Clinical Studies on a New Screening Assay for Anticancer Agents Using Nude Mice and Isotopic Evaluation

Yoshihiro Noso, Ken Niimi, Masahiko Nishiyama, Naoki Hirabayashi, Tetsuya Toge, Minoru Niimoto, and Takao Hattori

INTRODUCTION

In order to improve the response rate of chemotherapy, the most effective anticancer agents against individual human tumors should be prescribed. Recent attention has focused on the possibility of determining the sensitivity of individual human tumors to a therapeutic regimen by means of prior laboratory testing of the tumor. A great many in vitro and in vivo chemosensitivity tests have been applied to detect the most effective anticancer agents for the patients (1-9). However, the results of these chemosensitivity tests have not been completely satisfactory.

The development of xenograft techniques for the growth of human tumors in nude mice have yielded potentially more useful models of human tumors (10-16). It has been shown that human tumor xenografts retain many properties of the original tumor including morphological structure, biochemical characteristics, and sensitivity to anticancer agents (13, 14). The results of the sensitivity test using nude mice have correlated with agents tested clinically. It has become apparent that the human tumor-nude mouse system is of significant value in selecting useful clinical therapy.

A major practical limitation of this xenograft system is that tumor cells are relatively slow growing. Obviously, the use of these tumor xenografts for screening new agents, in addition to the evaluation of drug combination and schedule, would be extremely time consuming. Furthermore, the rate of macroscopic growth of tumors transplanted into nude mice is relatively low (6, 17) even if the nude mice are pretreated with any immunosuppressive procedures (18). The screening test is then limited, in some part, in patients with cancer whose tumors could be established in nude mice.

In order to improve this limitation of the screening test using nude mice, we have investigated the microscopic change of primary tumors transplanted s.c. into nude mice (6). Of 150 tumors removed from patients, 127 showed microscopic growth 10 days after implantation (84.7%). We have suggested the feasibility of evaluation of the sensitivity from the microscopic changes in primary tumors transplanted into nude mice (6). More recently, Houghton (19) suggested that the changes in the incorporation of [3H]dThd into tumor DNA was related to the response of chemotherapy in human tumor xenografts.

In order to improve this limitation of the screening test using nude mice, we have investigated the microscopic change of primary tumors transplanted s.c. into nude mice (6). Of 150 tumors removed from patients, 127 showed microscopic growth 10 days after implantation (84.7%). We have suggested the feasibility of evaluation of the sensitivity from the microscopic changes in primary tumors transplanted into nude mice (6). More recently, Houghton (19) suggested that the changes in the incorporation of [3H]dThd into tumor DNA was related to the response of chemotherapy in human tumor xenografts. Therefore, in a second group of studies, evaluation from ARG analysis was investigated (20). The evaluation from ARG was more quantitative and sensitive than histological evaluation. However, a major practical limitation of this ARG was the long exposure time required. For this purpose, evaluation from direct counts of [3H]dThd incorporation into the tumor was investigated as a new screening test of anticancer agents using nude mice (21).

MATERIALS AND METHODS

Mice

BALB/c nu/nu nude mice, 4-5 weeks of age, were purchased from Japan Clea Co. Ltd. (Tokyo, Japan). Mice were kept under specific pathogen free conditions, using laminar air flow racks (in the experimental animal center) and were fed sterile food and water ad libitum. Six- to 8-week-old mice (20-22 g) were used for the experiments.

Tumors

Tumor tissues were obtained from the primary site of human cancers resectively, minced into fragments (2 x 2 x 2 mm) with a scalpel and placed at 4°C in RPMI 1640 medium. Within 1 h, these fragments were transplanted s.c. into both flanks of ether-anaesthetized nude mice by a micro trocker (Japan Clea Co. Ltd., Tokyo). In order to observe the macroscopic growth of several human tumors, transplantation sites were observed twice a week. The tumors transplanted into the right flank of nude mice were extirpated on day 9 and histologically examined. The criterion for macroscopic growth was defined as growth of the tumor beyond 10 mm in diameter and that for microscopic assessment was the demonstration of numerous histologically viable cancer
cells. Pathological studies were done according to the General Rules for the Gastric Cancer Study in Surgery and Pathology in Japan (22).

