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In Vitro Chemosensitivity Test for Gastric Cancer Specimens Predicts Effectiveness of Oxaliplatin and 5-Fluorouracil

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Abstract. *Aim: Cisplatin plus 5-fluorouracil (5-FU) or S-1 is a standard therapy for gastric cancer (GC). However, cisplatin is emetic and potentially nephrotoxic. Oxaliplatin may be less toxic, but few basic data are available for this setting. Here, we evaluated oxaliplatin for GC, by testing surgical specimens. Materials and Methods: We evaluated effects of oxaliplatin and 5-FU, alone and in combination, on surgical specimens from 11 patients with GC, using collagen gel droplet embedded culture drug tests. Results: Oxaliplatin was less efficacious than 5-FU, and its synergistic effect was less in tumors highly sensitive to 5-FU than in those with low sensitivity. Tumor differentiation and drug sensitivity were not correlated. Conclusion: Although oxaliplatin monotherapy had little effect on GC, we saw a limited synergistic effect of oxaliplatin with 5-FU in 5-FU-sensitive patients. Collagen gel droplet embedded culture drug tests may predict this synergistic effect, and help select candidates for this or other regimens.*

S-1 is an important therapeutic agent for advanced gastric cancer (GC) (1), and as adjuvant chemotherapy for GC postoperatively (2, 3). However, some patients need more powerful antitumor effects. The SPIRITS trial in 2008 showed that adding cisplatin to S-1 led to longer overall (OS) and progression-free (PFS) survival than S-1 alone (2); this combination, or cisplatin with fluorouracil (5-FU) is now a standard first-line chemotherapy regimen (4-6). However, platinum-containing agents have a strong emetic effect, and can cause sensory neuropathy and nephrotoxicity, which complicate their use in outpatients. Oxaliplatin, like cisplatin, is a platinum-containing agent, but has no nephrotoxic effects. With fluorouracil and leucovorin or S-1, oxaliplatin is reportedly an effective agent against colorectal cancer (7-9), and advanced GC. In fact, a regimen of S-1 plus oxaliplatin has been shown not to be inferior to that of S-1 plus cisplatin with regard to PFS (10, 11).

S-1 is the most effective agent in GC treatment and is an important key drug (1). Thus in selection of drug to be added in combination with S-1, it is necessary to be careful. Although the enhancing effect of platinum-containing agents on 5-FU and S-1 has been shown through clinical trials, adverse effects from these combinations often curtail their use (5, 12, 13).

Although one *in vitro* study used five established cell lines to evaluate the effect of oxaliplatin alone on GC (14), we know of no studies that evaluated the synergistic effect of 5-FU and oxaliplatin using clinical samples. Therefore, in this study, we evaluated the additional efficacy of oxaliplatin when added to 5-FU, using an *in vitro* chemosensitivity test on resected GC specimens.

Materials and Methods

Patients. We used specimens resected from 11 patients who underwent surgery for advanced GC, eight males and three females (age range: 49-88 years), from July 2014 to March 2016. Their clinicopathological characteristics are summarized in Table I.

Clinical samples. Among the 11 specimens there were five differentiated adenocarcinomas and six undifferentiated adenocarcinomas. We obtained the primary tumor cells by stripping them off the surfaces of cancerous specimens at the end of surgery as empirical data suggest that the largest number of cancer cells can be collected from tissue surfaces (15). All samples were irrigated 10 times, with 40 ml of saline each time, without antibiotics. After irrigation, the samples were stored in Eagle's minimal essential medium (Gibco®, Thermo-Fisher Scientific, Waltham, MA, USA) at 4˚C until chemosensitivity testing.

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Key Words: Gastric cancer, CD-DST, synergetic effect, oxaliplatin, 5-FU.

