Individualized Adjuvant Chemotherapy Guided by Chemosensitivity Test Sequential to Extended Surgery for Advanced Gastric Cancer

MAKOTO IWAHASHI, MIKIHIITO NAKAMORI, MASAKI NAKAMURA, KOHEI NOGUCHI, KENTARO UEDA, YOSHIHIRO NAKATANI, TOSHIYASU OJIMA, KOICHIRO ISHIDA, TEIJI NAKA and HIROKI YAMAUE

Second Department of Surgery, Wakayama Medical University, School of Medicine, Wakayama, Japan

Abstract. Background and Objectives: Various adjuvant chemotherapy regimens have been proposed for patients with advanced gastric cancer; however, the majority of these trials failed to show a clear survival benefit over surgery alone. In this study, the feasibility and efficacy of a strategy of extended surgery combined with individualized adjuvant chemotherapy for advanced gastric cancer with serosal invasion and nodal involvement was examined. Patients and Methods: Sixty-four patients with advanced gastric cancer underwent gastrectomy with extended lymph node dissection. After surgery, a chemosensitivity test by MTT assay, using highly purified tumor cells, was performed, and the patients received individualized adjuvant chemotherapy on the basis of the results of this chemosensitivity test. Results: Overall survival in the chemosensitivity-guided chemotherapy (CSC) group was significantly better than the standard chemotherapy (SC) and the no-chemotherapy (NC) group (p<0.05). In patients with stage IV disease, the 5-year survival rate was 38.1% in the CSC group and 0% in the SC + NC group, respectively, with a significant difference being observed in the two survival curves (p<0.01). In patients with paraaortic node involvement, survival in the CSC group was significantly better than that in the SC + NC group (p<0.01). On the other hand, in patients without paraaortic node involvement, no survival difference was observed between the two groups. Conclusion: The strategy of extended surgery combined with individualized adjuvant chemotherapy offers a favorable survival outcome for advanced gastric cancer patients with serosal invasion and nodal involvement.

Gastric cancer is one of the leading causes of cancer-related death, especially in Asia, Africa and parts of Europe (1, 2). Extended lymph node dissection has been performed for gastric cancer in Japan, and the survival benefit of extended surgery has been demonstrated (3, 4). Nevertheless, the prognosis of patients with advanced gastric cancer has not been sufficiently improved by extensive surgery (5). Therefore, various adjuvant chemotherapy regimens have been proposed to improve the postoperative survival. However, there are only a few reports which show a clear survival benefit of adjuvant chemotherapy over surgery alone (6).

It is important to select anticancer drugs which are effective against cancer cells in order to avoid the unnecessary use of these drugs which may cause adverse effects, especially after curative operation. In this respect, in vitro chemosensitivity testing is important (7). A rapid colorimetric assay was described by Mosmann (8) for determining the ability of viable cells to convert a soluble tetrazolium salt, 3-(4,5-di-methylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), into an insoluble formazan precipitate. The MTT assay is a rapid and quantitative colorimetric system for determining the chemosensitivity of human tumor cells; however, the use of this assay for solid tumor tissues has been limited because of contamination by nonmalignant cells (9). In a previous study, we determined chemosensitivity in gastric cancer and colorectal cancer, using highly purified tumor cells, and showed a correlation between this sensitivity and clinical response (9-11). Since then, we have developed a treatment plan to improve the poor prognosis of patients with advanced gastric cancer. Gastrectomy, with extended lymph node dissection, was performed for patients with advanced gastric cancer showing serosal invasion and nodal involvement. After surgery, the MTT assay, using highly purified tumor cells, was performed,
and patients received individualized adjuvant chemotherapy on the basis of the results of the chemosensitivity test.

In the present study, the feasibility and the efficacy of the strategy of extended surgery combined with individualized adjuvant chemotherapy for advanced gastric cancer with serosal invasion and nodal involvement is examined in a prospective non-randomized manner.

Patients and Methods

Patients. Sixty-four patients with advanced gastric cancer, admitted to Wakayama Medical University Hospital, Japan, between 1991 and 1996, underwent gastrectomy with extended lymph node dissection. This extended surgery was indicated for patients with advanced gastric carcinoma showing serosal invasion or N2 lymph node metastases. Total gastrectomy was performed in 49 patients, distal gastrectomy was performed in 14 patients and pancreatocoduodenectomy was performed in 1 patient. The lymph nodes, located perigastrically, around the gastric artery, the hepatic artery, the splenic artery and the celiac artery and paraaortic nodes were extensively dissected in all the patients.

