ANTICANCER RESEARCH *39*: 313-318 (2019)
doi:10.21873/anticanres.13113

Assessment of the Applicability of Integrative Tumor Response Assays in Advanced Epithelial Ovarian Cancer

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Abstract. *Aim: To prospectively correlate clinical responses to second-line chemotherapy for recurrent epithelial ovarian cancer (EOC) with in vitro integrative tumor-response assay (ITRA) results. Patients and Methods: Forty-four patients with advanced EOC were enrolled from 2015-2017 at the Asan Medical Center, Seoul, Korea. ITRA comprised of two sequential histoculture drug response assays (HDRAs) of the tumor tissues. The first stage was HDRA with paclitaxel–carboplatin, paclitaxel, and carboplatin chemotherapy. The second stage was performed with surviving tumor cells from the first stage using topotecan, belotecan, gemcitabine, doxorubicin, ifosfamide, vinorelbine, and etoposide. Results: The median follow-up period was 23.35 (range=4-35.35) months. Eighteen patients (40.9%) completed the second-line chemotherapy, based on the ITRA results. The objective response rate was 38.9%. The clinical response rate was 50%; two patients (11.1%) had stable disease. The sensitivity of ITRA for predicting response was 85.7% (specificity=18.2%; accuracy=44.44%). Conclusion: ITRAs had acceptable applicability and may help choose second-line chemotherapy for patients with advanced EOC.*

Epithelial ovarian cancer (EOC) is one of the most challenging and lethal gynecological malignancies (1, 2) with a 5-year survival rate below 45% (3). The current standard of care for patients with advanced EOC is debulking surgery combined with paclitaxel and carboplatin chemotherapy (4). However, although patients have a good response to the initial treatment, most tend to experience relapse and develop resistance to platinum-based chemotherapy (5-7). Since platinum plays an essential role in the standard therapy for EOC, the development of platinum resistance implies a poor prognosis (8).

Anticancer therapy for recurrent ovarian cancer (ROC) is chosen based on platinum sensitivity (9). The standard of care for patients with platinum-sensitive ROC is platinum-based chemotherapy (10). In patients with platinum-resistant ROC, non-platinum-based agents, such as pegylated liposomal doxorubicin, topotecan, gemcitabine, and etoposide are preferred, which have similar clinical efficacies (11). There is no clear guideline on which anticancer therapy should be chosen.

The histoculture drug response assay (HDRA) is a test that evaluates chemosensitivity to a given chemotherapy agent *in vitro* before treatment is initiated, using tumor tissue obtained during surgery to determine the appropriate drug of choice (12). The advantages of this assay are its short duration and drug delivery similar to the physiological condition due to the maintenance of cell-to-cell or cell-to-substrate interaction and preservation of physiological tumor cell structure during the test (12-14). The accuracy of HDRA was reported to be between 74-92.1% in cancer of the head and neck, stomach, ovaries, and colon (12-17). However, with the pre-established HDRA method, only chemosensitivity to first-line therapies can be tested. Hence, once chemoresistance develops, it is impossible to search for alternative chemotherapies. Furthermore, if cancer recurs after the initial surgery and adjuvant chemotherapy, it is then difficult to obtain cancer tissue. To improve upon this issue, at our Center, we pertinently applied an *in vitro* chemosensitive assay based on HDRA: the integrative tumor-response assay (ITRA; Patent No. 10-1046883; the Korean Intellectual Property Office, Seoul, Korea), which was developed by Kim and Moon (18). ITRA is a new *in vitro* tumor chemosensitivity assay, performed under near-physiological conditions, that tests for sensitivity not only to first-line chemotherapy agents, but also

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Key Words: Epithelial ovarian cancer, tumor response assay, chemotherapy, histoculture drug response assay.

second-line agents that can be used in cases with chemoresistance to first-line drugs, using tumor specimens obtained during the initial surgery. ITRA is theoretically based on the HDRA procedure, with methods similar to those used in previous studies at our center (15, 16).

The current study aimed to evaluate the chemosensitivity of primary EOC in patients *via* ITRA using tumor tissue and to examine the real-world clinical outcomes.

