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A novel *in vitro* chemosensitivity test using materials collected by endoscopic biopsy

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The usefulness of chemosensitivity testing of cells collected by endoscopic biopsy using the adenosine triphosphate assay (ATP assay) was investigated for esophageal tumors. Correlation between this chemosensitivity test and other chemosensitivity tests was more than 80% in most combinations. The predictive rate of clinical sensitivity was 77.8% and of clinical resistance was 68.8%. The predictive accuracy was 72.0%. These results will extend the indication and usefulness of chemosensitivity testing in inoperable and preoperable cases.

Key words: ATP assay, biopsy, chemosensitivity test, endoscope, esophageal cancer.

Introduction

Chemotherapy plays an important role in the treatment of patients with digestive tract cancers. Although many regimens of chemotherapy have been employed to treat gastrointestinal cancers, the response rate of these cancers to chemotherapy is variable. To improve the response rate, *in vitro* and *in vivo* chemosensitivity tests have been developed and have shown good correlation with clinical response. For these tests, tissue samples are obtained at surgery. It is therefore almost impossible to treat gastrointestinal cancers before operation based on the results of chemosensitivity tests which can predict tumor response to anti-cancer agents. Large volumes of tumor are needed for the chemosensitivity tests and the results are obtained several days later and cannot be utilized before or perioperatively. Furthermore, the tests cannot be used in inoperable cases.

For advanced cases of esophageal cancer involving neighboring organs, we have performed bypass operations followed by multidisciplinary treatment including chemotherapy, radiotherapy, hyperthermia and immunotherapy.[1] In these cases, materials collected for chemosensitivity testing had to be collected by endoscopic biopsy. We explored *in vitro* chemosensitivity testing of cells collected by endoscopic biopsy of esophageal cancers in xenografts in nude mice. The ATP assay method of chemosensitivity testing was used because it could rapidly measure ATP levels in a small number of cells.[2,3] We also studied the clinical application in humans of this new chemosensitivity test.

Materials and method

Animal experiments

Five esophageal cancer (EH-1, 4, 5, 6 and 7) xenografts in nude mice, which have been established and maintained in our facility, were used. Five anti-cancer agents were used in the chemosensitivity test: mitomycin C (MMC), 5-fluorouracil (5-FU), adriamycin (ADM), *cis*-diaminedichloroplatinum (CDDP) and bleomycin (BLM). Proper drug concentration used in this chemosensitivity test, which has already been reported, was 1 g/ml for MMC, 10 g/ml for 5-FU, 0.4 g/ml for ADM, 2 g/ml for CDDP and 30 g/ml for BLM.[4]

Preparation of cell suspension and culture: The materials obtained from the xenografts in nude mice were minced with scissors, filtered through #100 stainless mesh, and centrifuged at 1500 rev/min for 5 min with RPMI 1640 media including 10% heat inactivated fetal bovine serum (HI-FBS, Gibco) and antibiotics (penicillin G 10^5 U/ml and streptomycin 100 mg/ml). These steps were repeated four

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times. In our original method the materials were digested with enzymes to obtain cancer cells; however, this enzymatic digestion was omitted here due to the small number of cells. The number of viable tumor cells was counted by the Trypan Blue dye exclusion method and the viable cells were prepared in concentrations of 10^2, 10^3, 10^4 and 10^5/160 μl. The microplate (Corning 25850) was incubated at 37°C in a humidified 5% CO atmosphere. Materials collected by biopsy forceps were prepared in the same manner, suspended in 4 ml total volume and cultured as above.

ATP assay: ATP level in the cells was measured by the biological luminous method. Briefly, 100 μl of the cell suspension were added to 200 μl of ATP releasing reagent (Laboscience Co. Ltd) and mixed with 200 μl of luciferin-luciferase reagent (Laboscience Co. Ltd). Intensity of fluoresence (RLU) was measured by Lumiphotometer (Laboscience Co. Ltd) for 15 s.

Inhibition rate of tumor growth by anti-cancer agents: Seven samples collected by biopsy forceps from the xenografts in nude mice were suspended in 4 ml total volume, and 200 μl of this suspension were poured into microplate wells. As a control, RPMI 1640 was used instead of anti-cancer agents. After 72 h of culture, the ATP level was measured and the ratio to the control was calculated.

Human studies

Thirty-one esophageal cancer cases consisting of 25 resectable cases and six unresectable cases who underwent bypass operations were tested. The materials were collected by endoscopic biopsy before operation and tested within two hours after collection.

