Applicability of Histoculture Drug Response Assays in Colorectal Cancer Chemotherapy

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Abstract. *Aim: The present study, using the histoculture drug response assay (HDRA) compared chemosensitivity with the clinical response of a treatment regime in patients with advanced colorectal cancer (CRC). Patients and Methods: A total of 324 patients with primary CRC were prospectively enrolled. HDRAs were performed using seven combinations of anticancer drugs, including 5-fluorouracil with leucovorin (FL), FL with oxaliplatin (FOLFOX), irinotecan (FOLFIRI), and their combinations with bevacizumab and cetuximab. Results: Among 324 HDRA results, tumor inhibition rates of regimes using FOLFOX (34.2-39.2%) were higher than those using FOLFIRI (24.2-32.7%, p<0.001). Out of 86 evaluated chemotherapeutic regimes, the correlation rate of HDRA to the clinical effect of chemotherapy was calculated to be 66.3% (57/86), with a 72.7% (40/55) sensitivity and a 54.7% (17/31) specificity. Conclusion: HDRA might be a feasible and useful technique for predicting therapy efficacy and selecting the appropriate anticancer regime for individual patients, notwithstanding its low accuracy.*

One of the major goals in cancer therapeutics is to increase the efficacy and reduce the toxicity by tailoring therapy to the needs of individual patients. Chemosensitivity and drug resistance studies use autologous viable tumors to evaluate susceptibility to specific agents *in vitro* and predict direct effects on that individual patient's tumor. Human tumor cloning assays (HTCAs), methyl thiazolyl-diphenyl-tetrazolium bromide (MTT) assays, and adenosine triphosphate (ATP) bioluminescence assays are well-known *in vitro* chemosensitivity and drug resistance assays (1-3). Among these assays, the histoculture drug response assay (HDRA) is a representative MTT assay, which has the advantage of more correctly reflecting the *in vivo* microenvironment (4, 5). Several clinical studies involving colorectal and gastric cancer have shown that inhibition rates obtained using HDRA can predict clinical responses to chemotherapy (6-10).

Anticancer drugs and established regimens in the adjuvant and palliative chemotherapy of advanced colorectal cancer (CRC) (11) are limited to 5-fluorouracil (5-FU) with leucovorin (FL), FL with oxaliplatin (FOLFOX) or irinotecan (FOLFIRI), and more recently, the use of targeted agents (cetuximab and bevacizumab). Combination chemotherapy including FOLFOX and FOLFIRI showed less than 50% efficacy in metastatic CRC (12). Few studies have evaluated chemotherapy and sensitivity assays in the context of CRC (7, 13). Therefore, we investigated the chemosensitivity to clinically used regimens using HDRA of samples from patients with advanced CRC, and compared their chemosensitivity with the observed clinical response.

Patients and Methods

Patients and tissue samples. A total of 324 patients with primary CRC were prospectively enrolled. All patients provided written informed consent and underwent surgery between December 2008 and May 2011 at the Asan Medical Center (Seoul, Korea). The baseline demographic and clinicopathological characteristics are listed in Table I. Eligibility criteria included histologically-proven colorectal adenocarcinoma, Eastern Cooperative Oncology Group performance status of 0 or 1, age of 75 years or less, and expectation of adjuvant or palliative chemotherapy. Patients were excluded if they had hereditary non-polyposis colorectal cancer and familial adenomatous polyposis, or had undergone preoperative chemotherapy or radiotherapy. Chemotherapeutic drugs were selected by oncologists with knowledge of the results of HDRA. The study protocol was approved by the Institutional Review Board of Asan Medical Center (Registration No: 2007-9009), in accordance with the Declaration of Helsinki.

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Table I. *Baseline demographic and clinicopathological characteristics.*

Characteristic	Total, n=324 (%)	Correlated pts, n=59 (%)	*p*-Value
Sex, male	203 (62.7)	39 (66)	0.656
Age, years	58.7±9.5	56.5±10.1	0.689
Preoperative serum CEA, >6 ng/ml	92 (28)	29 (49)	<0.001
Stage, I/II/III/IV	11/132/109/72	0/2/10/47	<0.001
Location, rectum	115 (35)	23 (39)	0.550
Differentiation, PD+mucinous	37 (11)	10 (17)	0.172
Lymphovascular invasion, yes	103 (32)	25 (42)	<0.001
Operation, palliative	43 (13)	37 (63)	<0.001

CEA, Carcinoembryonic antigen; PD, poorly differentiated. Cancer staging was performed based on the American Joint Committee on Cancer guidelines. In: AJCC Cancer Staging Manual, 6th edition (Greene FL, Page DL, Fleming ID, Fritz AG, Balch CM, Haller DG, and Morrow M eds.). New York, Springer, 2002.