Patients and Methods

Patients. This study was conducted with approval of the Institutional Review Board of Asan Medical Center (IRB number: 2012-0222). Forty-four patients with EOC were prospectively enrolled between March 2015 and December 2017. Informed consent was obtained from all patients. We included patients for whom we expected to use adjuvant chemotherapy during surgery, those with a histological diagnosis of epithelial carcinoma of the ovaries after primary cytoreductive surgery, those with International Federation of Obstetrics and Gynaecology (FIGO) stage IIIA to IVb cancer postoperatively, those with an Eastern Cooperative Oncology performance status ≤2, and ≤80 years old. Patients with a history of chemotherapy or radiotherapy preoperatively were excluded.

Primary cytoreductive surgery. All patients underwent complete staging surgery as the initial treatment. The complete staging surgeries included total abdominal hysterectomy, bilateral salphingo-oophorectomy, total omentectomy, pelvic and para-aortic lymphadenectomy, and tumorectomy of metastatic lesions. In cases with confirmed tumor invasion, resection surgery of tumor-invaded organs, such as colectomy, splenectomy, or liver segmentectomy, was also performed.

Chemotherapy selection. All patients received adjuvant chemotherapy with paclitaxel and carboplatin postoperatively. If combination therapy was unsuitable because of severe side-effects, underlying disease, or advanced age, single-agent paclitaxel or carboplatin therapy was given. After the initial chemotherapy cycle, patients with a platinum-free interval (PFI) of ≥12 months were considered to have platinum-sensitive recurrence; those with PFI ≥6 months but ≤12 months had partially platinum-sensitive recurrence, and those with PFI <6 months had platinum-resistant recurrence (19). Paclitaxel or carboplatin was chosen as the second-line chemotherapy for patients with platinum-sensitive recurrence. The main chemotherapy regimen was chosen based on ITRA results in patients with partially platinum-sensitive disease or those with platinum-resistant recurrent lesions or disease progression during first-line chemotherapy. For second-line chemotherapy, drugs with sensitivity higher than the inhibition rate of first-line chemotherapy as per the ITRA results were chosen, considering the patient's underlying disease and side-effects.

All 44 patients in this study underwent primary cytoreductive surgery. Tumor specimens were collected intraoperatively for ITRA. All patients underwent adjuvant chemotherapy: 43 with paclitaxel and carboplatin and one with carboplatin only because of hypersensitivity to paclitaxel. The disease progressed in 11 patients during first-line chemotherapy cycle, and one refused to participate further. Fifteen patients showed recurrence after initial chemotherapy, five of whom had platinum-sensitive recurrence. Ten patients had partially platinum-sensitive or platinum-resistant recurrence, two of whom refused to participate further and were lost to follow-up. Consequently, 18 patients underwent second-line chemotherapy based on the ITRA results (Figure 1).

ITRA procedure. *(i) Tissue preparation:* Approximately 200 mg of tumor tissue was obtained to confirm EOC on frozen biopsies; tissue was washed three times in 1-2% povidone-iodine (Besetine Solution, Hyundai Pharmaceuticals, Cheonan, Republic of Korea) and saline solution. The tissue was transported to our laboratory in tissue transport media (Hanks's balanced salt solution, 2% sodium bicarbonate, 10% fetal bovine serum (FBS), 1% penicillin-streptomycin, and 0.4% gentamycin) at 4˚C.

The above tissue was then washed three times or more in phosphate-buffered saline (PBS), 1% penicillin-streptomycin, and 0.4% gentamycin. A sterile tissue clamp and scalpel were used to dissect away all non-cancerous parts. The remaining tissue was moved to a new Petri dish and cut into 0.5-1.0 mm^3 pieces. Chemosensitivity was maintained under near physiological conditions by preserving the three-dimensional structure of the tissue rather than separating the cells.

The resected tissue was washed twice in an antibacterial culture solution [RPMI 1640 medium (Gibco, Grand Island, NY, USA), 2% sodium bicarbonate, 10% FBS, 1% penicillin-streptomycin, 0.4% gentamycin, 1% tetracycline, and 10% chloramphenicol (Carl Roth, Karlsruhe, Germany), 1× tetrazolium salt (MTS; Promega, Madison, WI, USA)] and cultured in a CO_2 incubator for 2 hours at 37˚C. The surviving tissue was then stained with tetrazolium salt (MTS; Promega, Madison, WI, USA), preventing non-specific drug reactivity to dead cells. The stained and selected tissues were moved to a Petri dish and cultured under CO_2 for 24 hours at 37˚C. Destained tissue samples of equal size were placed in a 96-well plate (TPP, Trasadingen, Switzerland) (Figure 2).

