Predicting the chemosensitivity of ovarian and uterine cancers with the collagen gel droplet culture drug-sensitivity test

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We investigated the utility of the collagen gel droplet culture drug-sensitivity test (CD-DST) for predicting the response of gynecological cancers to chemotherapy. Eighty-three cancer patients were enrolled in this study: 26 ovarian, 29 cervical and 31 endometrial cancers. The CD-DST was performed at various concentrations of drugs. We calculated the T/C ratio, where T is the total volume of the treated culture and C is the total volume of the control culture, and a T/C ratio of 50% or less was defined as sensitive *in vitro*. The efficacy rate (%) was defined as the number of cultures with a T/C ratio of 50% or less, divided by the total number of evaluable cultures. True-positive cases were defined as clinical responders (complete + partial responses) and true-negative cases were defined as clinical non-responders. The overall tumor evaluation rate was found to be 79.1%. The appropriate drug concentrations were selected as 1.0 μg/ml for cisplatin, 20.0 μg/ml for carboplatin, 1.0 μg/ml for paclitaxel and 0.1 μg/ml for docetaxel by the linear regression equations. The *in vitro* sensitivity for each drug showed a significant correlation with clinical response rates ($r = 0.592$, $p = 0021$). We therefore conclude that the CD-DST can be used to predict the response to anti-cancer drugs, and may also provide important information by contributing to the development of new chemotherapy regimens. *Anti-Cancer Drugs* 16:525–531 © 2005 Lippincott Williams & Wilkins.

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Introduction

Prediction of chemosensitivity has proved very useful for establishing individualized treatment for gynecological cancer patients. Previous studies have shown that several biomarkers may be predictors of chemosensitivity, including the overexpression or mutation of various oncogenes and tumor suppressor genes [1–3], apoptosis-related genes [4,5], mitotic activity [6] and expression of multidrug-resistant protein [7,8], but clinically useful predictors still remain to be established. Another approach has been to develop *in vitro* chemosensitivity tests, such as the human tumor clonogenic assay (HTCA) [9], thymidine incorporation assay (TIA) [10], succinic dehydrogenase inhibition test (SDI test) [11], MTT assay [12], three-dimensional agarose-based extreme drug resistance assay (EDRA) [13] and histoculture drug-response assay (HDRA) [14,15]. These *in vitro* chemosensitivity tests can serve for prediction of response to chemotherapy [9,10,14–16], but they are not widely used in clinical practice for various reasons, e.g. the HTCA and TIA tests need large tumor samples, the HTCA, SDI and MTT tests have low success rates of primary culture, and the HDRA requires a very high drug concentration in the culture medium.

Recently, we developed the collagen gel droplet culture drug-sensitivity test (CD-DST) [17], which satisfies the following requirements: (1) a high success rate of primary culture, (2) only a small number of cells are needed (1×10^5), (3) easy quantification of the anti-tumor effect without contamination by fibroblasts using imaging analysis [18], (4) maintenance of the original growth characteristics and (5) evaluation can be done using clinical concentrations of drugs.

Use of the CD-DST has been reported for breast, lung, stomach and colon cancers [17], but so far not for gynecological cancers. In the present study, we investigated the appropriate clinical concentrations of drugs for gynecological cancers, and studied the utility of the CD-DST for predicting the response of ovarian cancer and uterine cancer to chemotherapy.

