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Drug-sensitivity Testing in Patients with Human Oesophageal Squamous Cell Carcinoma

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To evaluate the response to chemotherapeutic agents against human oesophageal cancer, 19 samples were tested by the human tumour clonogenic assay (HTCA), 21 samples by subrenal capsule assay (SRCA) and 33 samples by SRCA with immunosuppressant (IS-SRCA). The evaluability rate of was 21% for HTCA, 95% for SRCA and 91% for IS-SRCA. No active agent was detected by HTCA, however, 29% of the drugs tested by SRCA and 22% by IS-SRCA were considered to be active. Histological analysis revealed substantial inflammatory infiltrates and poor tumour cell preservation with SRCA; however, infiltrates were minimal and there was a high degree of tumour cell preservation with IS-SRCA. The response rates of IS-SRCA were comparable with those of prior clinical tests for each drug. These results suggested that IS-SRCA is the most useful drug sensitivity test for human oesophageal cancer.
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INTRODUCTION

UNTIL RECENTLY, little information about the use of systemic chemotherapy for oesophageal cancer has been available. This type of carcinoma was thought to be completely refractory to chemotherapy; however, recently, considerable attention has been paid to the study of systemic chemotherapy with oesophageal cancer, and relatively good response rates have been obtained compared with other gastro-intestinal tumours [1]. However, the prognosis is still worse than for other types of gastrointestinal tumours because of the poor general condition in many patients and the rapidity of tumour progression. To improve the response rate to chemotherapy and prognosis of these patients, the selection of chemotherapeutic agent is very important not only with respect to the administration of active agents but also the avoidance of adverse effects of resistant agents. However, attempts to evaluate drug response prior to treatment in oesophageal cancer have not yet been reported.

The present study was designed to evaluate and compare the usefulness of human tumour clonogenic assay (HTCA), subrenal capsule assay (SRCA) and SRCA with immunosuppressant (IS-SRCA) as chemosensitivity tests in human oesophageal squamous cell carcinoma.

MATERIALS AND METHODS

Tumours

Fresh surgical specimens were obtained at the time of operation from patients with squamous cell carcinoma of the oesophagus treated at our department; 19 for HTCA, 21 for SRCA and 33 for IS-SRCA.

Mice

Immunocompetent male BDF_1 mice were obtained from NIHON CLEA (Kawasaki, Japan). Mice were kept in specific pathogen-free conditions and used for experiments when 6–8 weeks old.

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Human tumour clonogenic assay (HTCA)

The method used in this study was modified from that described by Hamburger and Salmon [2, 3]. Tumor specimens were cut into 1 mm pieces by scalpel in McCoy's 5A medium (Gibco, Grand Island, New York). Cell suspensions were prepared enzymatically. Briefly, the tumour fragments were placed into Hanks' balanced salt solution (Gibco) containing 0.02% DNase I (Sigma Chemical Co., St Louis, Missouri), 0.02% collagenase type I (Sigma) and 0.05% pronase E (Kaken Seiyaku, Tokyo, Japan) for 30 min at 37°C. After enzymatic digestion, cells were washed twice with phosphate buffered saline (PBS) and passed through a stainless steel mesh (100 G). Large nucleated cells were counted in a haemocytometer, and cell viability was measured by the trypan blue exclusion method.