(ii) ITRA first-line chemotherapy: The first-line chemotherapy agent was instilled in the wells of primary and secondary reaction plates, and PBS, which was used to wash tissue, was instilled in the control well. The samples were cultured for 72 hours. All chemotherapy agents were refrigerated, and drugs to be used were prepared in advance according to their combinations.

(iii) Analysis of first-line chemotherapy well and instillation of a second-line chemotherapy agent: The culture solution instilled with the first-line chemotherapy agent was discarded, and 100 μl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT; Sigma, St. Louis, MO, USA) was instilled in the primary wells and cultured in a CO2 incubator for 3-4 hours at 37˚C. The reaction plates in which the second-line chemotherapy agent was to be deposited were treated with an additional secondary chemotherapy agent and cultured for 48 hours. The culture solution treated with MTT in the first-line chemotherapy reaction plate was discarded, and 100 μl of dimethyl sulfoxide (Amresco, Solon, OH, USA) was added, which was shaken for 1-2 h for destaining. The destained solution was moved to a new 96-well plate and analyzed with an absorption spectrometer (VersaMax, Sunnyvale, CA, USA) at measurement and reference wavelengths of 570 and 630 nm respectively.

(iv) Analysis of the second-line chemotherapy well: The reaction plates treated with the second-line chemotherapy agent were destained, followed by analysis with absorption spectrometry. Inhibition ratio (IR) of 30% or above was deemed positive for tumor cell chemosensitivity according to the following equation:

$$IR\ (\%)=(1-T/C)\times 100$$

where T was the absorbance per gram of tumor tissue in the chemotherapy well and C was the absorbance per gram of tumor tissue in the control well.

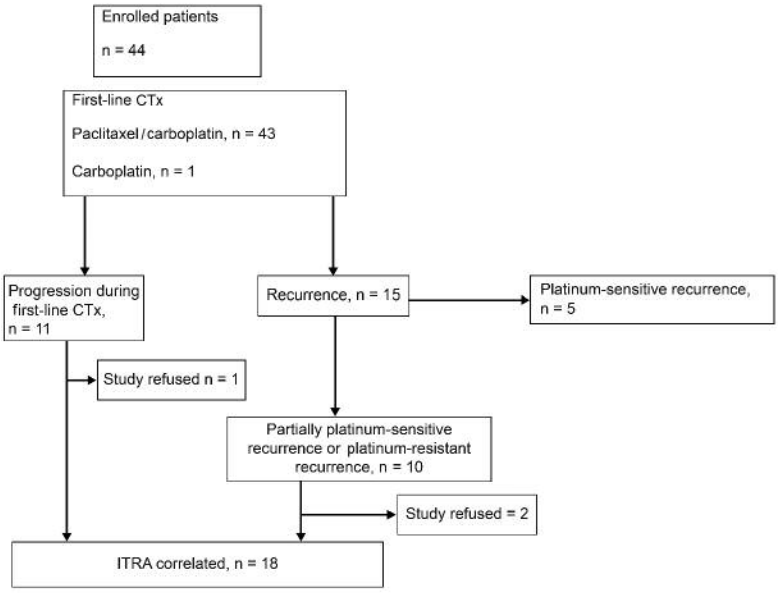

Figure 1. *Selection of patients with advanced epithelial ovarian cancer. CTx: Chemotherapy; ITRA: integrative tumor-response assay.*