Clinical correlation of HDRA. Patients with recurrent or remaining measurable tumor lesions were eligible for evaluation of the assay results by comparison to the clinical effects of chemotherapy. Among 324 patients, clinical correlation of HDRA was possible for 59 patients with palliative surgery (n=37), and with recurrent CRC (n=22). Out of these patients, 22 underwent more than two regimens of chemotherapy due to disease progression. Finally, 86 regimens of chemotherapy for 59 patients were analyzed for correlation with the HDRA results (Table I).

Tumor response evaluation. Treatment responses were assessed every three or four chemotherapy cycles by using consistent imaging techniques such as computed tomography (CT) and magnetic resonance imaging (MRI). According to the Response Evaluation Criteria in Solid Tumors (RECIST) (14), complete response (CR) was defined as the disappearance of all metastatic lesions, whereas a partial response (PR) was defined as a reduction of at least 30% of the sum of the longest diameter of metastatic lesions, with no evidence of new lesions. Progressive disease (PD) was defined as an increase of at least 20% of the sum of the longest diameters of either metastatic or recurrent lesions. Stable disease (SD) was indicated when neither a sufficient shrinkage to qualify for PR nor a sufficient increase to qualify for PD occurred. The primary endpoint of this study was a correlation between the HDRA results and the clinical response in the disease control rate (DCR). This was defined as positive for the effect of chemotherapy for tumor responses between CR and SD.

Investigated drug combinations and concentrations. The tested regimens included FL, FOLFOX, FOLFIRI, and combinations with biologically-targeted drugs. The targeted drugs used were bevacizumab (Avastin®; Roche, Basel, Switzerland) and cetuximab (Erbitux®; Merck, Munchen, Germany), FOLFOX with bevacizumab (FOLFOXA) and cetuximab (FOLFOXE), and FOLFIRI with bevacizumab (FOLFIRIA) and cetuximab (FOLFIRIE). The cut-off concentration of the five anticancer drugs used to determine the *in vitro* sensitivity and resistance was 50 μg/ml for 5-FU, 10 μg/ml for leucovorin, 40 μg/ml for oxaliplatin, 20 μg/ml for bevacizumab, and 20 μg/ml for cetuximab.

Histoculture drug response assay. Three sections of tumor tissues (0.5 cm in diameter) were freshly-harvested from surgically resected specimens, excluding the necrotic or non-viable portions. Tumor samples were aseptically washed in Hank's balanced salt solution (HBSS; Gibco, Gaithersburg, MD, USA), and HDRA was performed (15). Cancerous portions of the specimens were minced into pieces approximately 1 mm in diameter. Cancer tissues were further cut into 10-mg pieces, weighed on a chemical balance, and placed onto collagen gels (Gel Foam®; Pharmacia & Upjohn, Kalamazoo, MI, USA), immersed in 1 ml Roswell Park Memorial Institute 1640 (RPMI-1640) medium (Sigma, St. Louis, MO, USA) supplemented with 20% fetal calf serum and anticancer drugs in a 24-well plate. Six and four replicates were concurrently run for the control and treatment groups, respectively. After incubation for 72 h at 37˚C with 5% CO_2, 100 μl of 0.06% collagenase type I (Sigma) in HBSS and 0.2% MTT (Sigma) in PBS containing 50 mM sodium succinate (Wako Ind., Tokyo, Japan) were added to each well. Plates were incubated for another 4 h, the media were removed, and 0.5 ml dimethyl sulfoxide were added to each well to extract MTT formazan. Extracts from each well (100 μl) were transferred to a 96-well plate and absorbance was measured at 540 nm using a microplate reader (VersaMax, Sunnyvale, CA, USA). Samples with contamination or absorbance values of less than 15/g of control tumor tissue were classified as 'inappropriate'. The inhibition rate of tumor growth (IR) was calculated using the following equation: IR (%) = (1 − mean absorbance of treated wells per gram of tumor/mean absorbance of control wells per gram of tumor) ×100. In our study, the IR cut-off value for a positive response was previously determined to be ≥30% (16, 17).

Statistical considerations. A cross-table analysis employing Pearson's chi-square test or Fisher's exact test, as appropriate, was used to compare categorical variables. A *p*-value of 0.05 was considered statistically significant for all analyses, and all calculations were carried out using the SPSS software (version 13.0; SPSS, Chicago, IL, USA).