Correlation between results of the chemosensitivity test and clinical effects: Patients undergoing resection usually received hyperthermo-immuno-chemotherapy preoperatively (19 patients) and the remaining six had radio-hyperthermo-immuno-chemotherapy after palliative bypass surgery. Thirty mg of BLM, 10 mg of CDDP and 4 mg of ADM were used. They were suspended in 5 ml of lipiodol and administered into the submucosal layer around the tumor endoscopically. Lipiodol–BLM was injected once or twice with a 1-week interval followed by two or three injections of lipiodol–CDDP or ADM with the same interval. Thirteen resected cases and five bypass cases received hyperthermia and all bypass cases received irradiation. Protein-bound polysaccharide (PSK) and streptococcal preparation (OK-432) were used for immunotherapy.

In resected cases, clinical effects were judged by barium meal and endoscopy performed before operation; the cases were evaluated by our original criteria and classified from Grade 1 to Grade 6 (Table 1). If the grade was different between the barium meal and endoscopy, the higher grade was chosen. Grades 3 to 6 were estimated as positive effects. For the bypass cases, the clinical effect was evaluated by röntgenography, endoscopy and CT scan, and classified or divided into five categories by the standard criteria of clinical tumor response to anti-cancer agents. Briefly, a complete response (CR) required complete disappearance of all measurable disease for at least one month. A partial response (PR) 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 one month, and the absence of any new lesions during treatment. A minor response (MR) was defined as at least 50% or greater reduction that did not last one month. A progressive disease (PD) was defined as a greater than 50% increase in the size of the measurable disease. No change (NC) was defined as that between progressive disease and partial response. CR, PR and MR were estimated as positive effects.

Statistical analysis: The correlation coefficient was obtained from Pearson's formula. The linear

Table 1. Clinical criteria for the effect of preoperative therapy on resective esophageal cancer cases

Grade	Radiologic findings	Endoscopic findings
1 Progression	Enlargement	Enlargement
2 No change	No change	No change
3 Slightly effective	Slight widening of caliber	Slight depression of tumor elevation or slight widening of caliber
4 Moderately effective	Shortening of vertical extension or obvious widening of caliber	Obvious depression of tumor elevation or obvious widening of caliber
5 Markedly effective	Only rigidity	Only scar or erosion
6 Complete response	Disappearance	Disappearance

regression was obtained from the method of least squares.

Results

ATP level and cell number: ATP levels were measured in 1×10^2, 1×10^3, 1×10^4 and 1×10^5 cells collected from xenografts in nude mice and calculated by the Trypan Blue dye exclusion test. ATP level and cell number correlated well ($p < 0.01$), with coefficients ranging from 0.9954 to 0.9767. There was a linear correlation (Table 2). However, the standard deviation of ATP level in 1×10^2 cells measured by lumiphotometer was greater than for 1×10^3 cells ($p < 0.05$). Accurate measurement of ATP level was possible in cell numbers over 1×10^3.

ATP level and cell culture period: ATP levels in 1×10^2, 1×10^3, 1×10^4 and 1×10^5 cell suspensions were measured at 0, 24, 48, 72 and 96 h after culture. ATP levels in cancer tissues decreased until 24 h, and then gradually increased and reached a plateau at 72 h. However, in 1×10^2 cells, the increase of ATP was not observed in any cell culture periods. From these results, the optimum cell culture period was decided as being 72 h (Figure 1).

Table 2. Relationship between cell number and ATP levels in xenografts implanted in nude mice

Name	Linear regression	Correlation coefficient
EH-1	$Y = -3.96 + 1.01X$[a]	0.9767[b]
EH-4	$Y = -3.49 + 0.95X$	0.9926
EH-5	$Y = -3.77 + 0.98X$	0.9954
EH-6	$Y = -4.06 + 1.16X$	0.9951
EH-7	$Y = -3.95 + 1.02X$	0.9905

[a] $X = \log x$; x = cell number; $Y = \log y$; y = ATP amount (R.L.U.).
[b] $p < 0.01$.