Table I. *Patient and tumor characteristics.*

Patient	Age, years	Gender	Histological type	T	N	H	P	M	Stage
1	85	F	Differentiated	4a	0	0	0	0	IIB
2	69	M	Undifferentiated	4a	1	0	1	0	IV
3	60	F	Undifferentiated	4a	2	0	1	0	IV
4	49	M	Differentiated	4b	2	1	0	0	IIIC
5	51	M	Undifferentiated	4a	0	0	0	0	IIB
6	68	F	Differentiated	3	2	0	0	0	IIIA
7	77	M	Undifferentiated	4a	2	1	0	1	IV
8	82	M	Differentiated	4b	2	0	1	0	IV
9	88	M	Undifferentiated	3	2	0	0	0	IIIA
10	74	M	Differentiated	3	0	0	0	0	IIA
11	74	M	Undifferentiated	4a	3	0	0	1	IV

F: Female; M: male; T: depth of tumor invasion; 3: tumor invades the subserosa (SS); 4a: tumor invasion is contiguous to the serosa or penetrates the serosa (SE); 4b: tumor invades adjacent structures (SI); N: lymph node metastasis; 0: no regional lymph node metastasis; 1: metastasis in 1-2 regional lymph nodes; 2: Metastasis in 3-6 regional lymph nodes; 3: metastasis in 7 or more regional lymph nodes; H: hepatic metastasis; 0: no hepatic metastasis, 1: hepatic metastasis; P: peritoneal metastasis; 0: no peritoneal metastasis; 1: peritoneal metastasis; M: presence or absence and sites of distant metastasis; 0: no distant metastasis; 1: distant metastasis; Japanese classification of gastric carcinoma: 3rd English edition.

In vitro chemosensitivity test. The collagen gel droplet embedded culture drug test (CD-DST) was carried out according to the method reported by Kobayashi *et al*. (15-17). A portion from each tumor specimen was excised and sliced into thin sections. Each sample was treated with a dispersed enzyme cocktail EZ (Primaster® Reagent; Kurabo Industries, Osaka, Japan). The obtained cell suspensions were inoculated into collagen-coated flasks (CG Flasks; Kurabo Industries) and cultured in PCM-1 pre-culture medium (Primaster® Content) containing 10% fetal bovine serum at 37˚C in 5% CO_2 overnight. Next, the collagen gel was digested with 0.05% EZ and viable cancer cells were obtained. Type I collagen (Cellmatrix Type CD; Kurabo Industries Ltd. Osaka, Japan), 10× concentrated F-12 medium, and reconstitution buffer were mixed together in ice water at a ratio of 8:1:1 (Primaster® content). The prepared cancer cell suspensions were each added to the collagen solution at final densities of 1×10^5 cells/ml. Subsequently, the tumor cells in the collagen gel droplets were exposed to the treatment drugs at concentrations corresponding to the area under the curve (AUC) for drug concentration and time. Three drops of the collagen-cell mixture (30 μl/droplet) were placed in each well of a 6-well multiple plate on ice and allowed to gel at 37˚C in a CO_2 incubator; the final concentration was approximately 3×10^3 cells per collagen gel droplet. Dulbecco's modified Eagle's medium and F-12 medium (Gibco) containing 10% fetal bovine serum was overlaid on each well 1 h later and plates were incubated in a CO_2 incubator at 37˚C overnight. The *in vitro* chemosensitivity of these samples to oxaliplatin, 5-FU and combination of oxaliplatin and 5-FU was analyzed by CD-DST. The oxaliplatin concentration was 0.6 μg/ml, and that of 5-FU was 1 μg/ml (17). Contact time for the drugs was 24 hours. The AUC for oxaliplatin at 60-130 mg/m^2 is 17.3 μg h/ml (18). The AUC of our study was 14.4-17.3 μg h/ml. After removing the medium containing the anticancer drugs, each well was rinsed twice with 3 ml Hanks' balanced salt solution, overlaid with 4 ml PCM-2 medium (Primaster® content, serum-free medium), and incubated for another 7 days. At the end of the incubation, a neutral red solution (Wako Pure Chemical Industries, Ltd., Osaka Japan) was added to each well to a final concentration of 50 μg/ml, and cancer cell colonies in the collagen gel droplets were stained for 2 h. Each collagen droplet was fixed with 10% neutral buffered formalin, washed in water, air dried, and quantified using image analysis (Primage System, Solution Systems Inc., -Tokyo, Japan). This sensitivity test was conducted at LSI Medience Corporation laboratory (Tokyo, Japan).

