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Original Paper

The Usefulness of the ATP Assay with Serum-free Culture for Chemosensitivity Testing of Gastrointestinal Cancer

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In order to predict a patient's response to a drug prior to chemotherapy for gastrointestinal cancer, we developed an adenosine triphosphate (ATP) assay with serum-free culture (SF-ATPA) which enables fibroblast overgrowth to be suppressed. A total of 244 gastrointestinal cancer tissue samples were obtained from surgical resection. After enzymatic digestion, cells (2×10^4/well) were cultured for 72 h with continuous exposure to drugs (single or combined use), and cell viability was evaluated by measuring the intracellular ATP level. 208 of 244 samples (85%) were considered to be evaluable in terms of drug response. There were no differences in the evaluability rates among the tumour types. A drug was judged as active using the criterion of ≤50% reduction of the intracellular ATP level in a single-agent treated group compared with the level of the control. Similarly, in the combined drug treated group, drugs were considered as active using two different criteria (<30% reduction of the intracellular ATP level for two drugs and ≤20% for three drugs). Each tumour type had its own spectrum for chemosensitivity. Of 25 patients evaluated for assay–clinical correlations, 16 were examined by both single-agent and combined drug administration, and the predictive accuracy of the assay was higher for the combined drug than for the single agent (88% versus 69%, respectively). In 25 patients, the true positive rate was 64% and the true negative rate was 100%, yielding an overall predictive accuracy of 84%. These results suggest that SF-ATPA may be useful for predicting drug response in patients with gastrointestinal cancer.

Key words: adenosine triphosphate, serum-free culture media, gastrointestinal neoplasms, drug therapy, combination drug therapy

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INTRODUCTION

RECENTLY, CONSIDERABLE progress has been made in the study of systemic chemotherapy against gastrointestinal cancers. Several investigators have attempted to develop chemosensitivity testing that would predict the response of a tumour in an individual patient to a particular chemotherapeutic agent. This type of chemosensitive testing would not only help to identify the most effective drug to treat an individual patient, but also to avoid the adverse effects of ineffective agents [1].

However, chemosensitivity testing that evaluates tumour response prior to therapy has not yet found widespread clinical utility, mainly because of low reliability, low evaluability rates, high cost and poor correlation between the assay result and the clinical response [2, 3]. The low reliability of these conventional *in vitro* assay systems can be attributed largely to contamination by non-malignant cells such as fibroblasts or lymphocytes [4].

We developed a serum-free ATP assay (SF-ATPA) which enables the overgrowth of fibroblasts to be suppressed [5]. This study was designed to confirm the clinical usefulness of our system using a total of 244 clinical samples which were obtained from surgical resection of gastrointestinal neoplasms. We investigated the effect of single-agent and combined drug administration, and also examined the correlation between the results of this assay and the clinical response.

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MATERIALS AND METHODS

Tumours

All patients in this study had advanced gastrointestinal cancer. After written informed consent was obtained according to institutional guidelines, the patient's tumour was sampled. The tumour specimen was placed immediately in RPMI 1640 (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) containing 10% fetal bovine serum (Gibco Laboratories, Grand Island, New York, U.S.A.), 100 units/ml penicillin G and 100 μg/ml streptomycin (Gibco) and 200 mM glutamine (Gibco).

Drugs

Chemotherapeutic agents used in the SF-ATPA were cisplatin (CDDP, Nihon Kayaku, Tokyo, Japan), mitomycin C (MMC, Kyowa Hakko, Tokyo, Japan), 5-fluorouracil (5-FU, Kyowa Hakko), doxorubicin (DOX, Kyowa Hakko), vindesine (VDS, Shionogi and Company, Osaka, Japan) and etoposide (VP-16, Nihon Kayaku). The concentrations of these drugs are shown in Table 1. The drug concentrations used for single-agent administration were the peak plasma concentration (PPC) pharmacologically achieved in the patient's serum [6, 7] and empirical clinical evaluation of assay results (CDDP 2.0 μg/ml, MMC 1.0 μg/ml, 5-FU 50 μg/ml, DOX 0.4 μg/ml, VDS 0.1 μg/ml, VP-16 10 μg/ml), $10 \times$ PPC and $1/10 \times$ PPC. Usually, the three drug concentrations, as described above, were used, but only the median range of concentration (PPC level) was used when not enough cells were obtained.