Results

Tumor growth inhibition ratios and sensitivity of HDRA. Among 324 patients, tumor growth IR with regimens was the most significant for FOLFOXE, followed by FOLFOXA, FOLFOX, FOLFIRIE, FOLFIRIA, FOLFIRI, and FL. Significant differences were evident between FOLFOX and FOLFIRI, and FL ($p<0.001$, respectively; Figure 1A). The percentage of chemosensitive tumors was also highest for FOLFOXE, followed by FOLFOX, FOLFOXA, FOLFIRIE, FOLFIRIA, FOLFIRI, and FL (Figure 1B). There was no significant difference in tumor growth IR and percentage of chemosensitive tumors according to tumor location or stage.

Response of chemotherapy. Out of 86 evaluated chemotherapy modalities, all seven combinations of drugs were evaluated. In treatment responses, CR was 4% (n=3), PR was 33% (n=28), SD was 28% (n=24), and PD was 36% (n=31). Overall response rate and disease control rate were 38% and 64%, respectively. With further use of chemotherapy, response rate (RR) and DCR decreased (Table II).

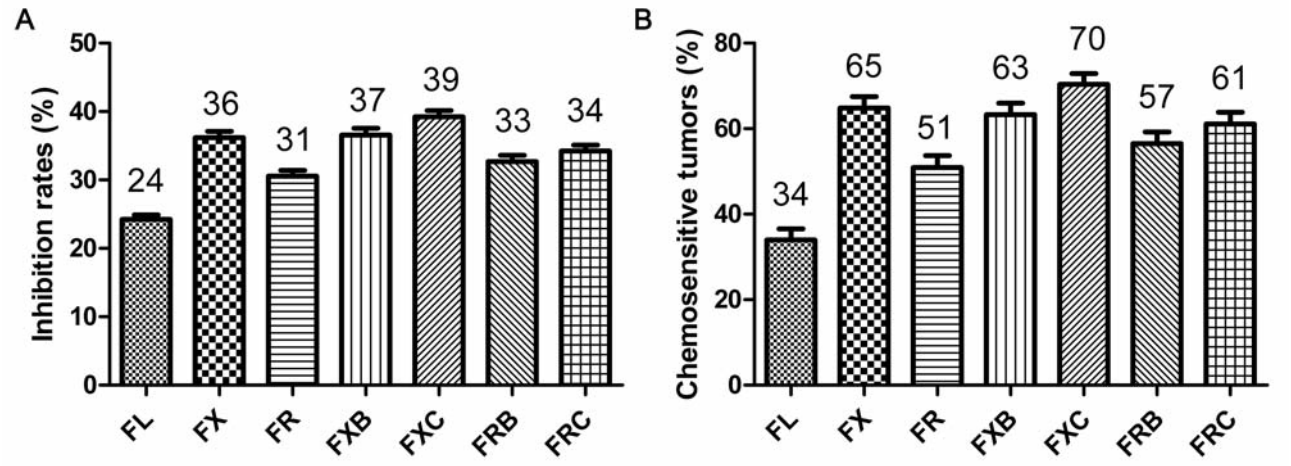

Figure 1. *A: Tumor cell inhibition rates of established regimens, alone and in combination with targeted agents. B: Proportions of chemosensitive tumors according to regimens. Inhibition rate and chemosensitivity of combination regimen of 5-fluorouracil, leucovorin, and oxaliplatin (FOLFOX) alone or combined with targeted agents, showed significantly greater effects than those of other regimens. FL, 5-Fluorouracil+leucovorin; FX, FL+oxaliplatin; FR, FL+irinotecan; B, bevacizumab; C, cetuximab. Data represent the means±SEM.*

Clinical correlations. In total, there were 14 false-positive, 15 false-negative, and 57 true-positive cases, giving a correlation rate of the HDRA to the clinical effect of chemotherapy of 66.3% (57/86), with 74.1% (40/54) true-positive and 53.1% (15/32) true-negative rates, and 72.7% (40/55) sensitivity and 54.7% (17/31) specificity (p=0.019). According to sequences of treatment, the correlation rate of first-line treatment was 67.6%, with 83.3% (20/24) true-positives and 30% (3/10) true-negatives. The correlation rate of HDRA to response after first-line treatment was 65.4%, with 66.7% (20/30) true-positives and 63.6% (14/22) true-negatives (p=0.048). In terms of chemotherapy regimen, the correlation rates of FOLFIRI regimens were higher than those of FOLFOX regimens and of regimens including targeted agents, which were not significant (Table III). When the RR was set to positive for effect of chemotherapy response, the correlation rate of HDRA for all chemotherapies decreased to 54.7% (47/86), with 42.6% (23/54) true-positives and 75.0% (24/32) true-negatives.