Double-agar culture was performed in 35 mm petri dishes. The lower layer contained 40 ml enriched McCoy's 5A, 10 ml 3% tryptic soy broth (Difco, Detroit, Michigan), 0.6 ml asparagine (6.6 mg/ml; Wako Pure Chemical Industries, Osaka, Japan) and 0.6 ml DEAE dextran (50 mg/ml; Pharmacia, Uppsala, Sweden). The enriched McCoy's 5A was prepared from the following: 500 ml McCoy's 5A, 25 ml heat-inactivated horse serum (HS; Gibco), 50 ml heat-inactivated fetal calf serum (FCS; Gibco), 5 ml glutamine (200 mmol/l; Wako), and 5 ml penicillin–streptomycin (Gibco). A 3% suspension of hot agar (Difco) was added to the enriched McCoy's 5A medium to make a final concentration of 0.5% agar. A volume of 1 ml of this suspension was then immediately dispensed into a petri dish and allowed to cool. The upper layer was prepared with enriched CMRL1066; 100 ml CMRL1066 (Gibco), 15 ml HS, 200 U insulin in 5 ml, 1 ml vitamin C (30 nmol/l; Kanto Chemical Co., Tokyo, Japan), 2 ml glutamine and penicillin–streptomycin. Immediately before plating, asparagine (0.6 ml/40 ml medium), DEAE dextran (0.3 ml/40 ml medium) and 5 mmol/l 2-mercaptoethanol (0.4 ml/40 ml medium) were added (double enriched CMRL). Cells were suspended in 0.9 ml double enriched CMRL, and 3% agar suspension was added and plated on top of the lower layer. Each petri dish contained 500 000 viable cells. The petri dishes were then incubated at 37°C in a humidified atmosphere with 5% CO_2. Colony growth was monitored during the course of the experiment using an inverted microscope. Colonies were defined as aggregates of 50 or more cells. For the quality control, day 0 control plates were examined and number of cell aggregates recorded (usually 2 or less). Maximum colony formation was reached between 14 and 21 days of culture, at which time their numbers were recorded. The numbers of colonies were determined as final count minus the count on day 0.

In vitro *drug exposure*

Tumour cells were exposed to chemotherapeutic agents at 37°C for 1 h before plating. The concentrations of the agents used are shown in Table 1. Cisplatin and peplomycin were donated by Nihon Kayaku (Tokyo, Japan). Vindesine was donated by Shionogi and Co. (Osaka, Japan). 5-Fluorouracil (5-FU) was purchased from Kyowa Hakkoh (Tokyo, Japan). Each drug was tested at a dose of one-tenth of the highest concentration pharmacologically achieved in patient serum.

Evaluation of the HTCA results

For HTCA, at least 30 colonies (TCFU) per control plate were required in order for an assay to be considered evaluable for measurement of drug effect. For a drug to be considered active, a decrease in TCFU of more than 50% is required.

Table 1. Drug concentration used in HTCA and drug dosage and administration schedule used in SRCA and IS-SRCA

Drugs	Concentration (μg/ml)	Dosage (mg/kg/injection)	Schedule (Day)
Cisplatin	0.20	10.0	1
Vindesine	0.01	5.3	1
5-Fluorouracil (5-FU)	1.00	150.0	1
Peplomycin	0.40	24.0	1,3,5
Mitomycin	—	10.0	1

Subrenal capsule assay (SRCA)

The SRCA methods followed the technique of Bogden *et al.* [4]. Briefly, about 1 mm^3 tumour fragments were implanted under the renal capsule of BDF_1 mice, and two diameters (length and width) of the implanted tumour were measured *in situ* (initial size) with a stereoscopic micrometer.

The animals with subrenal grafts were divided into groups of 4–6 each, and treatment groups were administered the different drugs intravenously to avoid direct contact between the drugs and the grafts.

At the end of the assay, each animal was killed and the tumour bearing kidney was removed and placed under the stereoscopic microscope for measurement of the final tumour size. Tumour size was represented by the mean of the length and width, as described by Bogden *et al.* [5, 6].

Subrenal capsule assay with immunosuppressant (IS-SRCA)

As described previously [4], in order to suppress the host immunoreaction, cyclophosphamide 180 mg/kg was injected subcutaneously into mice 36 h before tumour inoculation.

In vivo *drug treatment*

The drug dosage and administration schedule are shown in Table 1. Mitomycin was purchased from Kyowa Hakkoh. The dosage was the maximum tolerated dose that did not result in toxic death (weight loss <20%). All drugs were administered in 0.01 ml/g of 0.9% NaCl solution.

Evaluation of SRCA and IS-SRCA results

Change in tumour size (ΔTS), expressed as the mean tumour diameter on day 6 minus that on day 0 was calculated for each graft. For an experiment to be considered evaluable, control animals needed to show $\Delta TS \geq -0.05$ mm. A responsive tumour required a mean $\Delta TS \leq -0.1$ mm which was also a statistically significant decrease from the control by Student's *t*-test ($P < 0.05$). These criteria were minor modifications of those of Bogden *et al.* [4–6].

RESULTS

HTCA

Of 19 tumour samples, 10 samples formed more than five colonies. In four of 19 tumours, more than 30 colonies were observed in the control dish, providing an evaluable assay rate of 21%. No correlations could be demonstrated between cell viability or histologic type and probability of subsequent *in vitro* colony formation.