(v) Investigated drug combinations and concentrations: Standard drug combinations available for clinical use, as per the revised criteria of the Korean Health Insurance Review and Assessment Service from September 2014, were used. Second-line combinations were also chosen among drugs approved for reimbursement at the time of the study. The concentrations of drugs for primary chemotherapy were 25 μg/ml carboplatin (Neoplatin; Boryung Pharmaceuticals, Seoul, Korea), 5 μg/ml paclitaxel (Taxol; Bristol-Myers Squibb Pharmaceutical Korea, Seoul, Korea) for single therapy; and 25 μg/ml carboplatin with 5 μg/ml paclitaxel for combination. For single-regimen second-line drugs, 5 μg/ml topotecan (Hycamtin; GlaxoSmithKline Korea, Seoul, Korea), 20 μg/ml belotecan (Camtobell; Chong Keun Dang, Seoul, Korea), 50 μg/ml gemcitabine (Gemzar; Lilly Korea, Seoul, Korea), 6 μg/ml doxorubicin (Ildong Adriamicin; Ildong Pharmaceuticals, Seoul, Korea), 250 μg/ml ifosfamide (Holoxan; Bukwang Pharmaceuticals, Seoul, Korea), 7.5 μg/ml vinorelbine (Navelbine; Bukwang Pharmaceuticals), and 50 μg/ml etoposide (EPS; Boryung Pharmaceuticals) were used.

Evaluation of tumor status. Tumor status was evaluated *via* computed tomography (CT) after three cycles of chemotherapy. CT was also performed whenever clinical signs of disease progression were noted. Cancer antigen 125 was measured during every cycle. According to the revised Response Evaluation Criteria in Solid Tumors (RECIST) guideline (version 1.1, 2009) (20), a complete response (CR) was disappearance of all target lesions, partial response (PR) was defined as a greater than 30% decrease in the sum of longest diameters of the target lesions without a new metastatic lesion, progressive disease (PD) was defined as a greater than 20% increase in the sum of longest diameters of the target lesions, and stable disease (SD) was neither a PR nor PD. The objective response rate (ORR) was defined as CR plus PR. Since the drug used for SD is continued until PD occurs in actual clinical settings, the clinical benefit response rate was defined as the sum of CR, PR and SD. The primary endpoint was the correlation between ITRA results and CR, seen with the ORR.

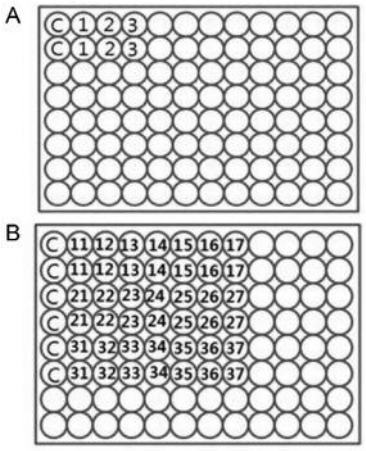

Figure 2. *Integrative tumor-response assay. The arrangement of 96-well plates with first- (A) and second-line (B) chemotherapy agents for advanced epithelial ovarian cancer. A: C: PBS control, 1: carboplatin, 2: paclitaxel, 3: carboplatin+paclitaxel. B: Two-digit coding is used, the first digit represents the first-line chemotherapy: 1#: Carboplatin, 2#: paclitaxel, 3#: carboplatin+paclitaxel; the second digit represents the second-line chemotherapy: #1: topotecan, #2: belotecan, #3: gemcitabine, #4: doxorubicin, #5: ifosfamide, #6: vinorelbine, #7: etoposide. For example, 11 represents first-line therapy with carboplatin and second-line with topotecan, while 25 represents first-line therapy with paclitaxel and second-line with ifosfamide.*

Statistical analysis. Descriptive statistics were used to analyze patient clinicopathological characteristics. To analyze the performance of ITRA, the sensitivity, specificity, accuracy, and negative and positive predictive values for predicting response were calculated, 95% confidence intervals for these rates using the exact binomial method.