The clinical stages of the 64 patients according to the TNM classification (4th edition) of malignant tumors by UICC were: 7 with stage Ib, 6 with stage II, 10 with stage IIIa, 12 with stage IIIb and 29 with stage IV. None of these patients had received any previous antitumor drugs. Surgical specimens were obtained from primary gastric lesions and the MTT assay was successfully performed in 38 patients. Informed consent was obtained from the patient and/or the family twice, in advance of operation and chemotherapy, in accordance with the guidelines of the Ethical Committee on Human Research, Wakayama Medical University, Japan.

Anticancer drugs. The antitumor drugs tested were cisplatin (CDDP), mitomycin C (MMC), doxorubicin (DOX) and 5-fluorouracil (5-FU). Each drug was diluted in complete medium containing 4 ml each of 25%, 15% and 10% Percoll (Pharmacia, Uppsala, Sweden) in complete medium. Centrifugation was performed at 15 xg for 7 min and tumor cells depleted of lymphoid cells were collected from the bottom and from the 25% interface. The cells thus prepared were primarily tumor cells, with less than 10% contamination by nonmalignant cells (9).

Purification of fresh human gastric cancer cells. Freshly excised tumor tissues were processed using enzymatic digestion, as previously described (10). Briefly, tumor tissues were dissected into small pieces, which were immersed in complete medium containing collagenase (2 mg/ml, type V-S; Sigma), hyaluronidase (10 units/ml, type IV-S; Sigma), and DNase-I (0.4 mg/ml; Sigma). After a 40-min incubation at 37°C, the cells were harvested and were centrifuged on Ficoll-Hypaque (specific gravity 1.077; Pharmacia, Uppsala, Sweden) in complete medium. Centrifugation was performed at 15 xg for 30 min, a tumor cell-rich fraction was collected from the 75% interface. The tumor cell-enriched suspension was then layered on discontinuous gradients containing 4 ml each of 25%, 15% and 10% Percoll (Pharmacia, Uppsala, Sweden) in complete medium. Centrifugation was performed at 15 xg for 7 min and tumor cells depleted of lymphoid cells were collected from the bottom and from the 25% interface. The cells thus prepared were primarily tumor cells, with less than 10% contamination by nonmalignant cells (9).

MTT assay. Chemosensitivity was assessed using the tetrazolium salt MTT (Sigma No. M2128) to measure the viability of tumor cells, as previously described (9, 10). Briefly, tumor cell suspensions (1x10^6 cells /ml) were added to each anticancer drug at a final concentration of Cmax x 10 in 96-well flat-bottomed microtiter plates (Corning No. 25860), and incubated at 37°C in a humidified 5% CO2 atmosphere for 96 h. The chemosensitivity assay was assessed in triplicate. Microtiter wells containing tumor cells without anticancer drugs were used as controls for cell viability, while wells containing only complete medium were used as controls for nonspecific dye reduction. After incubation, the plates were centrifuged, the supernatants were removed and MTT solution with 10 ÌM of sodium succinate was added to all the wells. The plates were incubated for an additional 4 h, and dimethyl sulfoxide (DMSO) was then added to all the wells; the mixtures were pipetted thoroughly to dissolve the dark blue crystals. The plates were then read on a microplate reader (Corona Electric, MTP-32) using a test wavelength of 570 nm and a reference wavelength of 630 nm. The control wells without tumor cells had an optical density (OD) of less than 0.005, and the samples in which the OD was over 0.1 were accepted for the assay. The inhibition rate was calculated as follows:

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\text{Inhibition rate} = \left(1 - \frac{\text{OD drug-treated}}{\text{OD control}}\right) \times 100
\]

The background of tumor cells (including dead cells) without addition of MTT had an OD of less than 0.012 after 96-h incubation, and the influence of dead tumor cells could therefore be ignored in the present study. The viability of tumor cells was maintained at 75-90%, during the 96-h incubation (10). The cut-offs were inhibition rates equal to or more than 74% (10).

Chemosensitivity test-oriented chemotherapy. The MTT assay was performed in 40 out of 64 patients, succeeding in 38. The patients received treatment according to the chemosensitivity guideline as follows: when sensitive drugs could be selected by the MTT assay, a single drug or combination of two or three drugs were chosen on the basis of these results; when no effective drugs were identified, patients primarily did not receive the adjuvant chemotherapy, or were treated with cisplatin and 5-fluorouracil (FP) at their request; when the chemosensitivity test could not be performed, the patients were treated with FP.

