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Single-cell Suspension Assay with an MTT End Point Is Useful for Evaluating the Optimal Adjuvant Chemotherapy for Advanced Gastric Cancer

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One hundred and forty-eight patients with gastric cancer admitted to Keio University Hospital between July 1988 and October 1992 underwent resection of the primary lesion, as well as single-cell suspension assay of fresh surgical materials with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay) for chemosensitivity evaluation. Fifty patients with histologically stage III or IV gastric cancer were enrolled in this study, among whom 10 received no chemotherapy after surgery while 40 received chemotherapy at equivalent dose levels after surgery. The patients given chemotherapy were divided into two groups consisting of an "Adapted" group treated with at least one agent identified as effective by the assay, and a "Non-adapted" group treated with agents to which the cells were not sensitive in the assay, in order to identify the optimal cut-off inhibition rate (IR) in MTT assay for evaluation of the appropriate adjuvant cancer chemotherapy after surgery. A cut-off IR of 30% was optimal for differentiating the survival rates between the "Adapted" and "Non-adapted" groups. Patients treated with drugs which showed more than 30% IR on their surgical specimens showed a better survival rate than patients treated with drugs which were ineffective in the assay.

Key words: MTT assay — Gastric cancer — Chemosensitivity test

Although many *in vitro* and *in vivo* methods have been reported to predict the clinical chemosensitivity of human tumors,[1,2] their clinical utility is not yet established. Using a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay devised by Mosmann,[3] we have reported high predictability of *in vivo* chemosensitivity of human tumor xenografts serially transplanted into nude mice. The influence of normal cells in fresh surgical specimens and the clinical usefulness of this method for predicting the effects of antitumor agents have also been reported.[4–6] Although the MTT assay has been employed on fresh surgical specimens in our department since July, 1988, the value of this assay was found to be limited to the prediction of true-negative cases for gastrointestinal tumors when applied to patients with inoperable advanced or recurrent cancer. In the present study, we assessed the optimal cut-off value of the inhibition rate (IR) in the MTT assay from our accumulated results in order to predict gastric cancer patients with a good prognosis.

PATIENTS AND METHODS

Patients One hundred and forty-eight patients with gastric cancer admitted to Keio University Hospital between July 1988 and October 1992 underwent surgery as well as MTT assay, and 50 patients in stage III and stage IV were analyzed in this study. For determination of macroscopic and microscopic stages of gastric cancer and the curative effect of surgery, "The General Rules for the Study of Gastric Cancer" in Japan were used.[7] The "General Rules" are broadly similar to the TNM classification.

The results of chemosensitivity testing and the survival rate of the 50 patients with gastric cancer were compared. The patients had all undergone curative or non-curative surgery. The cohort was divided into three groups, an "Adapted" group treated with at least one agent to which the tumor was sensitive as determined by the assay, a "Non-adapted" group treated with agents found to be ineffective in the assay, and a group without adjuvant cancer chemotherapy. The background factors of these groups, when the "Adapted" group was selected on the basis of a 30% cut-off IR, are shown in Table I. There was no statistically significant bias between the "Adapted" and "Non-adapted" groups, although the age of patients in the group without cancer chemotherapy was significantly higher than that of patients in the other two groups.

Adjuvant chemotherapy was conducted as follows after surgery, and other anticancer therapies were not combined. Mitomycin C at a dose of 30 mg/body i.v., adriamycin at a dose of 30 mg/body i.v. and cisplatin at a dose of 100 mg/body were given i.p. or i.v. These therapies were administered within 1 month after the operation, although the treatment schedule was adjusted according to the patients' condition, including age, body surface, and renal, hepatic and bone marrow functions.

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Table I. Background Factors of Patients with Stage III and IV Gastric Cancer

Factors	Adapted (21)	Non-adapted (19)	No chemotherapy (10)
Age	55.7±16.0	58.2±20.2	76.2±11.2
Sex (male:female)	9:12	15:4	7:3
Stage (II:III:IV)	0:4:17	0:5:14	1:2:7
H(+)	5	3	1
P(+)	8	5	3
S(+)	20	19	10
N(+)	19	14	9
Stage (III:IV)	6:15	7:12	5:5
s≥se	13	12	8
n(+)	20	15	5
Histological features (mod:por:muc:pap)	6:12:2:1	4:14:1:0	1:9:0:0
Surgery (curative:non-curative)	6:15	6:13	5:5

A total of 50 patients with stage III and IV advanced gastric carcinomas who underwent gastrectomy were divided into three groups, an "Adapted" group comprising 21 cases treated with at least one drug which was shown to be effective by MTT assay, a "Non-adapted" group comprising 19 cases given chemotherapy drugs ineffective in the assay, and a group without chemotherapy comprising 10 cases. For determination of macroscopic and microscopic stages of gastric cancer and the curability by surgery, the "General Rules" for the study of gastric cancer in Japan were used as a reference. There is no bias between the adapted and non-adapted groups by Student's t test for age, and by chi-squared test or Fisher's exact probability test for other factors. There is bias between the no chemotherapy group and the others for age.