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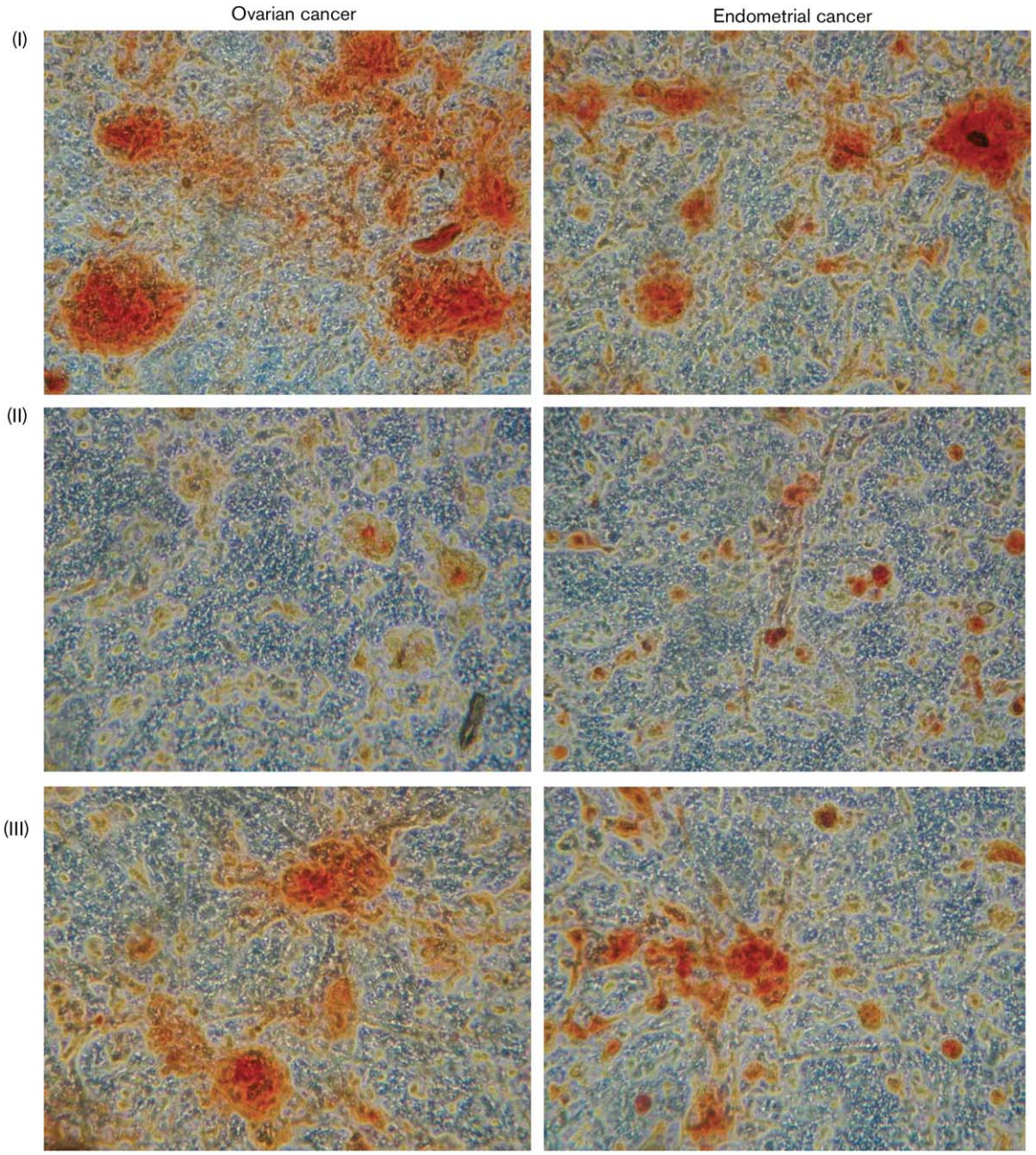
Materials and methods

Patients

Twenty-six patients with ovarian cancer, 29 patients with cervical cancer and 31 patients with endometrial cancer were studied. All patients had primary disease and were treated at the Department of Obstetrics and Gynecology of Hiroshima University Hospital between April 2002 and December 2003, and all of them gave informed consent. Among these 86 patients included, two or three samples per patient for the CD-DST were obtained by punch biopsy or uterine curettage in 11 patients with cervical cancer and five patients with endometrial cancer.