Combined drug effects were also observed by a combination of two or three drugs [two drugs: 5-FU, CDDP (FP); VP-16, CDDP (EP); VDS, CDDP (VP); three drugs: 5-FU, DOX, CDDP (FAP); 5-FU, DOX, MMC (FAM); 5-FU, VP-16, CDDP (FEP)]. For the combined drug assays, the PPC level was used in each combination regimen.

Culture method

Tumour samples were washed intensively with sterile saline and cut into 1 mm pieces in phosphate-buffered saline (PBS, Nissui). The tumour fragments were then placed in Hank's balanced salt solution (Gibco) containing 0.1% collagenase type I (Sigma Chemical Co., St. Louis, Missouri, U.S.A.), 0.02% DNase (Sigma) and 0.05% pronase E (Sigma) and incubated at 37°C for 30 min. After enzymatic digestion, the cells were washed twice with PBS and passed through a stainless steel mesh (100G). Large nucleated cells were counted in a haemocytometer, and cell viability was measured by the trypan blue dye exclusion method. The washed cells were suspended in S-clone SF-B (SF-B, Sanko Pure Chemical Co. Ltd., Tokyo , Japan) supplemented with 0.1% bovine serum albumin. The cells were then plated into a 96-well microplate at a concentration of 2.0×10^4 cells/180 μl medium/well, then 20 μl/well of the relevant drug was added (drug-free medium was added to control wells). The microplate was cultured for 72 h at 37°C in 5% CO_2 with concomitant exposure to drugs.

Table 1. Concentrations of antineoplastic drugs used for SF-ATPA

Drug	Concentrations (μg/ml)		
	$10 \times$ PPC	PPC	$1/10 \times$ PPC
Cisplatin (CDDP)	20	2.0	0.2
Mitomycin C (MMC)	10	1.0	0.1
5-Fluorouracil (5-FU)	500	50	5.0
Doxorubicin (DOX)	4.0	0.4	0.04
Vindesine (VDS)	1.0	0.1	0.01
Etoposide (VP-16)	100	10	1.0

PPC, peak plasma concentration.

Evaluation of the cell viability

Cell viability was evaluated by measuring the intracellular ATP level using bioluminescence as described by Kangas and associates [8]. Briefly, 100 μl of ATP-releasing reagent (Sigma) was added to 100 μl of the cell suspension in a polyethylene cuvette. After 100 μl of luciferin-luciferase (Sigma) was added, the fluorescence was measured immediately by the AutoLumat LB 953 luminometer (Berthold, Wildblad, Germany). The intracellular ATP level was calculated from the standard curve.

Evaluation of the assay results

In order for SF-ATPA to be considered to be evaluable, at least 2.0 nM ATP level was required in the control group. In the case of single-agent administration, the drugs were judged as sensitive when the intracellular ATP level was decreased more than 50% from the control level in the drug-treated wells containing the PPC level for each drug as described by Bertelsen and associates [6]. In the case of combined drug administration, antitumour activity is indicated by a drug-induced reduction of control ATP level as a percentage (%) of the control.

Clinical chemotherapy

Patients who had a measurable lesion underwent clinical chemotherapy. Complete response (CR) was defined as the disappearance of all clinical manifestations of the disease for more than 4 weeks. Partial response (PR) was defined as a 50% or greater decrease in the sum of the products of all perpendicular diameters of measurable lesions. Minor response (MR) was defined as a decrease of greater than 25% but less than 50% of the sum of the products of all perpendicular diameters of measurable lesions. No change (NC) was defined as any change from a decrease of less than MR to an increase of less than 25% in the size of any measurable lesion, with no appearance of new lesions and no worsening of the symptoms. Progressive disease (PD) was defined as an unequivocal increase of at least 25% in the size of any measurable lesion or the appearance of any new lesions. Patients who achieved CR, PR and MR were recorded as being "*in vivo* sensitive". Patients with NC or PD were recorded as being "*in vivo* resistant".