Table II. *Chemotherapy regimens and treatment responses according to chemotherapy timing.*

	Total, n=86 (%)	1st-line, n=34	2nd-line, n=40	3rd-line, n=10	4th-line, n=2
Chemotherapy					
FL	4 (5)	2	2	0	0
FOLFOX	28 (33)	12	15	1	0
FOLFIRI	37 (43)	17	17	3	0
FOLFOX+B	6 (7)	1	4	1	0
FOLFIRI+C	3 (4)	1	1	1	0
FOLFOX+B	1 (1)	1	0	0	0
FOLFIRI+C	7 (8)	0	1	4	2
Treatment response					
CR	3 (4)	0	3	0	0
PR	28 (33)	17	10	1	0
SD	24 (28)	10	11	1	2
PD	31 (36)	7	16	8	0
RR	38%	50%	33%	10%	0%
DCR	64%	79%	60%	20%	20%

FL, 5-Fluorouracil+leucovorin; FOLFOX, FL+oxaliplatin; FOLFIRI, FL+irinotecan; B, bevacizumab; C, cetuximab; CR, complete response; PR, partial response; SD, stable disease; PD, progressive disease; RR, response rate; DCR, disease control rate.

Discussion

To predict accurate responses to drugs, we chose the HDRA method among various *in vitro* assays. In the present study, the HDRA used three-dimensional (3-D) multicellular spheroids in tissue culture, which was an improvement from previous two-dimensional (2-D) monolayer culture methods. Our 3-D HDRA has the advantage of more accurately reflecting the *in vivo* microenvironment and the drug response of tumor cells, with maintenance of cell–cell and cell–extracellular matrix interactions compared to the 2-D method (4, 5). However, the accuracy for clinical correlation rate of our HDRA was relatively lower than those of previous reports, with 74-92.1% evaluated in head and neck, gastric, and colorectal cancer (6, 7, 10). Another well-known *in vitro* drug response assay is the ATP-based chemotherapy response assay (ATP-CRA), which has shown 68.8-90.0% accuracy in lung, ovary, and breast cancer (18-20). Moreover, the accuracy of the correlation of RRs was lower than those

Table III. *Clinical correlation of histoculture drug response assay (HDRA) results and clinical effects of chemotherapy according to treatment sequence and regimen.*

		Clinical drug response			True-positive or -negative rate (%)	Correlation rate (%)	
In vitro drug response		Positive	Negative	Total			*p*-Value
According to sequence							
1st - line (n=34)	Positive	20	4	24	83.3	67.6	0.394
	Negative	7	3	10	30.0		
≥2nd - line (n=52)	Positive	20	10	30	66.7	65.4	0.048
	Negative	8	14	22	63.6		
According to regimen							
FOLFOX (n=28)	Positive	13	7	20	65.0	64.3	0.231
	Negative	3	5	8	62.5		
FOLFIRI (n=37)	Positive	17	3	20	85	70.3	0.032
	Negative	8	9	17	52.9		
Targeted agent	Positive	7	3	10	70	58.8	0.644
(n=17)	Negative	4	3	7	42.9		

FOLFOX, 5-Fluorouracil+leucovorin+oxaliplatin; FOLFIRI, 5-fluorouracil+leucovorin +irinotecan.

of the DCRs in this study. Because of the relatively low correlation rate, it might be difficult to apply HDRA in current clinical practice. In 2004, the American Society of Clinical Oncology (ASCO) stated that the use of *in vitro* drug response assays to select chemotherapeutic agents for individual patients is not recommended outside of a clinical trial setting (21). Until 2011, ASCO guidelines had not changed because the evidence was not sufficient to support use of such assays in clinical practice (3).

There is a possible explanation for the low correlation rate of HDRA, implicating the complexity of anticancer regimens. In *in vitro* assays, drug responses and interactions of multiple drugs might be less predictable than those of a single drug. Use of combinations of anticancer drugs might contribute to complexity in the different interactions among these drugs, resulting in a poor correlation with the clinical outcome. Another possible reason for the low correlation rate is the low drug concentration and low cut-off value of the IR. We used 40 μg/ml of 5-FU, which was lower than previous reports (300 μg/ml), and a 30% cut-off value for IR, which was also lower than previous reports (40-60%) (7-10). Generally, low drug concentrations or low cut-off values of IR have the advantage of detecting a more sensitive drug rather than a drug which could produce more resistance (22). Thus, the frequent low true-negative rates of our results might reduce overall correlation rates.