Fig. 1



Representative results of CD-DST. (I) Control. The morphology of ovarian and endometrial cancer cells grown in collagen gel droplets on day 7. Cancer cell colonies were densely stained by neutral red. Fibroblasts showed weak staining by neutral red. (II) Sensitive. The *in vitro* sensitivity was expressed as the percentage T/C ratio, where T was the total volume of the treated group and C was the total volume of the control group. A T/C ratio of 50% or less was defined as sensitive *in vitro*. T/C ratio = 33.8% in ovarian cancer and T/C ratio = 37.8% in endometrial cancer at the concentration of 1.0 μg/ml for cisplatin. (III) Resistant. T/C ratio = 94.7% in ovarian cancer and T/C ratio = 78.6% in endometrial cancer at the concentration of 1.0 μg/ml for cisplatin.

Drugs

Cisplatin, carboplatin, paclitaxel (TXL) and docetaxel (TXT) were purchased from Bristol-Myers Squibb (Tokyo, Japan) and Aventis Pharma (Tokyo, Japan).

CD-DST

The test was performed with a CD-DST kit provided by Nitta Gelatin (Osaka, Japan), according to the method previously described [17].

In brief, each fresh surgical specimen was finely minced using a scalpel, suspended in HBSS and then treated with EZ Dispersion Enzyme Cocktail (containing 1.0% collagenase; Nitta Gelatin) and digested at 37°C for 2 h. The dispersed cancer cells were collected by centrifugation at 900 *g* for 3 min, filtered through a 80-μm nylon mesh, washed in HBSS, suspended in PCM-1 medium (Nitta Gelatin) and then incubated in a collagen gel-coated flask (CG-flask; Nitta Gelatin) in a CO_2 incubator at 37°C for 24 h. Then the collagen gel in the flask was dissolved by the EZ Dispersion Enzyme Cocktail and the viable cells adherent to the gel were collected for the sensitivity test.

Type I collagen (Cellmatrix Type CD; Nitta Gelatin), 10 × F-12 medium and reconstitution buffer were mixed together in ice water in a ratio of 8:1:1. The prepared tumor cell suspension was added to the collagen solution so that the former did not exceed 1/10th of the volume of the latter and the final density was 1×10^5 cells/ml. Three drops of this collagen–cell mixture (30 μl/drop) were placed in each well of a six-well multiplate on ice and allowed to set at 37°C in a CO_2 incubator. As a result, the final concentration was about 3×10^3 cells per collagen gel droplet. DF medium (3 ml; Nissui Pharmaceutical, Tokyo, Japan) containing 10% fetal bovine serum (FBS; Gibco, Gaithersburg, MD) was overlaid on each well 1 h later and incubation was performed overnight in a CO_2 incubator at 37°C.

Anti-cancer drugs were added at the following concentrations: 10.0, 2.0, 1.0, 0.5 and 0.2 μg/ml for cisplatin, 100, 20, 10, 5.0 and 2.0 μg/ml for carboplatin, 10.0, 1.0, 0.1 and 0.01 μg/ml for TXL, and 1.0, 0.1, 0.01 and 0.001 μg/ml for TXT. Then incubation was performed for 24 h. After removal of medium containing the anti-cancer drug, each well was rinsed twice with 3 ml of HBSS, overlaid with 4 ml of PCM-2 medium (serum-free medium; Nitta Gelatin) and incubated for a further 7 days. On the fourth day of the incubation, the medium was changed once. At the end of incubation, neutral red was added to each well for 2 h at a final concentration of 50 μg/ml to stain colonies in the collagen gel droplets. Then the collagen droplets were fixed with 10% neutral formalin buffer, washed in water, air dried and subjected to imaging analysis [18]. The growth rate of control cultures was calculated as the total volume on day 7 divided by the total volume on day 0.

We calculated the T/C ratio, where T was the total volume of the treated culture and C was the total volume of the control culture, at various concentrations for each anti-cancer drug. We also calculated the percentage of cultures with a T/C ratio of 50% or less, which were defined as sensitive *in vitro*, and the efficacy rate (%) was defined as the number of cultures with a T/C ratio of 50% or less divided by the total number of evaluable cultures. Then the efficacy rates at various concentrations were displayed on the vertical axis of a graph versus the concentration of an anti-cancer drug on the horizontal axis, and the linear regression equation was obtained ($y = ax + b$). Next, the known clinical response rates to anti-cancer drugs were substituted for y and the obtained x values were used as approximations (cut-off values) for clinically appropriate concentrations.