Table 2. Surgically resected samples investigated by SF-ATPA

Tumour type	No. of patients	No. of samples
Oesophagus	81	85
Stomach	61	63
Colorectum	42	43
Liver	11	11
Pancreas	19	19
Biliary tract	23	23
Total	237	244

Table 3. Evaluability rates and tumour growth by tumour type

Tumour type	Evaluability rates* (%)	ATP levels in control group† [nM] (range)
Oesophagus	73/85 (86)	23.7 ± 38.7 (0.0147–291.0)
Stomach	52/63 (83)	25.2 ± 35.2 (0.308–155.0)
Colorectum	35/43 (81)	36.7 ± 50.7 (0.2–242.0)
Liver	10/11 (91)	19.1 ± 21.7 (1.05–77.0)
Pancreas	18/19 (95)	37.7 ± 45.3 (0.904–132.0)
Biliary tract	20/23 (87)	37.4 ± 44.9 (0.762–168.0)
Total	208/244 (85)	28.5 ± 40.9 (0.0147–291.0)

*Evaluability criterion is defined as ⩾ 2.0 nM ATP level of the control group. † Values are expressed as mean ± S.D.

RESULTS

Clinical samples

A total of 244 fresh surgically resected samples were obtained from 237 gastrointestinal cancer patients treated in our department: 85 with oesophageal cancer, 63 with gastric cancer, 43 with colorectal cancer, 11 with hepatic cancer, 19 with pancreatic cancer, 23 with biliary tract cancer (Table 2).

Evaluability rates by tumour types

Table 3 shows the evaluability rates and tumour growth in the control group by tumour type. 208 of 244 samples (85%) were evaluable according to our criteria. All of the non-evaluable samples had low growth, and there were no fungal nor bacterial contamination.

There were no notable differences in the evaluability rates among the tumour types and there were no notable differences in the intracellular ATP levels in the untreated controls except for a slightly lower level in hepatic cancer.

In vitro *drug sensitivity (single-agent)*

Table 4 shows the sensitivity for each tumour type to several chemotherapeutic single agents at the PPC level. The sensitivity rates ranged from 8 to 40% (range, 11–40% for CDDP, 19–33% for MMC, 11–29% for 5-FU, 13–33% for DOX and 8–33% for VP-16, respectively). For each drug, some tumours showed specific sensitivity such as DOX and VP-16 in oesophageal cancer; MMC, DOX and 5-FU in gastric cancer; 5-FU in colorectal cancer; CDDP, MMC and DOX in hepatic cancer; VP-16, MMC and 5-FU in pancreatic cancer; and 5-FU and DOX in biliary tract cancer. The total sensitivity rates of a single agent in hepatic, gastric and pancreatic cancer were relatively higher than those in biliary tract, oesophageal and colorectal cancer.

In vitro *evaluation of drug combinations*

The antitumour activity for combination chemotherapy in oesophageal and gastric cancer are shown in Figure 1. In combined two-drug administration, median reduction rates (% of control) in oesophageal cancer were, for FP 42.6%, for EP 40.1%, for VP 49.3%; in gastric cancer were, for FP 40.4% and for EP 51.7%. In combined three-drug administration, median reduction rates (% of control) for FAP, FAM and FEP were 26.1%, 21.8% and 37.2%, respectively, in oesophageal cancer, 26.0%, 30.7% and 38.4%, respectively, in gastric cancer. In both oesophageal and in gastric cancer, three-drug combinations were more efficacious than two-drug combinations.

Correlations between ATP reduction rates of single-agent or combined drug used in the clinical treatment regimens and clinical results

25 patients were assigned to single-agent or combined drug clinical trials. Of these 25 patients, 7 received a combination of two drugs, and 9 received a combination of three drugs. Figure 2 illustrates the correlation between ATP reduction rates (% of control) of single-agent or combined drug used in the clinical treatment regimens and clinical results.

For assay/clinical correlations for single-agent or combined drug assay, cut-off levels of 50% for single-agent, 30% for two drugs and 20% for three drugs gave the best overall prediction of sensitivity, at 78%, 100% and 78%, respectively.