Table 2. Evaluability rate and sensitivity rate for SRCA and IS-SRCA

Assay	Evaluability rate* (%)	Sensitivity rate† (%) Cis-platin	Vin-desine	5-FU	Peplo-mycin	Mito-mycin
SRCA	20/21 (95)	9/20 (45)	4/20 (20)	6/19 (31)	4/19 (21)	5/19 (56)
IS-SRCA	30/33 (91)	9/30 (30)	3/29 (10)	4/26 (16)	2/22 (9)	12/28 (43)

*Criterion for evaluability: control for growth $\Delta TS \geq -0.05$ mm
†Criterion for sensitivity: tumour regression $\Delta TS \leq -0.1$ mm, and statistically significantly different from the control by Student's *t*-test ($P < 0.05$).

Two to four drugs were tested per specimen, resulting in 10 possible drug assays. In these 10 drug assays, no drug showed a colony kill of more than 50%.

SRCA and IS-SRCA

In order to evaluate the response of therapeutic groups, adequate growth of the tumour in control groups was obtained from 20 of 21 tumours (95%) for SRCA, and 30 of 33 tumours (91%) for IS-SRCA (Table 2). The chemosensitivity of these tumours to cisplatin, vindesine, 5-FU, peplomycin and mitomycin were tested. In both assays, tumours showed a relatively high sensitivity rate to cisplatin, mitomycin and 5-FU. The overall sensitivity rates were 32% for SRCA and 22% for IS-SRCA.

Histological analysis

21 samples for SRCA and 28 of 33 samples for IS-SRCA were included in a histological study focused mainly on residual tumour cells and the extent of inflammatory infiltration (Table 3).

In SRCA, inflammatory infiltrates were observed in 18 of 21 samples (86%). Tumour cell preservation was recognised in only nine samples (43%).

Table 3. Histological analysis of human oesophageal xenografts implanted under the renal capsule of BDF_1 mice

	Number of cases SRCA	IS-SRCA
Inflammatory cells		
−*	3	24
+	3	4
++	14	—
+++	1	—
Tumour cells		
−	12	1
+	7	14
++	2	8
+++	—	5

*: <1/4, +: 1/4–1/2, ++: 1/2–3/4, +++: >3/4.

In IS-SRCA inflammatory infiltrates were observed in 25–50% of the implant surface area in only four of 28 samples (14%), and in the remaining samples infiltration was minimal. Tumour cell preservation was recognised in 27 of 28 samples (96%), and in 13 cases (46%) tumour cells accounted for more than 50% of the area.

Correlations with clinical results

Of the 54 patients with evaluable tumour in each assay, 5 had clinically evaluable disease and received chemotherapy after tissue was taken for assay. 4 patients exhibited no change (NC) or progressive disease (PD), and one showed partial response (PR).

For HTCA, one assay–clinical correlation was possible. HTCA was predictive of the clinical resistance in this patient's tumour. For SRCA two assay–clinical correlations were possible. One was a false positive and the other was a true negative. For IS-SRCA two correlations were possible. One was a true positive and the other was a true negative.

DISCUSSION

Several *in vitro* tests have been developed in order to predict the response of an individual patient's tumour to a particular chemotherapeutic agent [7, 8]. However, in these rapid *in vitro* tests, inhibition of the overgrowth of fibroblasts is very difficult. Development of a two-layer semisolid agar system by Hamburger and Salmon was a major advance which enabled the study of individual human cancer specimens *in vitro* [2, 3]. A large number of studies have been performed on various types of tumour, and good correlations between assay results and clinical tumour response or lack of response have been reported. For HTCA, a major problem is that not all tumours will grow and form enough colonies *in vitro*. Von Hoff *et al.* [9] reported that with application of HTCA to 800 specimens, only 25% formed more than 30 colonies in control plates. They demonstrated that ovarian tumours, melanoma, colorectal and breast tumours formed colonies relatively well. However, those from stomach, lymphoma and leukaemia showed poor colony formation. In this study, oesophageal tumours were found to exhibit poor colony formation with an evaluability rate of 21%. These results were not due to technical problems, since we reported good colony forming rates in colorectal, lung and breast cancer, comparable with those of other reports [10]. As a result of these results, we discontinued the use of HTCA as a drug sensitivity test for oesophageal cancer.