Clinical responses were assessed according to WHO criteria, with patients who showed a complete response (CR) or a partial response (PR) being regarded as responders. *In vitro* sensitive in this assay treated with one agent was defined as clinical response (CR + PR) and *in vitro* non-sensitive treated with an agent shown to be ineffective by the assay was defined as clinical non-response (NC + PD).

Statistical analysis

The results of the CD-DST were compared between responders and non-responders to chemotherapy by Student's *t*-test and the χ^2-test. The relationship between the *in vitro* drug-sensitivity rate and the clinical response rate was analyzed by Pearson's correlation coefficient. $p < 0.05$ was considered significant for these analysis.

Results

Evaluability of tumors by the CD-DST

The overall tumor evaluation rate was found to be 79.1%, being 76.9% for 26 ovarian cancers, 75.9% for 29 cervical cancers and 83.9% for 31 endometrial cancers (Table 1). The reasons for failure were an insufficient number of viable tumor cells (less than 1×10^5 at the start of assay) and poor growth of control cells. Evaluation failed for six ovarian cancers, seven cervical cancers and five endometrial cancers, and there were no significant differences in evaluability between these cancers (χ^2-test).

Table 1 Evaluation rate of ovarian and uterine cancer cells in the CD-DST

	Ovary	Uterine cervix	Uterine endometrium	Total
No. of cancer tests	26	29	31	86
Evaluable cases	20	22	26	68
Evaluation rate (%)	76.9	75.9	83.9	79.1

Growth rate of primary cancer cells in collagen gel droplets

The mean growth rate of ovarian, cervical and endometrial cancer over 7 days of incubation was 2.30 ± 2.20, 4.32 ± 5.09 and 2.99 ± 3.44, respectively (Table 2).

The morphology of ovarian and endometrial cancer cells grown in collagen gel droplets is shown in Fig. 1. In Fig. 1(I), ovarian cancer cells and endometrial cancer cells grew in the collagen droplets to form three-dimensional spheres. The cells maintained their *in vivo*