Comparison of assay/clinical correlation determined by single-agent or combined drug assays

Table 5 shows a summary of clinical correlations by single agents or combined drugs derived from SF-ATPA.

Table 4. Sensitivity rates of single agent by tumour type

Tumour	Sensitivity rates* (%)						
	CDDP	MMC	5-FU	DOX	VP-16	VDS	Total
Oesophagus	15/70	13/63	11/68	14/57	14/57	4/42	71/357
	(21)	(21)	(16)	(25)	(25)	(10)	(20)
Stomach	11/51	13/47	12/52	12/50	8/38		56/238
	(22)	(28)	(23)	(24)	(21)		(24)
Colorectum	5/33	6/31	9/33	4/30	3/18		27/145
	(15)	(19)	(27)	(13)	(17)		(19)
Liver	4/10	3/9	1/9	3/9	2/8		13/45
	(40)	(33)	(11)	(33)	(25)		(29)
Pancreas	2/18	5/16	5/17	3/16	3/9		18/76
	(11)	(31)	(29)	(19)	(33)		(24)
Biliary tract	3/19	4/18	5/17	5/17	1/12		18/83
	(16)	(22)	(29)	(29)	(8)		(22)
Total	40/201	44/184	43/196	41/179	31/142	4/42	203/944
	(20)	(24)	(22)	(23)	(22)	(10)	(22)

*Sensitivity rate indicates the number of sensitive drug-treated samples/number of drug-treated samples using ⩾ 50% inhibition of the intracellular ATP level relative to the control at PPC level.

