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Original Article

In Vitro Chemosensitivity Using the Histoculture Drug Response Assay in Human Epithelial Ovarian Cancer

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The choice of chemotherapeutic drugs to treat patients with epithelial ovarian cancer has not depended on individual patient characteristics. We have investigated the correlation between *in vitro* chemosensitivity, as determined by the histoculture drug response assay (HDRA), and clinical responses in epithelial ovarian cancer. Fresh tissue samples were obtained from 79 patients with epithelial ovarian cancer. The sensitivity of these samples to 11 chemotherapeutic agents was tested using the HDRA method according to established methods, and we analyzed the results retrospectively. HDRA showed that they were more chemosensitive to carboplatin, topotecan and belotecan, with inhibition rates of 49.2%, 44.7%, and 39.7%, respectively, than to cisplatin, the traditional drug of choice in epithelial ovarian cancer. Among the 37 patients with FIGO stage Ⅲ/Ⅳ serous adenocarcinoma who were receiving carboplatin combined with paclitaxel, those with carboplatin-sensitive samples on HDRA had a significantly longer median disease-free interval than patients with carboplatin-resistant samples (23.2 vs. 13.8 months, $p < 0.05$), but median overall survival did not differ significantly (60.4 vs. 37.3 months, $p = 0.621$). In conclusion, this study indicates that HDRA could provide useful information for designing individual treatment strategies in patients with epithelial ovarian cancer.

Key words: chemosensitivity, carboplatin, HDRA (histoculture drug response assay), epithelial ovarian cancer

Ovarian cancer is the most common cause of death among gynecologic malignancies [1]. Approximately 90% of ovarian cancers are of the epithelial type, and most patients present with advanced-stage disease. The treatment of choice for patients with advanced ovarian cancer consists of adequate surgical cytoreduction, followed by platinum-based chemotherapy, which yields initial response rates of 70% to 80%, including a high proportion of complete responses. Most patients, however, eventually relapse and die of chemoresistant disease. Tumor cell sensitivity to chemotherapeutic agents depends on various factors, including cancer cell viability and complex genomic alterations. Usually, however, the choice of chemotherapeutic drug is based on clinical trials that determine whether a drug is effective in large numbers of patients. This approach ignores variations in individual patient conditions [2]. To improve the outcome in patients with ovarian cancers, it is important to select the chemotherapeutic agents most effective for each individual patient.

Although *in vitro* methods may accurately predict the chemosensitivity of human tumors *in vivo* [3–5], none of these methods has yet been applied clinically

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because of their technical complexity and the difficulty of obtaining evaluable tumor samples. In addition, although these methods can exclude ineffective drugs, they are less useful in the selection of effective drugs because true-positive rates tend to be lower than true-negative rates. These findings suggest the need for effective drug sensitivity tests that overcome these disadvantages. The histoculture drug response assay (HDRA) with the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) endpoint may be used to detect individual chemosensitivity in many types of cancer [6–10]. The HDRA is appropriate for the culture of many types of cancer cells because it allows these cells to be cultured in their natural three-dimensional architecture [10, 11]. Recently, HDRA has been reported to be clinically useful in patients with breast and gastrointestinal cancers [12, 13]. HDRA was also reported to be effective in predicting the response to chemotherapy in patients with ovarian cancer; moreover, using the HDRA to measure the response to cisplatin may be useful in optimizing cisplatin-based therapy, including combinations with doxorubicin and cyclophosphamide, for patients with ovarian cancer [14]. We therefore used the HDRA to assess the sensitivity of epithelial ovarian cancer cells to various chemotherapeutic agents, and we evaluated the correlation between HDRA results and the clinical responses of patients to carboplatin.

Patients and Methods

Patients. Fresh tissue samples were obtained from 79 patients with epithelial ovarian cancer who were treated at the Asan Medical Center between January 2004 and December 2007. The medical records and pathologic reports of these patients were reviewed retrospectively. We performed this retrospective study with the approval of the Institutional Review Board of Asan Medical Center to protect the patients' confidentiality. The median age of these 79 patients was 50 years (range: 19–74 years) and the median follow-up duration was 28.5 months (range: 0.7–79.5 months). Of the 79 patients, 17 (21.5%) had the International Federation of Gynecology and Obstetrics (FIGO) stage Ⅰ, 1 (1.3%) had stage Ⅱ, 52 (65.8%) had stage Ⅲ, and 9 (11.4%) had stage Ⅳ tumors. Fifty-five patients (69.6%) had serous adenocarcinomas, 6 (7.6%) had mucinous adenocarcinomas, and 4 (5.1%) had clear cell carcinomas. All patients underwent surgery, and 70 (88.6%) received platinum-based chemotherapy as an adjuvant treatment, including 57 patients treated with carboplatin and 13 with cisplatin (Table 1). Recurrence was reported in 46 patients (58.2%).