SRCA is an *in vivo* test system for *in situ* measurement of the growth of human tumour fragments implanted under the renal capsule of mice, which is used for determining the chemosensitivity of a tumour within a 6-day period [5]. The advantages of SRCA over HTCA are the high evaluability rate, the shorter assay period, and the lack of need for a special metabolic procedure for masked compounds. Griffin *et al.* [6] reported from the results of 1000 fresh surgical explants that the evaluability rate was 86%, the true positive rate was 82.2%, and the true negative rate was 94.1% in the 55 assay–clinical correlations.

We have previously performed SRCA for gastric and colorectal cancer, and the evaluability rates were 79 and 83%, respectively [10]. The evaluability rates of gastric and colorectal cancer were comparable with those of other reports; however, that of oesophageal cancer obtained in this paper was much higher. Bogden and Von Hoff reported on a comparative study of HTCA and SRCA [11]. Despite the large differences in these two assays, a high correlation of tumour sensitivity and resistance was

obtained (83%) when the same human tumours were tested against the same drugs in both SRCA and HTCA. We have reported the correlation between HTCA and SRCA in 21 tumours; the overall accuracy was 72% [10]. Therefore, SRCA seems to be a useful test for predicting the response to chemotherapeutic agents against human oesophageal cancer.

Subsequently, several investigators have demonstrated significant host cell infiltration under the renal capsule of immunocompetent mice, and have suggested that this infiltration could complicate the interpretation of chemosensitivity data obtained in the assay [12, 13]. We studied the histology of xenografts implanted under the renal capsule, and demonstrated that inflammatory infiltrates were observed in most samples and that tumour cell preservation was poor. From these results, we studied the effects of immunosuppressive agents, and reported the usefulness of cyclophosphamide pretreatment for SRCA against human oesophageal cancer [4]. In a basic experiment using nude mouse transplantable tumour xenografts, immunosuppressive effect of cyclophosphamide pretreatment was compared with those obtained after cyclosporine A treatment. Cyclophosphamide and cyclosporin A suppressed the host reaction until day 6 and day 12, respectively, however, the histological evaluation of the tumours on day 6 revealed no differences between the two groups. Furthermore, cyclophosphamide was less toxic than cyclosporin A and had no influence on the activities of chemotherapeutic agents, and its excellent immunosuppressive effect was also demonstrated in clinical samples. The antitumour activities were very similar to those of the nude mouse subcutaneous assay in the SRCA using immunocompetent mice with cyclophosphamide pretreatment. We compared the antitumor effects of several agents using HTCA and IS-SRCA simultaneously in three samples. The correlation between HTCA and IS-SRCA was very high (86%; data not shown). As the evaluability rate of HTCA was extremely low, we decided to use IS-SRCA as a chemosensitivity test for oesophageal cancer.

The evaluability rate for IS-SRCA was high enough to apply this assay as a clinical chemosensitivity test, and the response rate of this assay were comparable with those of prior clinical tests. In single agent phase II studies in oesophageal cancer, mitomycin and cisplatin showed relatively higher response rates than other chemotherapeutic agents [1]. The effectiveness of these agents was also predicted by IS-SRCA. Although the number of cases was small, a good clinical correlation was demonstrated by IS-SRCA. Several drug sensitivity tests show a good correlation between the results of assay and clinical response [6, 9]. However, most of these reports were retrospective studies and did not definitely show that a drug selected by the assay was superior to the selection made by an experienced oncologist. Recently, Von Hoff *et al.* [14] reported on a prospective randomised clinical trial, in which patients were treated either with the clinician's empirical choice of drug or with the best single agent based on *in vitro* sensitivity results. The response rate was significantly higher in patients whose treatment was based on the result of *in vitro* assay than in patients with empirically selected therapy. An improvement in response rate by drug sensitivity testing was clearly demonstrated. However, an improvement in the survival of advanced cancer patients was not obtained because most patients included in this study had had prior chemotherapy and their responses were limited to only partial response.

In addition, in case of adjuvant chemotherapy after curative resection, improvement in survival rate was obtained in the patients whose tumours were sensitive to the agents administered after surgery (data not shown). However, as the number of patients was very small, further prospective study is necessary.

The results of this study indicate that IS-SRCA is the most useful drug sensitivity test for human oesophageal cancer.

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