Anticancer Agents and Method of Sensitivity Test

The protocol of the NMI assay is shown in Fig. 1. Anticancer agents were administered on the 4th day after inoculation of tumors. Anticancer agents employed in these studies were 3 mg/kg of MMC, 75 mg/kg of 5-FU, 80 mg/kg of CPM, and 4.8 mg/kg of ADM, respectively. MMC, 5-FU, and ADM were purchased from Kyowa Hakko Kogyo Co. Ltd., Tokyo, and CPM were purchased from Shionogi Co. Ltd., Osaka. These agents were dissolved in 0.1 ml of saline solution and were administered i.p. except ADM, which was administered i.v. Control mice were given 0.1 ml of saline solution. For the determination of the sensitivity, 100 μCi of [3H]dThd was injected i.p. on the 9th day after inoculation of the tumor. One h later tumors were removed for determination of [3H]dThd uptake. After tumor weights were measured, an aliquot of these tumors was examined histologically. The acid-soluble isotope of a separate aliquot was removed by preserving tumors overnight in 5.0% trichloroacetic acid. Then, tumors were dissolved in NCS tissue solubilizer (Amersham Corporation, Arlington Heights, IL) at 50°C for about 6 h. After the tumors were dissolved, 5 ml of PCS-II liquid scintillation cocktail (Amersham) was added into each counting vial; the incorporation of [3H]dThd was counted on a liquid scintillation counter. Based on histologic comparison with the original tumor fragment, i.e., when there were very few viable tumor cells left in the control tumor fragment or when there was some evidence of infection in the control tumor, and based upon the positive relationship between tumor weight and uptake of [3H]dThd, when uptake was less than 200 cpm/mg in the control tumor, we decided that this is a nonevaluable case. The inhibition rate of incorporation of [3H]dThd into tumor cells by anticancer agents was calculated using the following formula:

\[
\text{Inhibition rate} = \frac{\text{Uptake of treated group (cpm/mg)}}{\text{Uptake of control group (cpm/mg)}} \times 100\%.
\]

When the inhibition rate was less than 50%, the tested anticancer agent was regarded as a tumor sensitive agent (Table 1).

Analysis of Assay-Clinical Correlation

Correlation between Sensitivity Test and Clinical Effects: Retrospective Analysis. Retrospective and prospective clinical studies were performed to determine the usefulness of NMI assay for the prediction. In our department, the patients with gastric cancer are treated according to a standard dosage and schedule with two combined chemotherapeutic agents, which are MMC and Futraful [N1(2’-tetrahydrofuryl)-5-fluorouracil] as adjuvant chemotherapy (23, 24). Correlation between the sensitivity of anticancer agents and the end result after gastrectomy in incurresectable resectable gastric cancer of Stage I V (22) was investigated retrospectively.

Correlation between Sensitivity Test and Clinical Effects: Prospective Analysis. On the basis of the promising results of the retrospective analysis of the clinical correlation, a prospective study was initiated. All patients involved in this study had advanced and inoperable gastrointestinal cancer. Correlation between the sensitivity of anticancer agents and the end result after chemotherapy according to the NMI assay sensitivity test was investigated. The following criteria were arbitrarily defined: (a) tumor-sensitive group, in which the tumor was sensitive to anticancer agents; (b) tumor-resistant group, in which the tumor was resistant to anticancer agents; and (c) nontested group, in which the tumor was not tested for chemosensitivity. For the analysis of a patient’s tumor response to anticancer agents, standard criteria of clinical tumor responses and regression were employed (25). A complete response required complete disappearance of all measurable disease for at least 1 month. A partial response was defined as at least 50% or greater reduction in the sum of the products of greater and lesser diameters of all measurable lesions lasting at least 1 month and absence of any new lesions during treatment. A progressive disease was defined as a greater than 50% increase in size of measurable disease. A no change was defined as between progressive disease and partial response. When a patient exhibited complete response or partial response after treatment, all drugs used for chemotherapy were defined as tumor-sensitive agents, or when a patient showed no change or progressive disease, all agents were defined as tumor-resistant agents.

Actuarial survival rates were calculated with Cutler-Ederer’s method (26). Concerning statistical analysis, the χ² test was applied.

RESULTS

Comparison of Macroscopic Growth Rates and Microscopic “Take” Rates of Primary Human Tumors Transplanted into Nude Mice. Human tumors derived from 124 patients with cancer were transplanted primarily into nude mice and the results of macroscopic growth and microscopic findings are shown in Table 2. Although most of the tumors derived from colon or esophagus were successfully transplanted and grew progressively in nude mice, macroscopic growth was observed in only 52 out of 124 primary tumors (41.9%). In all these tumors, however, microscopic “take” was observed constantly by histologic examination in 110 out of 124 primary tumors on the 9th day after inoculation (88.7%).