Fig. 2

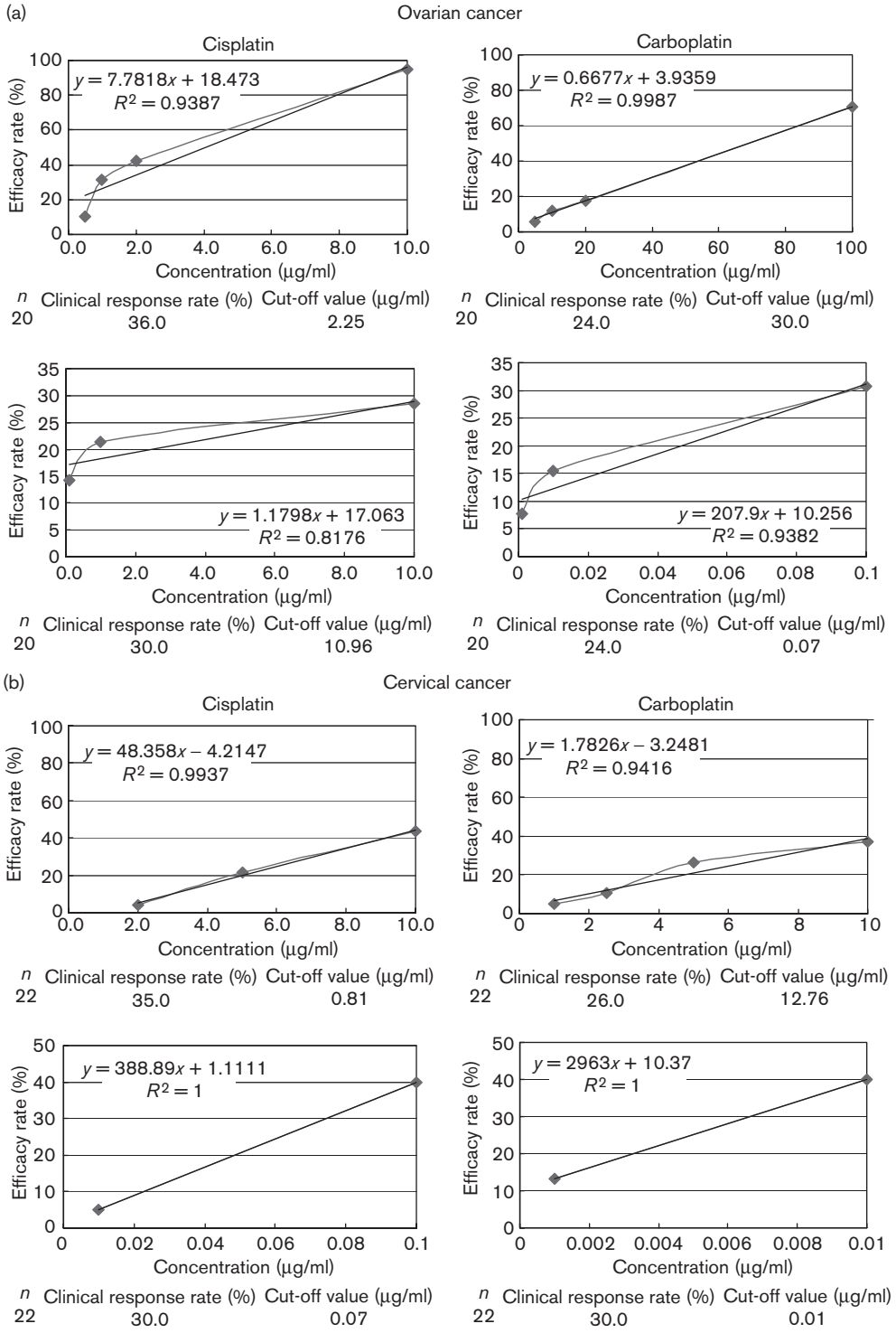
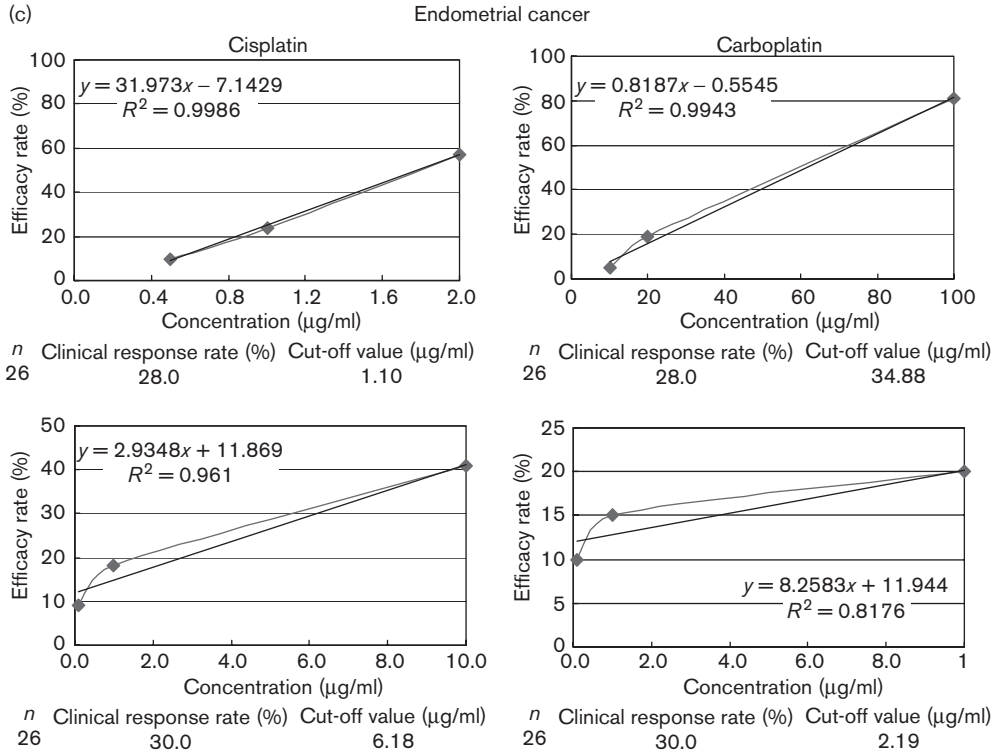