Table II. *Results of chemosensitivity tests on gastric cancer specimens. The in vitro chemosensitivity effect of 5-fluorouracil (5-FU) and oxaliplatin, alone and in combination, was expressed as the inhibitory rate (IR), which was calculated using the following formula: IR (%) = (C−T)/C × 100, where T was total colony volume of the treated group and C was that of the untreated control group. A combination/5-FU ratio ≥1.2 indicates a positive synergistic effect.*

Patient	5-FU	Oxaliplatin	Combination	Ratio (combination/5-FU)
1	19.1	0	21.2	1.11
2	17.2	4.5	32.0	1.86
3	18.0	11.2	14.6	0.81
4	50.4	17.4	46.0	0.91
5	42.9	18.5	43.4	1.01
6	9.5	6.2	4.0	0.42
7	6.8	16.4	20.2	2.97
8	45.2	25.6	47.9	1.06
9	27.1	32.4	31.5	1.16
10	40.7	27.8	50.8	1.25
11	21.9	4.4	28.4	1.30

Combination: 5-FU plus oxaliplatin.

The *in vitro* chemosensitivity effect was expressed as the inhibitory rate (IR), which was calculated by the following formula: IR (%)=(C−T)/C ×100, where T was total colony volume of the treated group and C was that of the untreated control group. To quantify the effect of the combination regimen, we used the

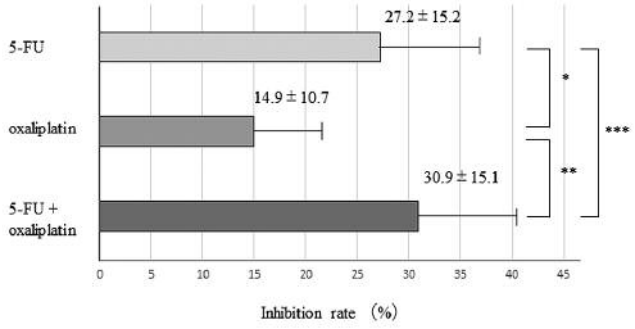

Figure 1. *Inhibitory rates using 5-fluorouracil (5-FU), oxaliplatin, and 5-FU in combination with theoxaliplatin. Data are shown as the mean±SD. Significantly different at *p=0.0095, **p=0.0014 and ***p=0.1035.*

following ratio: combination/5-FU. We considered a ratio ≥1.2 to indicate a positive synergistic effect.

Results

Table II shows the IRs for 5-FU, oxaliplatin, and their combination for each of the 11 patient specimens. Whereas IR values varied for 5-FU, those for oxaliplatin were low overall, and significantly lower than those for 5-FU (p=0.0095) and the combination regimen (p=0.0014). However, the IRs for 5-FU and the combination did not significantly differ (p=0.1035; Figure 1).

Synergistic effects were observed in four out of 11 cases (36.3%; patients 2, 7, 10 and 11) based on IRs for 5-FU. Four patients (patiens 4, 5, 8 and 10) were highly sensitive to 5-FU (mean IR ≥27.2%), among whom only patient 10 showed a synergistic effect from oxaliplatin. Among the five patients not responding to 5-FU (patients 1, 2, 3, 6, 7 and 11), we observed synergetic effects in three (patients 2, 7 and 11). We did not see a significant correlation between tumor differentiation and IR using 5-FU or oxaliplatin, or their combination (Table III).

Discussion

In this study, we evaluated the synergistic effect of 5-FU and oxaliplatin using an *in vitro* chemosensitivity test on surgical GC specimens. We had three novel findings: (i) the synergic effect of the combination of oxaliplatin and 5-FU is limited *in vitro*; (ii) among patients whose GC is sensitive to 5-FU, additional synergistic effects from adding oxaliplatin to 5-FU are unlikely; and (iii) tumor differentiation does not correlate with synergetic effects of these drugs.