The histoculture drug response assay. HDRA procedures were performed as described [10]. Fresh tumor tissue samples were obtained from each patient and transported to the laboratory at 4℃ in Hank's balanced salt solution (HBSS; Gibco, Gaitherburg, MD, USA). Tumor tissues were cut into approximately 10-mg pieces, and viable parts were selected based on staining methods. The tumor tissues were placed onto collagen sponge gels (Gel Foam; Pharmacia & UpJohn Inc., UK) in 24-well plates and incubated for 24 h in RPMI 1640 medium (Sigma, St. Louis, MO, USA) containing 20% fetal calf serum at 37℃ with 5% CO_2. The next day, chemotherapeutic agents were added to each well, and the plates were incubated for 72 h. The concentration of each of the 11 drugs used in this study was determined in our laboratory based on previous determinations of 50% growth inhibition of tumor cells (IC_{50} values): 75 μg/mL paclitaxel, 50 μg/mL carboplatin, 10 μg/mL cisplatin, 75 μg/mL docetaxel, 10 μg/mL topotecan, 20 μg/mL

Table 1 Clinicopathologic characteristics of patients

Characteristic	No. of patients
Total no. of patients	79
Median age (years)	50 (19–74)
Median follow-up duration (months)	28.5 (0.7–79.5)
FIGO stage	
Ⅰ	17
Ⅱ	1
Ⅲ	52
Ⅳ	9
Histology	
Serous adenocarcinoma	55
Mucinous adenocarcinoma	6
Clear cell carcinoma	4
Endometrioid adenocarcinoma	2
Others	12
Adjuvant treatment modalities	
No adjuvant treatment	7
Carboplatin-based chemotherapy	57
Cisplatin-based chemotherapy	13
Not known	2

belotecan [15], 20 μg/mL irinotecan, 250 μg/mL ifosfamide, 50 μg/mL etoposide, 6 μg/mL adriamycin, and 50 μg/mL gemcitabine. As a control, each tumor tissue sample was cultured with phosphate-buffered saline (PBS) in the absence of chemotherapeutic agents.

After the tumor samples were incubated with drugs for 3 days, 100 μL PBS containing 0.1 mg/dL collagenase (type I) and 5 mg/dL MTT (Sigma) were added to each well, and the plates were incubated at 37℃ for an additional 4 h. The resulting formazan crystals were extracted from the wells with dimethyl sulfoxide, and the optical density was measured with a plate reader (SPECTRA max 340PC, Molecular Devices) at 540 nm. The inhibition rate (IR) was calculated using the formula:

$IR = (1\text{-}T/C) \times 100$, where

T is the mean absorbance of the treated tumor/g

C is the mean absorbance of the control tumor/g.

The cut-off values of the HDRA IR for deciding the chemosensitivities of tumor samples to several chemotherapeutic agents were not well defined. We therefore determined these cut-off values by comparing HDRA results with clinical outcomes.

Assessment of chemosensitivity in the HDRA and clinical response. To determine the carboplatin IR cut-off value in the HDRA related to clinical prognosis, we selected patients with stage Ⅲ or Ⅳ epithelial ovarian serous adenocarcinoma who had received at least 3 cycles of chemotherapy with carboplatin combined with paclitaxel. Disease-free intervals were analyzed in groups of patients divided by IR intervals of 10 using multiple comparison tests and Cox regression tests. Clinical responses were evaluated using the Response Evaluation Criteria in Solid Tumors (RECIST) criteria [16]. The disease-free interval was measured from the pathologic confirmation of epithelial ovarian cancer to documented progressive disease. In patients without recurrence, the disease-free interval was defined as the period from pathologic diagnosis to last follow-up. Patients responsive to initial carboplatin-based chemotherapy and who did not experience a recurrence within 6 months after finishing chemotherapy were defined as carboplatin-sensitive. Descriptive statistics were used to analyze chemosensitivity in the HDRA and clinical characteristics, and the Kaplan-Meier method was used to compare chemosensitivity and clinical outcome. Statistical significance was defined as $p < 0.05$. SPSS v15.0 was used for all statistical analyses.

Results

The ability of 11 chemotherapeutic agents to inhibit the growth of epithelial ovarian cancer tumor samples was tested using the HDRA. The IRs of paclitaxel and carboplatin, the chemotherapeutic agents of choice for treating ovarian cancer, were 32.2% and 49.2%, respectively. Cisplatin, a traditional platinum agent, had an IR of 34.7%. In addition, 2 topoisomerase I inhibitors, topotecan and belotecan, had high IRs of 44.7% and 39.7%, respectively (Fig. 1). Chemosensitivities, however, did not differ by histopathologic type or FIGO stage.