Table I. *Clinicopathological characteristics of patients, n=44.*

Characteristics	Value
Age at initial diagnosis, years	
Median (range)	54 (25-79)
Follow-up period, months	
Median (range)	23.35 (4-35.5)
Body mass index, kg/m^2	
Median (range)	22.25 (15.35-34.99)
Parity, n	
Median (range)	2 (0-3)
Initial CA-125, U/ml	
Median (range)	1,003.95 (30.6-9,834)
FIGO stage, n (%)	
IIIa	2 (4.5%)
IIIb	5 (11.4%)
IIIc	16 (36.4%)
IV	21 (47.7%)
Surgical procedure, n (%)	
Debulking operation	44 (100%)
Histological type, n (%)	
Serous	37 (84.1%)
Other	7 (15.9%)
Residual tumor, n (%)	
<1 cm	30 (68.2%)
≥1 cm	14 (31.8%)
Progression, n (%)	
During 1st-line chemotherapy	11 (25.6%)
Recurrence, n (%)	
No	18 (40.9%)
Yes	15 (34.1%)
Death from disease, n (%)	
No	36 (81.8%)
Yes	8 (18.2%)

A Chi-squared test was used for a cross-table analysis to compare categorical variables. A p-value less than 0.05 was deemed statistically significant. All statistical analyses were performed with SPSS software, version 21.0 (SPSS, IBM Corp., Armonk, NY, USA).

Results

Forty-four patients met this study's criteria. The median follow-up period was 23.35 months (range=4-35.5 months). The clinicopathological characteristics of patients are summarized in Table I. All patients underwent primary cytoreductive surgery. Among them, 84.1% were diagnosed with the serous type of ovarian carcinoma, and 68.2% with a residual tumor size of less than 1 cm underwent optimal debulking surgery. Eleven patients (25.6%) showed tumor progression during the first chemotherapy cycle after surgery. Fifteen patients (34.1%) had recurrent tumor after adjuvant chemotherapy, and eight patients (18.2%) died of their disease.

Table II shows the mean IR for the *in vitro* chemosensitivity of ITRA. The mean IR when paclitaxel and carboplatin were used as first-line therapy was 53% (range=19-79%).

Table II. *Average tumor inhibition rate (IR) in the integrative tumor-response assay.*

Chemotherapy line	Drug	Mean IR (range), %
First	Paclitaxel–carboplatin	53 (19-79)
Second	Topotecan	66 (12-96)
	Belotecan	67 (25-97)
	Gemcitabine	63 (20-95)
	Adriamycin	68 (35-95)
	Ifosfamide	67 (10-95)
	Vinorelbine	57 (11-91)
	Etoposide	68 (19-96)

Table III. *Main second-line chemotherapy.*

Drug	N (%)
Pegylated liposomal doxorubicin	8 (44.4)
Belotecan	6 (33.3)
Topotecan	4 (22.2)

Adriamycin and etoposide had the highest mean IR at 68% (range=19-96% and 35-95%, respectively) among the second-line chemotherapy agents.

Based on the results of ITRA, pegylated liposomal doxorubicin was the most frequently used second-line chemotherapy agent, followed by belotecan (Table III).

Table IV shows the treatment response to second-line chemotherapy. The ORR was 38.9%. Four patients (22.2%) showed a CR and three (16.7%) had PRs in target lesions (according to the RECIST criteria). The clinical benefit response rate was 50%, including two patients (11.1%) with SD. The sensitivity of ITRA was 85.7%, specificity was 18.2%, negative predictive value was 66.7%, positive predictive value was 40%, the accuracy was 44.44% (Table V).

Advanced-stage tumors (p=0.045) and serous-type tumors by histology (p=0.005) were related to poor chemo-responsiveness towards first-line chemotherapy. No variables were significantly related to the patient's responsiveness towards the second-line chemotherapy.

Discussion

We evaluated patients with primary ovarian cancer in terms of chemosensitivity *via* ITRA using tumor tissue and attempted to determine the real-world applicability of ITRAs. This study showed that ITRAs had acceptable applicability.

After the standard first-line treatment including surgery followed by platinum-based chemotherapy for advanced stages of ovarian cancer, patients showed high overall response rates

Table IV. *Treatment response to second-line chemotherapy.*

	Second-line chemotherapy n=18	
	n	%
Clinical response (RESCIST 1.1)		
CR	4	22.2
PR	3	16.7
SD	2	11.1
PD	9	50
Objective response rate		
CR+PR	7	38.9
Clinical benefit response rate		
CR+PR+SD	9	50

CR: Complete response; PR: partial response; SD: stable disease.