Cell number and number of biopsy samples: ATP levels of 3, 5 and 7 samples collected by endoscopic biopsy forceps were measured in EH-5, 6, 7 xenograft

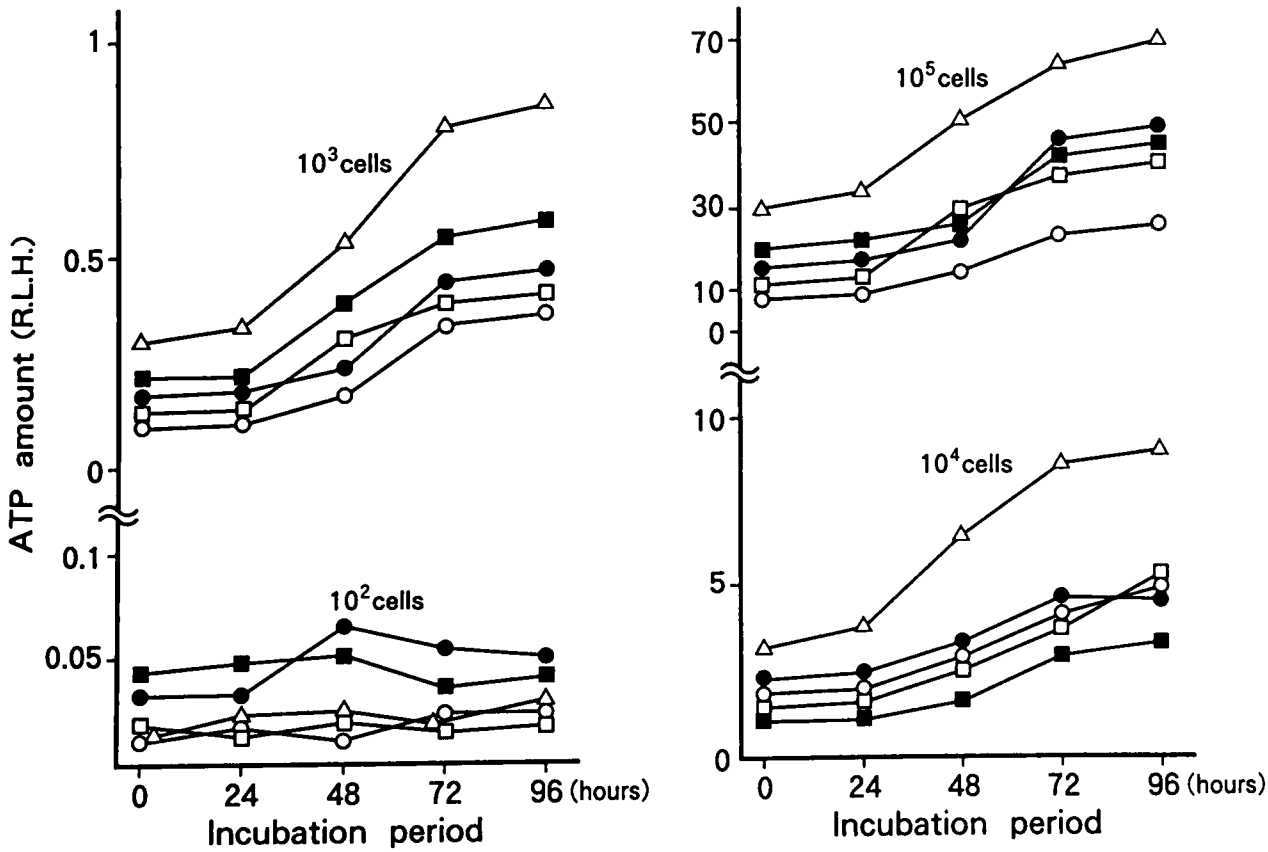

Figure 1. ATP amount and cell culture period. ○ EH-1 ($n = 25$); ● EH-4 ($n = 25$); □ EH-5 ($n = 25$); △ EH-6 ($n = 25$); ■ EH-7 ($n = 25$).

Table 3. Cell number estimated from ATP level

Tumor name	Sample number	ATP level (R.L.U.)	Estimated cell number ($\times 10^4$)
EH-5	3	3.92 + 0.58	1.18 + 0.26
	5	5.81 + 0.86	1.96 + 0.28
	7	7.65 + 0.66	2.38 + 0.66
EH-6	3	4.78 + 0.72	0.28 + 0.03
	5	9.02 + 0.63	0.43 + 0.02
	7	22.38 + 3.85	0.92 + 0.04
EH-7	3	3.38 + 0.21	0.38 + 0.04
	5	11.80 + 3.86	1.10 + 0.36
	7	14.53 + 5.44	1.33 + 0.67

tumors without cell culture. The results were applied to the formula of each tumor shown in Table 3. As a result, the average cell number was presumed to be 0.28×10^4 to 1.18×10^4 in 3 samples, 0.43×10^4 to 1.96×10^4 in 5 samples, and 0.92×10^4 to 2.38×10^4 in 7 samples. Because it was not distressing for the patients to have 7 samples taken clinically, this was decided to be the number of biopsy samples.