Fig. 2 *Continued*



Appropriate concentrations of anti-cancer drugs. The efficacy rate (%) was defined as the total number of T/C ratios of 50% or less divided by the total number of evaluable cultures. The efficacy rates at various concentrations were displayed on the vertical axis of a graph versus the concentration of an anti-cancer drug on the horizontal axis and the linear regression equation was obtained ($y=ax+b$). The known clinical response rates were substituted for y and the obtained x values were used as appropriate concentrations (cut-off values).

Table 2 Growth rate of primary cancer cells in collagen gel droplets

	Organ		
	Ovary ($n=20$)	Uterine cervix ($n=22$)	Uterine endometrium ($n=26$)
Growth rate[a] (mean ± SD)	2.30 ± 2.20	4.32 ± 5.09	2.99 ± 3.44

[a]Growth rate = total volume on day 7/total volume on day 0.

characteristics and were densely stained by neutral red. On the other hand, fibroblasts showed bipolar extension and weak staining by neutral red in the control culture on day 7. In Fig. 1(II and III), sensitive and resistant cases are shown in the treated culture containing 1.0 μg/ml for cisplatin.

Appropriate concentrations of anti-cancer drugs

The efficacy rates at various concentrations of each anti-cancer drug are shown in Fig. 2 for ovarian, cervical and endometrial cancer, while the already published clinical response rates for each anti-cancer drug are shown in Table 3 [19–31]. Using the obtained linear regression equations ($y = ax + b$) for various cancer specimens, the known clinical response rates to various anti-cancer drugs for gynecological cancers were substituted for y and the appropriate drug concentrations obtained as x values (cut-off value) were selected as 1.0 μg/ml for cisplatin, 20 μg/ml for carboplatin, 1.0 μg/ml for TXL and 0.1 μg/ml for TXT.

In vitro drug sensitivity

The *in vitro* sensitivity data are shown in Table 3. The *in vitro* sensitivity of the individual drugs showed a significant correlation with the clinical response rates according to WHO criteria (Fig. 3, $r = 0.592$. $p = 0.0211$). The *in vitro* sensitivity of ovarian cancer to cisplatin was higher than that for carboplatin, TXL and TXT, but there were no significant differences between these drugs. There were also no significant differences between the response of cervical and endometrial cancer to cisplatin, carboplatin, TXL and TXT. Comparison between the different organs also showed no statistically significant differences.

Discussion

Prediction of the response to chemotherapy is very useful to establish individualized treatment for patients with

Table 3 ***In vitro*** **drug sensitivity and clinical response rates of the individual drugs**

Organ/sensitivity	*N*	Cisplatin	Carboplatin	TXL	TXT
Ovary	20				
sensitivity rate (%)		40.0 (8/20)	25.0 (0/20)	30.0 (6/20)	20.0 (4/20)
clinical response [19–21] (range)		36.0 (25.0–53.8)	24.0 (24.0–38.4)	36.0 (29.8–35.0)	36.0 (24.0–30.0)
Uterine cervix	22				
sensitivity rate (%)		36.4 (8/22)	36.4 (8/22)	45.5 (10/22)	31.8 (7/22)
clinical response [22–28] (range)		35.0 (23.0–44.0)	26.0 (15.0–28.0)	30.0 (17.3–31.0)	30.0 (12.5–34.3)
Uterine endometrium	26				
sensitivity rate (%)		26.9 (7/26)	23.1 (6/26)	30.8 (8/26)	23.1 (6/26)
clinical response [29–31] (range)		28.0 (28.0–32.0)	28.0 (28.0–30.0)	30.0 (30.0–35.7)	30.0 (none)

ovarian cancer and uterine cancer. In this study, we investigated the clinical value of the CD-DST as an *in vitro* chemosensitivity test for gynecological cancers.