Evaluable Rates of NMI Assay. Three hundred and thirty fresh human tumor specimens were tested by NMI assay. The following tumors were tested: 130 gastric carcinomas, 67 colorectal carcinomas, 35 breast carcinomas, 32 esophageal carcinomas, 17 hepatobiliary tract carcinomas, and 49 other carcinomas. In 270 out of 330 carcinomas, chemo sensitivity testing was evaluated by this method (81.8%). Evaluability rates varied from 94.3% in breast carcinoma, to 67.2% in colorectal carcinoma (Table 3).

<p>| Table 2 Macroscopic growth rates and microscopic “take” rates of human tumors in nude mice |
|-------------------------------------------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Origin of cancer cel</th>
<th>Entered cases</th>
<th>Macroscopic growth (%)</th>
<th>Microscopic “take” (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td>62</td>
<td>15 (24.2)</td>
<td>53 (85.5)</td>
</tr>
<tr>
<td>Colon and rectum</td>
<td>23</td>
<td>17 (73.9)</td>
<td>22 (95.7)</td>
</tr>
<tr>
<td>Esophagus</td>
<td>11</td>
<td>8 (72.2)</td>
<td>10 (90.9)</td>
</tr>
<tr>
<td>Hepatobiliary tract</td>
<td>11</td>
<td>6 (54.5)</td>
<td>11 (100)</td>
</tr>
<tr>
<td>Breast</td>
<td>8</td>
<td>3 (37.5)</td>
<td>7 (87.5)</td>
</tr>
<tr>
<td>Other</td>
<td>9</td>
<td>3 (33.3)</td>
<td>7 (77.8)</td>
</tr>
<tr>
<td>Total</td>
<td>124</td>
<td>52 (41.9)</td>
<td>110 (88.7)</td>
</tr>
</tbody>
</table>

Table 1 Evaluation of sensitivity. When the inhibition rate was less than 50%, the tested anticancer output was considered to be sensitive.

<table>
<thead>
<tr>
<th>Evaluation</th>
<th>Inhibition rate*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sensitive</td>
<td>≥50%</td>
</tr>
<tr>
<td>Resistant</td>
<td>&lt;50%</td>
</tr>
</tbody>
</table>

* Inhibition rate (%) = \( \frac{\text{uptake of drug treated group (cpm/mg)}}{\text{uptake of control group (cpm/mg)}} \) × 100.
Results of the Sensitivity Test. Results of the sensitivity test of anticancer agents on human tumors are summarized in Table 4. The rate of positive tumor sensitivity against all tumors was 21.9% in MMC, 12.2% in 5-FU, 27.4% in CPM, and 23.6% in ADM, respectively. The tumor sensitivity of anticancer agents varied according to the type of cancer. The most tumor-sensitive agent for gastric cancer was CPM and that for colorectal cancer and breast cancer was ADM.

In gastric cancer, correlation with the sensitivity to anticancer agents and morphological features of gastric cancer (such as Borrmann type and histological pattern) were investigated (Table 5). MMC showed about a 30% response rate in any type of histological pattern. A type of scirrhous tumor seemed to be chemoresistant and the rate of response to each anticancer agent was decreased. There was a wide variation in the tumor chemosensitivity of histologically similar tumors.

Correlation between Tumor Sensitivity Test and Clinical Effects: Retrospective Analysis. Correlation between sensitivity to anticancer agents and the end results after gastrectomy in noncurative Stage IV gastric cancer was investigated retrospectively. Actuarial survival curves of the tumor-sensitive, tumor-resistant, and nontested groups are shown in Fig. 2.

Survival rates of the tumor-sensitive group (24 patients) was significantly higher than that of the tumor-resistant group (28 patients) ($P < 0.01$). Survival rates of the nontested group was lower than that of the tumor-sensitive group but higher than that of the tumor-resistant group.

Correlation between Sensitivity Test and Clinical Effects: Prospective Analysis. Correlation between the tumor sensitivity to anticancer agents and the end result after chemotherapy was investigated prospectively in advanced and inoperable gastrointestinal cancer patients. Actuarial survival curves of the three groups are shown in Fig. 3. Out of 19 cases, the 50% survival time of the tumor-sensitive group (11 patients) was longer than that of the tumor-resistant group (eight patients). From a prospective correlation study carried out on 25 patients whose tumors could be measured to evaluate the tumor response, this screening assay correlated with clinical response (overall agreements, 76%) with specific agreements of sensitivity and resistance of 37.5 and 94.1%, respectively (Table 6).