Inhibition rate of tumor growth by anti-cancer agents: Average ATP levels in the seven biopsy samples were 13.8, 6.4 and 22.9 RLU in EH-1, 4, 5; the number of cells was estimated to be over 1×10^4 and was sufficient for assessment. The inhibition rates revealed over 50% inhibition in EH-1 and EH-4 by BLM and ADM, and in EH-5 by BLM (Table 4).

Table 4. Results of chemosensitivity test using samples collected by endoscopic biopsy forceps

Tumor name	Inhibition rate (%)				
	MMC	CDDP	5-FU	ADM	BLM
EH-1	35.5	37.2	6.0	67.1	95.9
EH-4	18.3	11.9	39.8	65.2	84.0
EH-5	13.7	16.4	32.3	45.7	97.1

Clinical evaluable rate and positive rules: Thirty-one esophageal cancer cases were tested and the percentage of evaluable patients was found to be 93.5% (29/31). When the reduction of ATP level in the group treated with anti-cancer agents was more than 50%, the tested anti-cancer agent was regarded as a tumor-sensitive agent. The sensitivity rates to anti-cancer drugs in 29 cases of esophageal cancer were 6.9% for MMC, 27.2% for CDDP, 27.2% for 5-FU, 6.9% for ADM, and 37.5% for BLM.

Comparison with other chemosensitivity tests: Table 5 shows the correlation rate between the chemosensitivity test using ATP assay for collected samples by endoscopic biopsy and other chemosensitivity tests which were performed in the same case using the resected materials. There was a >80% agreement, except in column 3 for 5-FU in the nude mouse isotope assay (NM-IA), subrenal capsule assay (SRCA) and ATP assay using resected tumor materials.

Correlation between chemosensitivity test and clinical effects: Of the 19 cases with esophageal resection, 9 (47.4%) were sensitive and 10 (52.7%) were resistant to chemotherapy (Table 6). Of six bypass cases, half were sensitive and the rest were resistant to the chemotherapy (Table 7). The predictive rate of clinical sensitivity was 77.8% and that of clinical resistance was 68.8% in the total number of cases. The overall predictive accuracy was 73.3% in resection cases, 66.7% in bypass cases and 72.0% in all cases, respectively (Table 8).

Table 5. Correlation rate between chemosensitivity test for samples collected by endoscopic biopsy and other chemosensitivity tests

Chemosensitivity tests	Anti-cancer agents			
	MMC	CDDP	5-FU	ADM
NM-IA	15/18[a](83.3)[b]	15/18(83.3)	14/18(77.8)	
MTT	11/13(84.7)	11/13(84.7)	11/13(84.7)	11/13(84.7)
SRCA	14/15(93.3)	12/15(80.0)	10/15(66.7)	12/15(80.0)
ATP	15/16(93.8)	13/16(81.3)	10/16(62.5)	13/16(81.3)

NM-IA: nude mouse isotope assay; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay; SRCA; subrenal capsule assay; ATP: adenosine triphosphate assay using resected tumor materials.
[a] Number represents no. of positive cases vs those of tested cases.
[b] Positive cases as percentage of tested cases.

Table 6. Clinical response in esophageal cancer: resectable cases

	X-ray finding	Endoscopic finding	Total finding
Grade 1	1	1	1(5.3)
2	10	8	9(47.4)
3	4	4	4(21.1)
4	2	4	4(21.1)
5	1	0	1(5.3)
6	0	0	0
Total	18	17	19(100)

Discussion

Current chemosensitivity tests for anti-cancer agents use materials collected at the operation, because large volumes of material are needed. Chemosensitivity tests are therefore not possible with patients who do not undergo surgery due to advanced stage or high risk.

We have chosen cases with a bypass operation for far advanced esophageal cancer followed by intensive radio-immuno-chemotherapy.[1] These are the cases which most require effective and intensive chemotherapy based on the results of chemosensitivity tests. In these cases, materials can only be collected by endoscopic biopsy. We therefore investigated the possibility of using materials collected by endoscopic biopsy for chemosensitivity testing.

The two major problems with chemosensitivity testing of biopsy samples are the small number of cells obtained and contamination. To test the former, the ATP assay was chosen, because it has been reported to be convenient for small numbers of cells (for example 5×10^2 to 1×10^3).[2,3] Our results show that it is possible to measure ATP levels accurately when the cell number is over 1×10^3. The level of ATP accurately reflects the viability of cells.[2,3] Firefly luciferine was used to measure ATP level. ATP reacts with luciferin in the presence of luciferase causing radiation. The biologically luminous amount was measured with a Lumiphotometer for 15 s. This is the simplest and quickest method among the various chemosensitivity tests.[4]

The second problem was resolved by washing the cells five times with the RPMI 1640 containing penicillin G and streptomycin. These procedures prevented contamination during cell culture for 72 h.