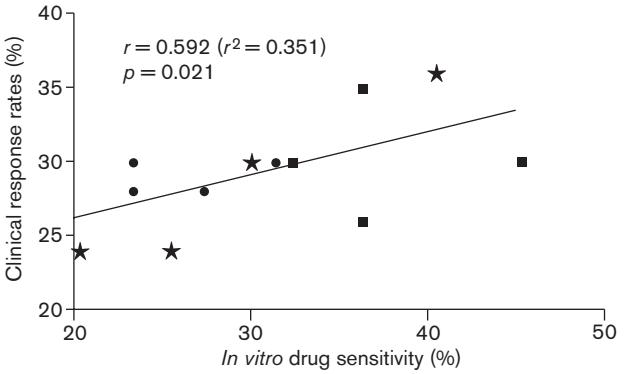
We obtained a higher success rate using the CD-DST (79.1%) in ovarian and uterine cancers compared with other *in vitro* chemosensitivity tests (35.2–97%) [9,16,32,33]. The original CD-DST method required 5×10^4 cells/well [34], while the number was reduced to 5×10^3 cells/well in the present assay. Even though the number of biopsy specimens available was small, the CD-DST method was still feasible. This is an advantage over the HTCA or TIA, which both require 5×10^5 cells/well. In our study, tissue samples were obtained by punch biopsy or uterine curettage rather than by open surgical biopsy, in 11 patients with cervical cancer and five patients with endometrial cancer.

Growth of the cancer cells was good in the collagen gel droplets (Fig. 1), being significantly higher than the growth rates reported previously in soft agar or capillary cloning systems [35]. The CD-DST method allowed prolonged stable primary culture when compared to monolayer culture. The collagen gel matrix method has also been reported by Freeman *et al.* [36] and others [36–38] as another three-dimensional culture technique. They placed ultrathin sections of tumor tissue on gelatin sponge, which achieved favorable growth during long-term culture. Hoffman *et al.* [39] and Furukawa *et al.* [14] established the HDRA using this culture method. However, the HDRA requires high anti-cancer drug concentrations, approximately 20–30 to several hundred times of the area under the curve (AUC) observed *in vivo*. In contrast, the CD-DST method uses concentrations similar to *in vivo* drug levels, thus ensuring that the mode of action is similar [40].

Another significant advantage of the CD-DST is that imaging analysis can be done to differentiate cancer cells from fibroblasts in the collagen gel droplets [18].

In the present study, the known clinical response rates to anti-cancer drugs were substituted for y by using the obtained linear regression equation ($y = ax + b$) (Fig. 2) and we selected the appropriate concentrations of anti-cancer drugs as 1.0 μg/ml for cisplatin, 20 μg/ml for carboplatin, 1.0 μg/ml for TXL and 0.1 μg/ml for TXT in the case of ovarian and uterine cancer. The *in vitro* sensitivity of tumors for each drug also showed a significant correlation with the already published clinical response rates for each anti-cancer drug in gynecological cancers ($r = 0.592$, $p = 0.021$) (Fig. 3).

Fig. 3



Correlation with the *in vitro* drug sensitivity of the individual drugs and the clinical response rates: stars, ovarian cancer; squares, cervical cancer; circles, endometrial cancer.

As a result, the CD-DST method as well as modern non-clonogenic assays like the ATP assay [41] overcome the previous clonogenic technical and theoretical limitations of chemosensitivity testing. Since *in vitro* sensitivity to various anti-cancer drugs was similar to the clinical response rate, the CD-DST method seems to be clinically useful for prediction of the response of ovarian and uterine cancer to chemotherapy.

In conclusion, the CD-DST can be used to predict the response to cisplatin, carboplatin TXL and TXT, and may also provide important information by contributing to the development of new chemotherapy regimens. A future study on the efficacy of drug combinations and a large number of patient's predictions and prognoses is needed to confirm the clinical utility of the CD-DST.

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