DISCUSSION

In this study, $[^{3}H]$dThd was used for the evaluation of human tumor sensitivity against anticancer agents. The influence of 5-FU on the incorporation of $[^{3}H]$dThd in tumor cells is well known. The kinetic profile of 5-FU action on the incorporation of $[^{3}H]$dThd in the tumor has been followed (27) and three phases of the response have been described (27). The initial phase of response to 5-FU, which lasted 6 to 12 h, consisted of a rapid rise in $[^{3}H]$dThd incorporation. The second phase of the response to 5-FU was characterized by a rapidly falling level of $[^{3}H]$dThd incorporation. The third, or recovery phase following 5-FU, was identified by an increase in the incorporation of $[^{3}H]$dThd into DNA, reflecting the disappearance of the block in DNA synthesis in surviving cells (27). In order to avoid the increase of $[^{3}H]$dThd incorporation in tumor cells by 5-FU, we administered $[^{3}H]$dThd on 5th day after administration of 5-FU.

From the preliminary studies by autoradiography, more than 90% of the radiolabeled were tumor cells and less than 10% of the radiolabeled cells were interstitial tissue cells (data not shown). The incorporation into the interstitial tissue cells does not influence the direct counts of $[^{3}H]$dThd in the whole tumor xenografts in nude mice.

Our assay does include both cytostatic and cytotoxic effects by anticancer agents. The incorporation of $[^{3}H]$dThd in the tumor has been investigated by autoradiography techniques (20). In our assay, $[^{3}H]$dThd was administered in nude mice to evaluate the chemosensitivity test 5 days after administration of anticancer agents. During these 5 days, tumor cells sensitive to the anticancer agent died and cell structures were broken histologically. These tumor cells did not uptake $[^{3}H]$dThd. Tumor cells affected cytostatically by anticancer agents did not show the accumulation of $[^{3}H]$dThd from studies of the autoradiography.

In 270 of 330 cancers, chemosensitivity was evaluated by the NMI assay. This assay proved to afford to sufficiently high evaluable rate (81.8%). Thus, the NMI assay sensitivity test could be applied to more patients suffering from cancer. The data obtained by the NMI assay showed that individual tumors originating from the same organ did not always show the same chemosensitivity. Fodstad et al. (28) and Fujita et al. (16) also reported that in human tumors transplanted in nude mice, there was a wide variation in the chemosensitivity of histologically similar tumors. In gastric cancer, it is difficult to predict the chemosensitivity of individual tumors by the form of tumor, type of infiltration, or histological pattern.

In our assay, sensitivity of colorectal carcinomas against ADM was higher and that against 5-FU was lower than the clinical response rate. Regardless of the mode of action of anticancer agents in mice, all anticancer agents were administered in nude mice with the same schedule to evaluate the chemosensitivity at the same time; the one injection and dose of each agent was 1/3 of each LD$_{50}$ value. The protocol of the chemosensitivity test for antimetabolites such as 5-FU should be modified, taking into account the kinetics of antitumor activity.

In the analysis of predictions of clinical responses, the cor-

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### Table 3 Evaluated cases of NMI assay

<table>
<thead>
<tr>
<th>Origin of Cancer Cell</th>
<th>Entered Case</th>
<th>Evaluated Case</th>
<th>Rate of evaluation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td>130</td>
<td>108</td>
<td>83.1</td>
</tr>
<tr>
<td>Colon and rectum</td>
<td>67</td>
<td>45</td>
<td>67.2</td>
</tr>
<tr>
<td>Breast</td>
<td>35</td>
<td>33</td>
<td>94.3</td>
</tr>
<tr>
<td>Esophagus</td>
<td>32</td>
<td>27</td>
<td>84.4</td>
</tr>
<tr>
<td>Hepatobiliary tract</td>
<td>17</td>
<td>16</td>
<td>94.1</td>
</tr>
<tr>
<td>Other</td>
<td>49</td>
<td>41</td>
<td>83.7</td>
</tr>
<tr>
<td>Total</td>
<td>330</td>
<td>270</td>
<td>81.8</td>
</tr>
</tbody>
</table>