Concerning the relationship between the number

Table 7. Clinical response in esophageal cancer: bypass cases

No.	Tumor sensitivity					Chemotherapy	Clinical response[a]
	MMC	CDDP	5-FU	ADM	BLM		
1	–	–	–	–	+	BLM, FT-207	NC
2	–	–	–	–	–	BLM, CDDP, FT-207	PR
3	–	–	–	–	–	BLM, CDDP, FT-207	NC
4	–	–	–	–	–	BLM, CDDP, FT-207	PD
5	–	–	–	–	+	BLM, FT-207	MR
6	–	–	+	–	+	BLM, FT-207	PR

[a] PD: progressive disease; NC; no change; MR; minor response; PR: partial response.

Table 8. Correlation between tumor sensitivity and clinical response

	Assay/clinical				Predictive of clinical sensitivity	Predictive of clinical resistance	Overall predictive accuracy
	S/S	S/R	R/S	R/R			
Resective case	5	1	4	9	5/6(83.8)	9/13(69.2)	14/19(73.7)
Bypass case	2	1	1	2	2/3(66.7)	2/3(66.7)	4/6(66.7)
Total	7	2	5	11	7/9(77.8)	11/16(68.8)	18/25(72.0)

S: Sensitive; R: resistant.

of viable cells and the ATP level, a positive correlation was found in tumors using linear regression. Saeki.[4] reported the same result in human material. Maehara *et al.*[5] and Ichihashi *et al.*,[6] in HeLa cells and Ehrlich ascitic tumor respectively, reported a positive relationship. All of our results with chemosensitivity testing using biopsy materials from xenografts in nude mice support this finding.

If the inhibition rate by the anti-cancer agents was over 50%, the results of the chemosensitivity test were estimated as 'positive' in our experiment. With these criteria, EH-1 and EH-4 were positive for ADM and BLM, and EH-5 for BLM. In comparison with another chemosensitivity test using the same materials of nude mouse xenografts, the nude mouse isotope assay showed the same results with all drugs. In addition, subrenal capsule assay showed similar results except for the chemosensitivity for ADM in EH-1.

Of our 31 cases tested clinically, 29 cases (93.5%) were evaluable. This was a higher evaluable rate than for other chemosensitivity tests;[7,8] in two cases the tests were repeated tests and showed a good correlation. Comparison with another chemosensitivity test using materials from the same case also revealed good correlation. These results suggest the clinical usefulness of this chemosensitivity test as well as that of other chemosensitivity tests. The correlation between sensitivity testing and clinical effect is most useful. This new chemosensitivity test showed 73.3% accuracy in resective cases and 66.7% in bypass cases. Fukui *et al.*[9] showed almost the same predictive accuracy of 71% in SRCA testing in esophageal cancer cases; this might be lower in other types of cancer.[10,11]

These results show that the ATP assay on materials collected by endoscopic biopsy can be used for chemosensitivity testing. Since the result of this test is already available three days after biopsy, it is possible to use effective drugs earlier in preoperative or inoperable cases.

Conclusions

The usefulness of chemosensitivity testing of cells collected by endoscopic biopsy using the ATP assay was investigated in esophageal tumors. Our conclusions were as follows.

(1) ATP amount and cell number correlated well ($p < 0.01$) with coefficients ranging from 0.9954 to 0.9767.

(2) Cell culture showed that ATP amount in cancer tissues decreased until 24 h and then gradually increased and reached a plateau at 72 h in more than 1×10^3 cells.

(3) The average cell number collected by endoscopic biopsy was estimated to be $0.28–1.18 \times 10^4$ in 3 samples, $0.43–1.96 \times 10^4$ in 5 samples and $0.92–2.38 \times 10^4$ in 7 samples.

(4) The correlation rate between the chemosensitivity test using ATP assay for collected samples by endoscopic biopsy and other chemosensitivity test showed an agreement of over 80%.

(5) The predictive rate of clinical sensitivity was 77.8% and that of clinical resistance was 68.8% in the total 25 cases. The overall predictive accuracy was 73.3% in 19 resection cases, 66.7% in 6 bypass cases and 72.0% in all cases.

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