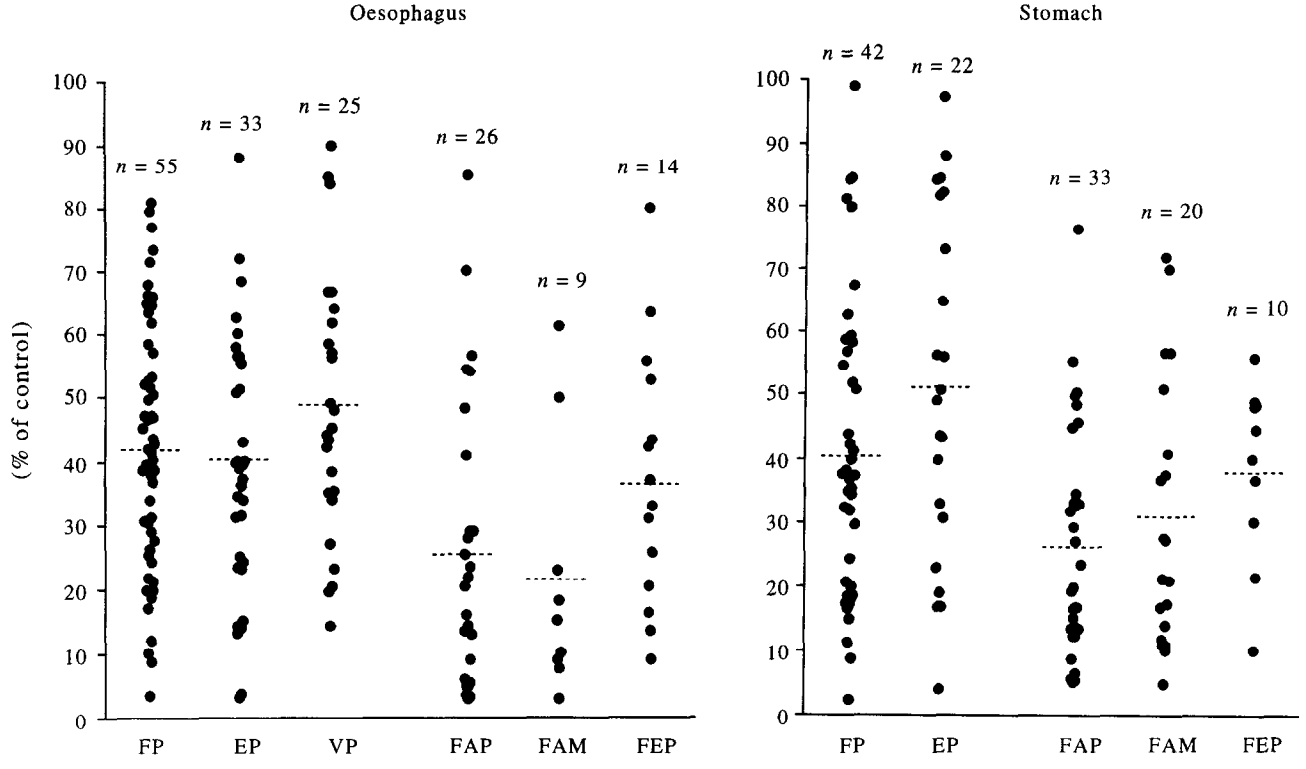

Figure 1. Reduction rates of control ATP level for combination chemotherapy in oesophageal and gastric cancer. Both in oesophageal and in gastric cancer, three-drug combinations were more effective than two-drug combinations.

Correlations between the SF-ATPA and clinical results in the retrospective study were observed for single agents (all patients) and drug combinations (7 patients with two-drug and 9 patients with three-drug administration).

16 patients were evaluated for clinical response by both single-agent and combined drug administration. For single-agent administration, the true positive rate (TPR) was 50% (5/10) and the true negative rate (TNR) was 100% (6/6), whereas for combined drug administration, the TPR was 71% (5/7) and the TNR was 100% (9/9). The overall predictive accuracy of the assay was higher for combined drug treatment [88% (14/16)] than for single-agent treatment [69% (11/16)].

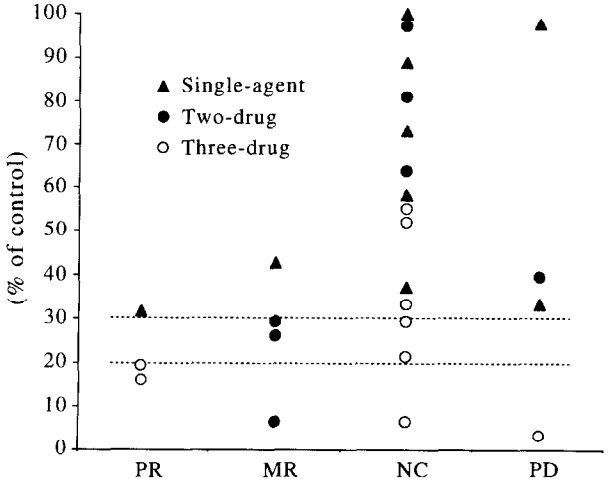

Figure 2. Correlation between ATP reduction rates and clinical effects. For the combined drug assay, a cut-off level of 30% for two drugs and 20% for three drugs gave the best overall prediction of sensitivity.

A total of 25 correlations between SF-ATPA and clinical response were reconsidered. A drug combination was judged as sensitive in the assay if the same drugs used in the clinical treatment regimen were tested and found to be sensitive, or at least one drug used in the treatment regimen was judged as sensitive if the combination was not tested. Of these 25 assay–clinical correlations, 7 were true positives, 4 were false positives and 14 were true negatives. The true positive rate was 64% and the true negative rate was 100%, yielding an overall predictive accuracy of 84%.

Good correlations were observed between SF-ATPA and clinical effect in oesophageal cancer, gastric cancer, colorectal cancer and hepatic cancer. However, the correlations appeared to be poor in pancreatic and biliary tract cancer.

DISCUSSION

Several assays for *in vitro* sensitivity tests have been developed in order to predict tumour response to chemotherapy. A tetrazolium (MTT) colorimetric assay [9, 10], ATP assay [11, 12] and [^{3}H]thymidine uptake method [13] are representative examples of these tests. Recently, the ATP assay and MTT assay have been used widely as simple sensitivity tests. The advantages of these assays are their short assay period, technical simplicity and the requirement of a relatively small number of cells.