Six out of 38 patients did not receive chemosensitivity-guided chemotherapy after extended surgery of their own volition, although suitable drugs had been identified by the MTT assay.

Forty-two patients received adjuvant chemotherapy after surgery. Thirty-two patients were treated on the basis of the results of the MTT assay (chemosensitivity-guided chemotherapy group; CSC), while 17 patients received standard chemotherapy without any chemosensitivity information (standard chemotherapy group; SC). Patients were individually treated with the protocols shown in Figure 1. On the other hand, 15 patients did not receive any chemotherapy after surgery (no-chemotherapy group; NC).
Statistical analysis. Quantitative results were expressed as mean±standard deviation of the mean. Statistical analysis was performed by ANOVA and Fisher’s test. Background factors were compared using the t-test, the Mann-Whitney U-test and the χ² test. The survival rates were estimated using the Kaplan-Meier method, and the differences were analyzed by using the log-rank test, to compare the resulting curves of the treatment groups. Multivariate analysis was examined according to Cox’s proportional hazard model. A p-value of <0.05 was considered to be statistically significant. StatView 5.0 software (Abacus Concepts, Inc., Berkeley, CA, USA) was used for all statistical analyses.

Results

Patient characteristics. The clinicopathological characteristics of the patients were shown in Table I. There were no significant differences in background factors which were considered to be related to prognosis between the CSC group and the SC + NC group.

Chemosensitivity of patients with advanced gastric carcinoma. The MTT assay was performed in 40 out of 64 patients who received extended surgery, succeeding in 38, but failing in 2 patients because of low OD. The success rate of this assay was 95%. The chemosensitivity of the patients with advanced gastric carcinoma is shown in Table II. At a drug concentration of Cmax x 10, the inhibition rates of tumor cells for each of the four drugs was around 65%. There was no significant difference in chemosensitivity between differentiated and undifferentiated types.

Effect of chemosensitivity-guided adjuvant chemotherapy on survival of gastric cancer patients. The 5-year survival rate was 56.3% in the CSC group and 28.1% in the SC + NC group, respectively, presenting a significant difference in the two survival curves (p<0.05) (Figure 2A). The difference between the two groups was remarkable in patients with advanced stage. In patients with stage III disease, the 5-year survival rate was 66.7% in the CSC group and 30.0% in the SC + NC group, respectively, although there was no statistically
significant difference in survival ($p=0.19$) (Figure 2B). In patients with stage IV disease, the 5-year survival rate was 38.1% in the CSC group and 0% in the SC + NC group, respectively, with a significant difference being observed between the survival curves ($p<0.01$) (Figure 2C). To analyze the characteristics of patients who benefited from the adjuvant chemotherapy, patients with stage IV were divided into two groups according to the existence of paraaortic lymph node metastases. In patients with paraaortic lymph node metastases, the 5-year survival rate was 42.9% in the CSC group and 0% in the SC + NC group, respectively, and there was significant difference in the survival curves ($p<0.01$) (Figure 3A). On the other hand, in patients without paraaortic lymph node metastases, no survival difference was observed between the two groups (Figure 3B).

For patients with stage IV disease, a significant difference in the survival curves was observed between the CSC group and either the SC group or the NC group ($p<0.05$), although the survival difference between the SC group and the NC group was not significant (Figure 4).

**Multivariate analysis of risk factors for prolonged overall survival.** Multivariate analysis of risk factors for prolonged overall survival was examined according to Cox’s proportional hazard model. The risk ratio of each factor is

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**Table II. Chemosensitivity of patients who received extended surgery.**

(n=38)

<table>
<thead>
<tr>
<th>Inhibition rates (%)</th>
<th>CDDP</th>
<th>MMC</th>
<th>ADR</th>
<th>5-FU</th>
</tr>
</thead>
<tbody>
<tr>
<td>All cases</td>
<td>66±23</td>
<td>66±21</td>
<td>64±23</td>
<td>69±25</td>
</tr>
<tr>
<td>Differentiated type</td>
<td>68±19</td>
<td>66±21</td>
<td>65±21</td>
<td>68±25</td>
</tr>
<tr>
<td>Undifferentiated type</td>
<td>64±28</td>
<td>67±22</td>
<td>62±25</td>
<td>70±25</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± standard deviation (SD).
Differentiated type: well- or moderately- differentiated tubular adenocarcinoma, papillary adenocarcinoma.
Undifferentiated type: poorly-differentiated adenocarcinoma, signet-ring cell carcinoma.