As regards fluoropyrimidines, tegafur was given p.o. at a dose of 600–800 mg/body daily until progression of the disease or until interruption owing to adverse effect, or 500 mg of 5-fluorouracil per body was administered i.v. daily for 5 days starting usually from 2 weeks after surgery. There were no statistically significant differences in dose levels administered between the adapted and non-adapted groups, although the total administered dose was naturally higher in the patients who survived longer.

Preparation of tumor cells Tumor specimens resected aseptically from primary or metastatic lesions were transported to our laboratory as soon as possible in Hanks' balanced salt solution containing 100 U of penicillin, 100 μg of streptomycin and 1.0 mg of Fungizone per ml.

Anticancer agents Four anticancer agents were used for the assay: mitomycin C (MMC), doxorubicin (DXR), and 5-fluorouracil (5-FU) were purchased from Kyowa Hakko Kogyo, Co. Ltd., Tokyo, and cisplatin (DDP) was purchased from Bristol-Myers Squibb Co. Ltd., Tokyo.

MTT assay The assay method of Mosmann[3] was used with some modifications, as reported previously.[4-6] Each tumor specimen was minced quickly with scissors in Hanks' balanced salt solution, added to an enzyme cocktail containing 0.02% collagenase, 0.02% DNase and 0.05% pronase, and stirred in a water bath for 30 min. The solution was filtered and centrifuged for 5 min at 1,000 rpm, then the supernatant was removed and RPMI1640 containing 20% fetal calf serum (FCS) was added to the pellet. The resulting single-cell suspension was diluted to $2\times10^5-1\times10^6$ cells/ml, and 100 μl of the cell suspension and 100 μl of drug solution were plated in 96-well plates (MS-8096F, Sumitomo Bakelite Co., Ltd., Tokyo). The final concentrations of MMC, DXR, 5-FU and DDP were 10, 10, 50 and 25 μg/ml, respectively. After the plates had been incubated at 37°C in a humidified atmosphere of 95% air/5% CO_2 for 48 h and centrifuged at 3,000 rpm for 5 min, the supernatant was removed, and 10 μl/well MTT (Sigma Chemical, St. Louis, MO) dissolved in 5 mg/ml phosphate buffered saline (PBS) and 10 μl/well sodium succinate dissolved in 0.1 mol/liter PBS and filtered through a 0.45-μm membrane filter (Millipore, Bedford, MA) were added to each well. Following an additional 4-h incubation, 150 μl/well dimethyl sulfoxide was added to dissolve the formazan salt and the plates were shaken for a few minutes. Absorbance of each well was read on a model EAR Easy Reader (SLT-Labinstruments, Austria) at 530–640 nm. IR was calculated as [1−(absorbance of treated group − absorbance of blanks)/(absorbance of control group − absorbance of blanks)] ×100 (%).

Statistical analysis Chi-squared test and Student's t test were used for statistical analysis of bias in the background factors of the patients, and Fisher's method was applied for groups consisting of less than 5 cases.

The efficacy rate was calculated as [cases evaluated as sensitive by supposed cut-off IR/total cases] × 100 (%). Survival rates were calculated according to the Kaplan-Meier method.[8] The significance of differences in the Kaplan-Meier survival curves was analyzed by using the generalized Wilcoxon test.

RESULTS

***In vitro* sensitivity to anticancer drugs** Table II shows the efficacy rates of the drugs against gastric cancer specimens at various cut-off inhibition rates (IR). When a cut-off IR of 50% was used, the efficacy rate ranged from 2.7–13.3%; the effect of 5-FU was markedly underestimated at 2.7%, whereas the efficacy rates for MMC, DXR, and DDP matched those reported clinically.[9] When the cut-off IR was set at 30%, the efficacy rate of 5-FU was increased to 10.7%, which is similar to the clinical efficacy rate, and the rates for the other three drugs ranged from 21.3–34.7%.

Chemosensitivity assay and survival of patients with advanced gastric cancer after gastrectomy The total of 50 patients with stage III or IV advanced gastric carcinomas who underwent gastrectomy were divided into three groups, an "Adapted" group comprising 21 cases, a "Non-adapted" group comprising 19 cases, and a group without chemotherapy comprising 10 cases (Table I). The survival rates of the patients with advanced gastric carcinomas were compared between the "Adapted" group treated with at least one drug which was effective in the assay, and the "Non-adapted" group treated with drugs that were ineffective in the assay. No statistically significant difference was observed between these two groups when the cut-off IR was 50%. However, when the cut-off IR was set at 30%, the "Adapted" group showed a significantly ($P<0.01$) better prognosis than the "Non-adapted" group and the group without chemotherapy, whereas there was no significant difference between the "Non-adapted" group and the group without chemotherapy (Fig. 1, Table III).