### Table 4 Chemotherapeutic response rate of human tumor transplanted in nude mice

<table>
<thead>
<tr>
<th>Origin of Cancer cell</th>
<th>No. of cases</th>
<th>5-FU (%)</th>
<th>CPM (%)</th>
<th>ADM (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stomach</td>
<td>108</td>
<td>26/108 (24.1)</td>
<td>17/108 (15.7)</td>
<td>31/108 (28.7)</td>
</tr>
<tr>
<td>Colon and rectum</td>
<td>45</td>
<td>8/45 (17.8)</td>
<td>3/45 (6.7)</td>
<td>7/45 (15.6)</td>
</tr>
<tr>
<td>Breast</td>
<td>33</td>
<td>8/33 (24.2)</td>
<td>6/33 (18.2)</td>
<td>8/33 (24.2)</td>
</tr>
<tr>
<td>Esophagus</td>
<td>27</td>
<td>6/27 (22.2)</td>
<td>1/27 (3.7)</td>
<td>5/27 (18.5)</td>
</tr>
<tr>
<td>Hepatobiliary tract</td>
<td>16</td>
<td>3/16 (18.8)</td>
<td>1/16 (6.3)</td>
<td>6/16 (37.5)</td>
</tr>
<tr>
<td>Other</td>
<td>41</td>
<td>8/41 (19.5)</td>
<td>17/41 (41.5)</td>
<td>6/33 (18.2)</td>
</tr>
<tr>
<td>Total</td>
<td>270</td>
<td>59/270 (21.9)</td>
<td>33/270 (12.2)</td>
<td>74/270 (27.4)</td>
</tr>
</tbody>
</table>
CHEMOSENSITIVITY TEST USING NUDE MICE

Table 5 Correlation with morphological features and chemotherapeutic response of gastric tumor

The histological features were made according to the General Rules for Gastric Cancer Study in Surgery and Pathology in Japan.

<table>
<thead>
<tr>
<th>Tumor form</th>
<th>No. of cases</th>
<th>Tumor sensitivity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MMC (%)</td>
<td>5-FU (%)</td>
</tr>
<tr>
<td>Borrmann I</td>
<td>2</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Borrmann II</td>
<td>44</td>
<td>9 (20.5)</td>
</tr>
<tr>
<td>Borrmann III</td>
<td>37</td>
<td>7 (18.9)</td>
</tr>
<tr>
<td>Borrmann IV</td>
<td>21</td>
<td>3 (14.3)</td>
</tr>
</tbody>
</table>

Histological pattern

- pap, papillary adenocarcinoma; tub1, well-differentiated tubular adenocarcinoma; tub2, moderately differentiated tubular adenocarcinoma; por, poorly differentiated adenocarcinoma; muc, mucinous adenocarcinoma.

- * Borrmann I, polyloid type; Borrmann II, ulcerative type; Borrmann III, diffusely spreading type; Borrmann IV, superficial spreading type.

Fig. 2. Actuarial survival curves of advanced gastric cancer patients in stage IV (retrospective study). (O), tumor-sensitive group, which was treated with sensitive agents; •, tumor-resistant group, which was treated with resistant agents; Δ, nontested group, which was treated without chemosensitivity tests. Survival rate of the tumor sensitive group (24 patients) was significantly higher than that of the tumor resistant group (31 patients) (P < 0.01; generalized Wilcoxon test).

Fig. 3. Actuarial survival curves of advanced and inoperable gastrointestinal cancer patients (prospective study). (O), tumor-sensitive group; •, tumor-resistant group; Δ, nontested group. Survival rate of the tumor sensitive group (11 patients) was significantly higher than that of the nontested group (17 patients) (P < 0.01; generalized Wilcoxon test).

The prediction of clinical sensitivity and resistance by our assay seems to be similar to other in vitro assays such as the clonogenic assay. However, our assay has several merits compared with the clonogenic assay: (a) evaluation of masked compounds; (b) availability of the solid tumor itself containing an intact cell membrane and present as a heterogeneous tumor cell population; and (c) evaluation is higher than other assays.

Although our prediction of clinical resistance of anticancer agents was as high as that of the clonogenic assay, our prediction of clinical sensitivity was not high (38%). The prospective study was performed with far advanced, inoperable cancer patients of terminal stage. In general, gastrointestinal cancer is one of the most difficult cancers to treat. Thus, this could be the limitation of our clinical prediction of sensitivity. Moreover, patients were actually treated with a combination of several anticancer agents. The comparison of activity of combined anticancer agents in our assay with the results clinically obtained will have to be thoroughly investigated in future studies.

From these results, it seems reasonable to conclude that the NMI assay is a useful screening assay to identify appropriate agents for the treatment of patients with cancer. However, there is still a need to develop better sensitivity assays for antimetabolites and to continue research in order to find more sufficient assays to predict clinical sensitivity to anticancer agents.

REFERENCES

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