However, the major problem associated with these assay systems is the contamination by non-cancer cells, such as fibroblasts or lymphocytes, which may overproliferate in culture and complicate the assay results. Koechli and associates [14, 15] reported that the growth of non-malignant cells was inhibited using an agar/McCoy underlayer and agarose-

Table 5. SF-ATPA/clinical correlations

Patient no.	Tumour	Sample	Drug sensitivity*						Clinical therapy	Clinical response					Correlation	
			CDDP	MMC	5-FU	DOX	VP-16	COM		PRI	LYM	HEP	PUL	Overall	SIN	COM
1	Oesophagus	PRI	–	–	–	–	+		EP		MR			MR	TP	
2	Oesophagus	LYM	+	–	–	+	–		FP	PD	NC			PD	FP	
3	Oesophagus	LYM	–	–	–	–	+	FP(–)	FP	NC				NC	TN	TN
4	Oesophagus	PUL	–	–	–	–	–	FP(–)	FP	PD				PD	TN	TN
5	Oesophagus	PRI	–	–	–	–	–	FP(–)	FP	NC				NC	TN	TN
6	Oesophagus	PRI	+	–	+	+	–	FP(+)	FP		MR			MR	TP	TP
7	Oesophagus	LYM	+	–	+	+	–	FEP(+)	FEP	PR	NC			PR	TP	TP
8	Oesophagus	PRI	–	+	+	+	+	FP(+)	FP	MR				MR	TP	TP
9	Stomach	PRI	–	–	–	–			MMC		NC	NC		NC	TN	
10	Stomach	PRI	–	–	–	–	+		FAP			NC		NC	TN	
11	Stomach	PER	+	+	+	+	–	FAP(–)	FAP		NC			NC	FP	TN
12	Stomach	PER	–	–	–	+		FAM(–)	FAM	NC	NC	NC		NC	FP	TN
13	Stomach	PRI	–	+	–	+	+	FAP(+)	FAP	PR	PR	PR		PR	TP	TP
14	Colon	PRI	+	–	+	–	–	FP(+)	FP			MR		MR	TP	TP
15	Colon	PRI	–	–	–	–			MMC			NC	NC	NC	TN	
16	Colon	PRI	–	+	–	–	–		5-FU				NC	NC	TN	
17	Liver	PRI	–	+	–	+			FAM	PR				PR	TP	
18	Pancreas	LYM	+	–	–	–			FM	PD				PD	TN	
19	Pancreas	HEP	–	+	–	+		FAP(+)	FAP			PD		PD	FP	FP
20	Pancreas	LYM	+	+	+	+	+	FAP(+)	FAP	NC	NC			NC	FP	FP
21	Pancreas	PLE	–	–	–	–		FP(–)	FP		NC		NC	NC	TN	TN
22	Pancreas	LYM	–					FAP(–)	FAP	NC	NC			NC	TN	TN
23	Biliary tract	PRI	+	–		+			FAP		NC			NC	FP	
24	Biliary tract	HEP	–	–	–	–	–	FAP(–)	FAP	NC		NC		NC	TN	TN
25	Biliary tract	LYM	–	–	+	–		FAP(–)	FAP	NC	PR	NC		NC	FP	TN

Sample: PRI, primary; LYM, lymph nodes; PUL, pulmonary; PER, peritoneum; HEP, hepatic; PLE, pleura. Clinical therapy: EP, VP-16/CDDP; FP, 5-FU/CDDP; FEP, 5-FU/VP-16/CDDP; FAP, 5-FU/DOX/CDDP; FAM, 5-FU/DOX/MMC; FM, 5-FU/MMC. Response: MR, minor response; PD, progressive disease; NC, no change; PR, partial response. Correlation: SIN, single; COM, combination; TP, true positive; FP, false positive; TN, true negative; FN, false negative.

*–, resistant; †+, sensitive.

coated wells, and good correlation was observed between the ATP viability assay and clinical response in breast cancer. Using discontinuous Ficoll–Hypaque and Percoll gradients, Yamaue and associates [4] also demonstrated that purified tumour cells showed high sensitivity to antitumour drugs compared with non-malignant cells. These reports suggest that suppression of the growth of these benign cells is a great advantage for *in vitro* chemosensitivity testing and increases the reliability of these assays.