The synergistic effect of oxaliplatin with 5-FU is limited *in vitro*, and (as described above), oxaliplatin alone had little effect on GC in this *in vitro* study. Only four patients showed

Table III. *Inhibitory rates (%) by histological type and treatment with 5-fluorouracil (5-FU), oxaliplatin and their combination.*

Histological type	n	5-FU	Oxaliplatin	Combination
Differentiated	5	33.0±17.7	15.4±12.1	34.0±20.5
Undifferentiated	6	22.3±12.1	14.6±10.5	28.4±10.1
p-Value		0.27	0.91	0.53

synergistic response to oxaliplatin combined with 5-FU in this study. Whereas IRs varied for 5-FU among our 11 patients, the IRs for oxaliplatin were low as a whole. Thus, in GC, the antitumor effect of oxaliplatin is expected to have a synergistic effect with 5-FU.

Although the response rate of ovarian cancer to oxaliplatin has been reported to be 29% (19), the reported response of colorectal cancer was only 10% (20).

The synergistic effect between oxaliplatin and 5-FU has been observed in colon, breast and ovarian cancer *in vivo* and *in vitro* (21, 22), but the mechanism that underlies their interaction is unclear. Moreover, identifying patients who can benefit from this combination is difficult. Among patients with advanced colorectal cancer, PFS and OS were reportedly equivalent in the FOLOFOX7 regimen, which has a modified oxaliplatin schedule, to those in the standard FOLOFOX4 regimen. The FOLFOX 7 is a modified regimen and the administration schedule of oxaliplatin as Stop-and-Go has been changed according to adverse events. Furthermore, patients with colorectal cancer who respond well to FOLFOX are also expected to respond well to 5-FU plus leucovorin without oxaliplatin (23). Therefore, although addition of oxaliplatin to a standard regimen may be beneficial, such patients should be personally assessed.

Tumor differentiation did not correlate with response to 5-FU, oxaliplatin or their combination. Although Eriguchi *et al*. reported poorly differentiated colorectal cell lines to be the most sensitive to oxaliplatin (14), as far as we are aware, no clinical trial has shown an effect of oxaliplatin with regard to tumor differentiation (24-28).

Furthermore, which patients can benefit from the addition of oxaliplatin is not known, and no basic data have been reported to predict responses to combination therapy. Even in our study, less-differentiated adenocarcinoma did not appear to respond well to oxaliplatin. However, addition of oxaliplatin is recommended in clinical trials, so it should not be omitted from a patient's regimen without clear reasons for doing so.

CD-DST uses a three-dimensional culture embedded in type I collagen gel and, unlike other chemosensitivity tests, antitumor activity is evaluated at drug concentrations similar to those obtained in humans (15-17). We, therefore, consider

CD-DST to be the best choice for evaluating synergistic drug effects using specimens *in vitro*.

This study had several limitations. Firstly, the sample size was small. However, we used genuine clinical samples as opposed to cell lines; this is a strength of this study. Secondly, because the number of clinical samples was limited, drug effects were evaluated only for certain concentrations and contact times, and other variables could not be tested. However, the AUCs in this study were set as they would be with clinical settings. Thirdly, for ethical reasons, this was inevitably an experimental study *in vitro* and was not reflected in patients' treatments. Nevertheless, this study potentially offers a means of assessing the appropriateness of treatment for individual patients for more personalized management of GC.

In conclusion, limited synergistic effects of oxaliplatin may be expected in 5-FU-responsive patients. Candidates for 5-FU monotherapy or 5-FU combined with oxaliplatin should be carefully selected; use of CD-DST may help predict response to such therapy.

Informed Consent

Informed consent was obtained from all individual participants included in the study.

Conflicts of Interest

The Authors declare that they have no conflict of interest.

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