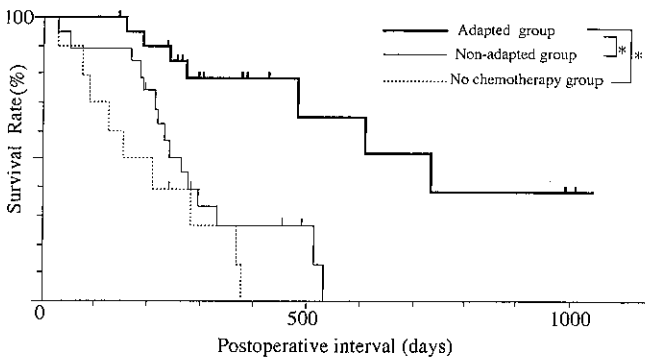

Fig. 1. The survival rates of patients with advanced gastric carcinomas were compared between an "Adapted" group (21 cases) treated with at least one drug shown to be effective by MTT assay, and a "Non-adapted" group (19 cases) given chemotherapy with drugs ineffective in the assay. When the cut-off IR was set at 30%, the "Adapted" group showed a better prognosis than the "Non-adapted" group and the group without chemotherapy (10 cases) with significant differences at $P<0.01$, whereas there was no significant difference between the "Non-adapted" group and the group without chemotherapy (*: $P<0.01$).

Table II. Sensitivity to Each Antitumor Agent at Various Cut-off IR Values

IR cut-off (%)	MMC (%)	DXR (%)	5-FU (%)	DDP (%)
10	64.0[a)]	45.3	40.0	50.7
20	52.0	33.3	28.0	36.0
30	34.7	21.3	10.7	24.0
40	21.3	13.3	4.0	20.0
50	10.7	9.3	2.7	13.3
60	5.3	1.3	2.7	4.0
70	2.7	0	0	2.7
80	2.7	0	0	2.7

The efficacy rates for the drugs against gastric cancer specimens were calculated at various cut-off inhibition rates (IR). When cut-off IR was set at a lower value, the efficacy rate of each drug became higher. The effect of 5-FU was markedly underestimated at 2.7% at 50% cut-off IR, but when the cut-off IR was set at 30%, the efficacy rate of 5-FU was increased to 10.7%, which is similar to the clinical value.
a) Efficacy rate in 75 gastric cancer specimens.

Table III. Ratio of Adapted Group and Non-adapted Group and *P* Value by Generalized Wilcoxon Test Determined at Various IR Cut-off Values

IR cut-off (%)	Adapted	Non-adapted	*P* value
10	34	6	0.1663
20	29	11	0.0522
25	23	17	0.0116
30	21	19	0.0053
35	14	26	0.1142
40	12	28	0.2362
50	9	31	0.0914
60	4	36	0.0624

The survival rates of patients with advanced gastric carcinomas, calculated according to the Kaplan-Meier method, were compared between the "Adapted" group treated with at least one drug shown to be effective by the MTT assay and the "Non-adapted" group given chemotherapy with drugs ineffective in the assay. The *P* value was calculated by using the generalized Wilcoxon test. When the cut-off IR was set at 30%, the "Adapted" group showed significantly ($P<0.01$) better prognosis than the "Non-adapted" group.

DISCUSSION

It is generaly agreed that advanced gastrointestinal carcinomas are uncontrollable by conventional regimens of antitumor drugs, and that no standard adjuvant cancer chemotherapy has yet been established clinically. In this situation, chemosensitivity testing would seem to have an important role for evaluating the most appropriate cancer chemotherapy after surgery.[10, 11]

We have reported high evaluability with the MTT assay in comparison with the human tumor clonogenic assay (HTCA).[6, 9, 11] The present study also indicated a high evaluability rate of 82.6%, which was similar to our previous result (88.6%).[6] However, the value of this assay was limited to elimination of antitumor agents ineffective for individual patients with gastrointestinal carcinomas, when applied to advanced and recurrent gastric and colon cancers. Since some randomized prospective studies have indicated that postoperative adjuvant chemotherapy improves the prognosis of patients,[12] and the efficacy rate of agents against advanced measurable gastric cancer is limited to 20–30%,[13, 14] it is essential to predict the most effective chemotherapy for individual patients after surgery. This assay will also be beneficial for eliminating ineffective forms of chemotherapy which would be harmful to patients by suppressing their immune system and/or reducing their quality of life.

In the present study, we assessed the optimal cut-off IR of the MTT assay to predict those patients with a favorable prognosis after surgery for advanced gastric cancer. It was found that a cut-off IR of 30% would be more appropriate for evaluating adjuvant cancer chemotherapy than the currently used value of 50%, when the present cut-off concentrations of drugs are used. This might be the reason why a small amount of residual cancer cells after surgery can be controlled by "less effective" anticancer agents, in comparison with far-advanced carcinomas.

In conclusion, the MTT assay with a cut-off IR of 30% would be useful for evaluating the adequacy of adjuvant chemotherapy for gastric carcinomas.

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