 5).

The data after September 2000, when taxanes were added to MTTA, were also analyzed. The adapted group for taxanes (n=11) demonstrated better survival than the non-adapted group (n=11), which represented a statistically significant difference (p=0.045, by the log-rank test) (Figure 6). For patients with P, the taxane-adapted group (n=5) also showed better survival than the non-adapted group (n=4) (p=0.022, by the log-rank test) (Figure 7).

Discussion

Although it is thought that adjuvant chemotherapy following surgery for the treatment of AGC may protect against the development of metastases or recurrent disease, a definite benefit for adjuvant chemotherapy after surgery has yet to be confirmed in gastric cancer (14). Indeed, there is a high incidence of local or distant recurrence after histologically curative surgery for AGC (15). While an effective chemotherapy may control AGC recurrence after removal of the main tumor, a standard regimen with good efficacy for most AGC cases remains to be established. As a result, AGC patients may suffer from drug toxicity from chemotherapies that are ineffective against the tumor and, therefore, do not improve the survival or quality of life (QOL) of AGC patients after surgery.

We have previously reported the usefulness of MTTA in predicting chemosensitivity in AGC patients (5-11) and concluded that the potentially effective drugs predicted by MTTA are likely to be clinically effective in the prevention of cancer growth after surgery and improve the survival time of AGC patients. Given the ability to select effective drugs and avoid ineffective drugs in advance, it is probable that MTTA will become a standard step in adjuvant chemotherapy against AGC after surgery.

As previously reported, an adapted patient group showed improved survival compared to non-adapted and no chemotherapy groups of stage III gastric cancer patients (11). The present study confirmed this result for stage III AGC. These findings suggest that microscopic cancer cells may remain after surgical resection, which might then support local cancer growth or metastatic tumor formation in the absence of an effective chemotherapeutic drug therapy. It is hoped that adjuvant chemotherapies that include drugs shown to be effective against the particular cancer by MTTA would then inhibit tumor recurrence and improve patient survival. However, our study showed no benefit of MTTA for stage IV AGC patients, with improved patient survival demonstrated for MTTA only after further classification of stage IV AGC patients according to the presence of P. Thus, stage IV AGC patients classified according to N stage rather than P or H would probably not be expected to show improved outcomes by adjuvant chemotherapy selected on the basis of MTTA results.
Recently, gastric cancer, which has been shown to exhibit low chemosensitivity similar to other solid tumors, has been reported to be successfully treated with high response rates by novel anticancer drugs such as the taxanes (16-18). Previous MTTA-based studies have shown that the taxanes exhibit a different spectrum of antitumor activity against gastric cancer cells compared to conventional anticancer agents, suggesting that they may be useful as novel anticancer agents due to their unique mode of action (19). Similar to the present study, the benefit of the taxanes in the treatment of AGC with P has been reported in several studies (9-12). In the present study, detailed analysis of AGC patients treated with taxanes revealed that the taxane-adapted group with P exhibited statistically better survival than the taxane-non-adapted group, which supported the idea that the taxanes exhibit a different spectrum of antitumor activity from conventional drugs such as 5-FU, MMC or CDDP. Thus, taxanes were able to improve the survival time of AGC patients with P, when the tumors were sensitive to taxanes as determined by MTTA. Our results also suggest that MTTA can be used to assess novel anticancer drugs as well as conventional anticancer drugs.
Conclusion

In this retrospective study, chemosensitivity testing based on MTTA was confirmed to be useful in the selection of effective drugs and the elimination of ineffective drugs, thereby improving AGC patient survival. In addition, the clinical indication of taxanes based on MTTA also showed survival benefit, suggesting the effectiveness of MTTA for the evaluation of novel anticancer agents such as the taxanes. It is hoped that further investigation into the clinical use of MTTA for other novel anticancer drugs will significantly improve AGC patient survival after surgery.

References


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