It has been reported that normal cells, such as fibroblasts, are unable to grow in serum-free culture systems, while malignant cells can grow with some nutrients. Andreotti and associates [16] observed the selective growth of malignant cells in serum-free culture sytem using cytology before and after culture for 124 specimens. In the culture conditions of the SF-ATPA, non-malignant cells do not contribute relevant ATP levels when treated samples are compared to controls on day 3 [5]. We have previously reported that among five serum-free media tested (S-clone SF-B, SF-H, SF-O, MCDB152 and ASF301), SF-B shows the most potent tumour cell growth almost to the level of serum supplemented medium [5]. This advantage may depend on the components of the medium and the addition of bovine serum albumin. The medium of SF-B is composed of a mixture of RPMI 1640, Dulbecco's MEM and Ham's F-12 medium and is supplemented with insulin, transferine, ethanolamine and 2-mercaptoethanol.

The major reason why we selected the ATP assay is that the intracellular ATP level was significantly correlated with cell number and viability, [^{3}H]thymidine incorporation and the results of clonogenic assay [8]. Maehara and associates [17] reported that the ATP assay has a higher sensitivity than the succinate dehydrogenase inhibition (SDI) test for the prediction of cell viability.

Moreover, the ATP assay could be performed with small samples, such as those obtained from endoscopic biopsy [16]. With this assay, a repeat determination of ATP can be carried out at various times of incubation [18]. The incubation time of 72 h for SF-ATPA was based on our preliminary experiments (data not shown). As the intracellular ATP level of a cell is markedly decreased and the nuclei and cytoplasm become swollen and flattened in the presence of mitomycin C on day 3, the biological activity is probably decreased, even if cells are proved to be viable in the novel dye-exclusion method [17].

In the SF-ATPA, tumour cell growth and the evaluability rate were adequately high for any tumour type. The evaluability rate of this assay (85%) nearly equalled that of the MTT colorimetric assay for gastrointestinal tumours and other malignant tumours reported by Yamaue and associates (87.2%) [4].

With regard to the sensitivity rate for single-agent therapy, the most active antitumour drug was different for each tumour. The results show that each tumour has its own spectrum of *in vitro* drug sensitivities.

A problem in retrospective analyses of predictive tests for chemotherapy is that the predictive test evaluates single-agent activity, while the majority of the patients with gastrointestinal cancer receive multidrug combination chemotherapy. The predictive accuracy of the assay depends on the criteria used to define *in vitro* sensitivity. As for assay–clinical correlations for this study of 25 patients, we feel that the chosen cut-off levels of 50% for single-agent, 30% for two drugs and 20% for three drugs were suitable, although further studies are necessary to evaluate the best cut-off levels for single-agent or combined drug assay. However, for 16 patients evaluated for assay–clinical correlations by both single-agent and combined drug therapy, the predictive accuracy of the assay was higher with the latter than with the former. Moreover, the effects of drug combination *in vitro* were observed mostly as synergistic in this assay (data not shown). Bertelson and associates [6] reported, in a review of 1582 *in vitro* chemosensitivity tests, that 21 out of 289 patients were found to be sensitive to the combined drug assay, when no single drug was found to be active. Therefore, it is necessary for *in vitro* assays to investigate not only single-agent sensitivities, but also the combination sensitivities of the same drugs which may be used in clinical treatment. Aapro [19] reported that better definition of the conditions under which to test multidrug combinations and better techniques for *in vitro* assays should allow for predictive combined drug testing in the near future.

Obviously, SF-ATPA has greater potential usefulness for patients with gastrointestinal cancers. Particularly in oesophageal, gastric, colorectal and hepatic cancer, good correlations were observed between SF-ATPA and clinical response. However, in pancreatic and biliary tract cancer, the correlations appeared to be poor because of the low clinical response rate (0%) and the complexity of the drug delivery. We are not fully satisfied with the overall predictive accuracy of 84% for accurate prediction of cell viability. The correlations presented in this study were derived from a retrospective analysis. Prospective and randomised controlled trials must demonstrate an unequivocal patient survival advantage from the use of SF-ATPA in selecting chemotherapeutic drugs for patients before it can be routinely applied to the selection of chemotherapeutic agents.

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