Previous studies of *in vitro* drug response assays analyzed chemosensitivity and drug resistance using a single drug (6, 7, 10). However, combinations of drugs are usually used in clinical settings, particularly for patients with advanced or metastatic CRC. The application of targeted agents, including cetuximab and bevacizumab, is rapidly increasing. With this in mind, we designed seven combinations of anticancer drugs currently used in clinical settings. The results of this study have the advantages of easy applicability to clinical practice and of a wide choice of chemotherapy regimens among various possible combinations of drugs. To our knowledge, there is no previously reported *in vitro* correlation study that showed drug responses of established regimens. The present investigation showed the characteristically higher chemosensitivity of FOLFOX regimen than those of FOLFIRI or FL regimens. In clinical practice, response rates to FOLFOX and FOLFIRI regimens were also higher than those to FL regimen in the adjuvant or palliative setting of CRC chemotherapy, which was similar to our findings (12).

It was difficult to apply HDRA results to the cases after first-line chemotherapy because surviving tumor cells after chemotherapy might obtain new traits in response to previous anticancer drugs. In terms of chemotherapy sequence, RR of first-line treatment with FOLFOX and FOLFIRI for metastatic CRC therapy was known to be 40-50%, and with FL it was approximately 20%, which decreased after first-line treatment (12). Thus, selecting chemotherapy regimens after first-line chemotherapy depends on drug resistance rather than drug sensitivity, and new regimens not previously used should be considered. As clinical correlation after first-line treatment had a similar accuracy to those of first-line treatment in preliminary analyses, we could apply HDRA results to cases after first-line treatment. To overcome and explain this theoretical limitation, we are currently investigating a new clinical trial that will determine if the integrative tumor response assay (ITRA) can identify drug responses for both first- and second-line chemotherapy.

The treatment of metastatic CRC has become increasingly complex, as treatments have changed over the past decade. During that time, treatment has evolved from single-agent 5-FU to combination chemotherapy, and more recently to the inclusion of monoclonal antibodies (11). Most patients undergone combination chemotherapy with targeted agents were involved in various other clinical trials, and the presence of a V-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog (*K-RAS*) mutation was checked in all cases, who were to undergo cetuximab treatment. We allocated four chemotherapy regimens with targeted agents among seven regimens to consider the application of this drug. Although clinical application of HDRA results for this drug was evaluated only in a small number of cases with recurrence, the efficacy of HDRA was relatively high because of the very high cost of this drug. Future approaches in guiding therapy will rely on understanding the molecular differences between tumors and patients, and in incorporation of global evaluations of genomic polymorphisms and molecular profiling of tumors. Tailoring optimal combinations of drugs to specific patients may require a combination of *in vitro* assays on viable tumor samples and molecular analyses of patient samples (23).

This study had some limitations. Firstly, the sample size was small, which is a common problem also dealt with in previous *in vitro* assays for clinical correlation (6, 7, 10, 18-20). Secondly, including cases after first-line treatment produced heterogeneity of study groups due to the drug response of the original tumor cells, which might distort results of *in vitro* drug responses after previous chemotherapy. Thirdly, this study also has a fundamental limitation related to the *in vitro* assay. That is, in tumor cell cultures there is an absence of other host-derived cells in the assay that may influence responsiveness and exhibit genomic differences that may affect the metabolism of specific compounds (24). As we used combinations of anticancer drugs, the complexity of interactions among these drugs may increase the unpredictability of the *in vitro* assay. This problem should be addressed through further development of improved culture systems.

In conclusion, HDRA might be further developed to be a feasible and useful technique for predicting therapy efficacy and selecting the appropriate anticancer regimen for individual patients despite its relatively low accuracy.

Author Contributions

Study proposal, design, analysis, writing of manuscript, and responsibility for correspondence: Jin Cheon Kim.
Study design, analysis, and writing of manuscript: Yong Sik Yoon.
Data collection and clinical association analyses: Chan Wook Kim,
Laboratory process for drug response assays: Dong Hyung Cho, Seon Ae Roh, Moon Bo Kim.
Interpretation and advice on the oncology aspects of the study: Gyu Pyo Kim, Yong Sang Hong, Tae Won Kim.

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