Table V. *Correlation between integrative tumor-response assay (ITRA) and clinical response to second-line chemotherapy in patients with recurrent ovarian cancer.*

	Clinical response, n		
ITRA, n	Positive**	Negative	Total
Positive*	6	9	15
Negative	1	2	3
Total	7	11	18

Sensitivity: 85.7%, specificity: 18.2%, negative predictive value: 66.7%, positive predictive value: 40%, accuracy: 44.44%. *Inhibition rate ≥30%. **Complete response or partial response.

of 30-50% (21, 22). However, the 10-year relative survival rate was only 10-20%, implying that most patients experience relapse, develop chemoresistance, and eventually die. Therefore, most patients are eligible for second-line chemotherapy (23). Considering the characteristics of ovarian cancer, generally agreed upon a first-line chemotherapy agent, and the high recurrence rate of tumors requiring a second-line agent, we developed ITRA for patients with advanced EOC.

ITRA is not a simple repetition of HDRA, but an evaluation of chemosensitivity towards second-line therapy in tumor cells that survived the first-line therapy. The theory of ITRA is based on the assumption that the genetic traits of *in vitro* and *in vivo* tumor cells in patients with recurrent cancer would be the same (18).

In this study, the ORR and CRs were similar to or slightly higher than those reported in previous studies (24-27). According to a multivariate analysis by Blackledge *et al*. of 92 patients enrolled in five phase II chemotherapy trials, the response rate to second-line chemotherapy was about 10% in patients with a treatment-free interval (TFI) ≤6 months and 29% in those with TFI of 7-12 months (28). Most patients initially undergo treatment with platinum-based chemotherapy; thus, the PFI and TFI are often considered equivalent in patients with recurrent ovarian cancer. According to Pujade-Lauraine *et al*., in patients with a TFI <12 months, the response rates to second-line chemotherapy ranged from 24-35% (24).

In this study, the sensitivity of ITRA was high and its specificity was low. Sensitivity is considered equivalent to the true-positive rate (29), and the high sensitivity in this study signifies that the chemotherapy agent selected based on ITRA results had a high rate of clinical efficacy. Therefore, ITRA may help with screening to choose the second-line drug. The specificity was relatively low, and this may have been affected by the fact that all three patients who refused to participate further in the study had a low IR to second-line therapy by ITRA.

In a study on metastatic colorectal cancer, the sensitivity of ITRA was 44.4%, specificity 75%, and accuracy 61.9% (18). The difference between metastatic colorectal cancer and ovarian cancer might be due to the characteristics of each cancer type. As mentioned by Yoon *et al*. in a study on ITRA, the use of ITRA is limited because *in vivo* conditions, including circulating tumor cells or cancer stem cells related to chemoresistance, cannot be reproduced completely (18).

This study has certain limitations. The first is the choice of second-line chemotherapy agents. The seven drugs chosen as second-line agents were those used clinically approved by the Korean government at the time ITRA was developed. At that time, the Korean government had not yet approved pegylated liposomal doxorubicin, which is now widely used. After it was included in the reimbursement scheme, pegylated liposomal doxorubicin was chosen instead of doxorubicin, but this was not updated in the ITRA protocol. Additionally, combination therapy including bevacizumab is now eligible for reimbursement as a first-line chemotherapy regimen. Cisplatin is also sometimes used in combination with other drugs. Adding combination therapies to ITRA would provide more useful and significant results. The short-term follow-up period and small sample size are also limitations of this study. According to our previous study, there was a significant difference in recurrence and progression-free survival between paclitaxel/carboplatin-sensitive and resistant patients by HDRA (16). In this study, the relationship between the result of first stage of ITRA and such outcomes was difficult to establish because of the short-term follow-up period and small sample size. However, this study is significant because as far as we are aware it is the first prospective study on ITRA for patients with EOC. Furthermore, it showed the applicability of ITRA, since the response rate to second-line therapy, chosen based on the ITRA results, was similar or slightly higher than that seen in previous studies (24-27), and it has a high sensitivity. Studies with a larger sample size and longer follow-up periods should be performed to confirm the efficacy of ITRA.

In conclusion, although ITRA has a relatively low accuracy and specificity, it is feasible and applicable and may be a useful tool to help physicians choose second-line chemotherapy regimens in patients with advanced EOC.

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Received October 31, 2018
Revised December 3, 2018
Accepted December 4, 2018