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shown in Table III. Chemosensitivity-guided chemotherapy was an independent risk factor for overall survival.

Discussion

Surgical resection is the most common approach for the treatment of patients with advanced gastric carcinoma. Although, the benefit of D2 lymphadenectomy is yet to be clarified (13, 14), Japanese surgeons have established D2 lymph node dissection (15), and some specialized centers in other countries have shown the benefits of D2 lymph node dissection (16-22). Furthermore, some Japanese specialists have performed a pilot study of extended D4 lymph node dissection (removal of paraaortic nodes in addition to D2 dissection) for patients with advanced gastric carcinoma (23, 24). Nevertheless, the prognosis of the patients who have paraaortic node involvement is still poor (23, 24). Therefore, we attempted to improve the postoperative survival of such patients by the combination of extensive surgery with adequate adjuvant chemotherapy.

To date, various adjuvant chemotherapy regimens have been proposed; however, the majority of trials have failed to show a clear survival benefit over surgery alone (25, 26), although several meta-analyses have shown the survival benefit of adjuvant chemotherapy after curative surgery compared with surgery alone (27-29). The most recent study conducted by the Japanese Clinical Oncology Group (JCOG) also could not show the efficacy of adjuvant chemotherapy (30).

Bearing the problem of drug resistance in mind, the MTT assay has proven to be a rapid and quantitative colorimetric system for the determination of the chemosensitivity of tumor cells, correlating with clinical response (9-11, 31, 32). This strategy was employed in choosing the regimen of adjuvant chemotherapy for advanced gastric cancer patients who underwent gastrectomy.

In this study, survival of the patients in the CSC group was significantly better than that in the SC + NC group. The difference between the two groups was more remarkable in patients with advanced stage, especially in patients with stage IV disease (5-year survival rate: 38.1%). This result is
consistent with previous reports (7, 33, 34). In stage IV, it was patients with paraaortic node involvement who benefited from the adjuvant chemotherapy. These results suggest that adjuvant chemotherapy after extended surgery might prolong the survival of patients who possibly have micrometastatic lesions which were not resected during operation. Noteworthily, when the SC + NC group was divided into its two counterparts, a significant difference in the survival curves of stage IV patients was observed not only between the CSC group and the NC group, but also between the CSC group and the SC group. Adjuvant chemotherapy in the SC group failed to show a survival advantage over surgery alone, although cisplatin and 5-fluorouracil (FP) were used in most of this group. Therefore, it is suggested that the chemosensitivity test based on the MTT assay using highly purified tumor cells was useful in choosing effective anticancer drugs for adjuvant chemotherapy, resulting in a favorable survival outcome.

Several studies are in agreement with our viewpoint. Kubota et al. have reported that prediction of chemosensitivity using a histoculture drug-response assay would potentially contribute to patient survival in gastric cancer (33). The collagen gel droplet embedded culture-drug sensitivity test (CD-DST) has been recently developed, and it has been reported that CD-DST can predict the response to chemotherapy with a high accuracy in breast cancer patients (35). Each method of chemosensitivity testing, including our method, has merits and demerits. Quite recently, so-called tailor-made chemotherapy has been developed using biomarkers such as multiple drug-resistant protein (MRP)-1 (36) and dihydropyrimidine dehydrogenase (DPD) (37), but superiority to bioassays are yet to be established (35).

New anticancer drugs have been recently developed, and some of them, such as TS-1, CPT-11, paclitaxel and docetaxel, are already available for gastric cancer. TS-1, in particular, is a novel oral anticancer drug and has been reported to be very effective in a phase II clinical trial (38). These new drugs, as single or combined regimens, will surely play major roles in chemotherapy against gastric cancer. However, with efficacy rates less than 50%, potential adverse effects and high costs, correct selection by chemosensitivity testing is obviously desirable.

In conclusion, chemosensitivity testing of individual gastric cancer with highly purified tumor cells using the MTT assay was useful in choosing effective anticancer drugs for adjuvant chemotherapy. The strategy of extended surgery combined with individualized adjuvant chemotherapy offers a favorable survival outcome for advanced gastric cancer with serosal invasion and nodal involvement.

References

Iwahashi et al: Individualized Adjuvant Chemotherapy Sequential to Surgery for Gastric Cancer


32 Ikeda M, Iwahashi et al: Individualized Adjuvant Chemotherapy Sequential to Surgery for Gastric Cancer

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3459