Among the 37 patients with FIGO stage Ⅲ/Ⅳ serous adenocarcinoma who were receiving carboplatin combined with paclitaxel, those with carboplatin-sensitive samples on HDRA had a significantly longer median disease-free interval than those with carboplatin-resistant samples (23.2 vs. 13.8 months, $p < 0.05$), but the median overall survival did not differ significantly between these 2 groups (60.4 vs. 37.3 months, $p = 0.621$).

We also assessed the response and prognosis relative to IRs in the HDRA among the 37 patients with stage Ⅲ/Ⅳ serous epithelial ovarian cancer who had

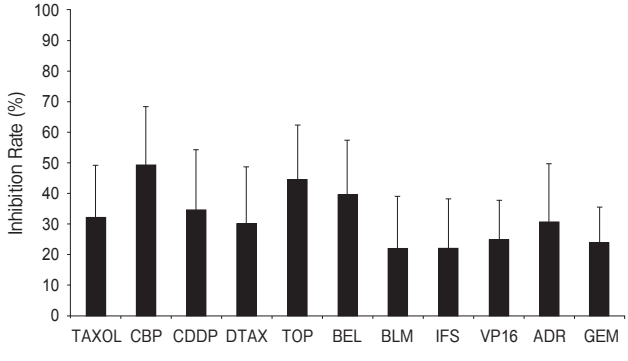

Fig. 1 Results of *in vitro* chemosensitivity tests using the HDRA in ovarian cancer. Data are expressed as mean ± standard deviation; (1) TAXOL: *paclitaxel*, IR 32.2 ± 17.6 (2) CBP: *carboplatin*, IR 49.2 ± 18.8 (3) CDDP: *cisplatin*, IR 34.7 ± 19.6 (4) DTAX: *docetaxel*, IR 30.4 ± 18.6 (5) TOP: *topotecan*, IR 44.7 ± 18.5 (6) BEL: *belotecan*, IR 39.7 ± 17.8 (7) BLM: *bleomycin*, IR 22.1 ± 16.8 (8) IFS: *ifosfamide*, IR 22.1 ± 16.1 (9) VP16: *etoposide*, IR 25.0 ± 12.9 (10) ADR: *adriamycin*, IR 30.8 ± 18.9 (11) GEM: *gemcitabine*, IR 24.0 ± 12.1.

been treated with at least 3 cycles of carboplatin chemotherapy. Multiple comparison tests and Cox regression analysis showed that 50 μg/mL carboplatin was the best cut-off value for IR in the HDRA (40%) that affected the patient disease-free interval, which is the most important clinical outcome. Patients whose samples showed a $<40\%$ IR in response to 50 μg/mL carboplatin had a relative risk (RR) of 2.95 ($p < 0.05$, confidence interval (CI) 1.23–7.06) compared with patients whose samples showed a $\geq 40\%$ IR in response to this carboplatin concentration. Using this cut-off value, we divided the 37 patients into 2 groups based on their sensitivity or resistance to carboplatin in the HDRA, with 22 patients included in the carboplatin-sensitive group and 15 in the carboplatin-resistant group. The 2 groups had similar clinical and pathological characteristics except for their chemosensitivity to carboplatin (Table 2), and their response rates based on RECIST criteria were similar (93.3% (14/15) vs. 90.9% (20/22), $p = 0.651$) (Table 3). The 2 groups also showed similar carboplatin-sensitivity in clinical outcomes ($p = 0.157$) (Table 3). The median disease-free interval was 23.2 months (range, 6.3–55.3 months) in carboplatin-sensitive patients and 13.8 months (range, 4.9–35.6 months) in carboplatin-resistant patients ($p < 0.05$) (Fig. 2). The median overall

Table 2 Comparison of the clinicopathologic characteristics of patients with carboplatin-sensitive and -resistant tumors in the HDRA

	Carboplatin-Sensitive ($n = 22$)	Carboplatin-Resistant ($n = 15$)	p value
Median age (years)	49 (25–73)	53 (30–74)	0.154†
Median follow-up duration (months)	37.8 (6.3–64.7)	28.0 (7.7–79.5)	0.085†
Inhibition rate to carboplatin	57.3 ± 14.2	28.7 ± 8.5	<0.000†
FIGO stage			0.951‡
ⅢB	2	1	
ⅢC	12	8	
Ⅳ	8	6	
Histology			—
Serous adenocarcinoma	22	15	
Residual tumor			0.785‡
<1 cm	17	11	
≥1 cm	5	4	

† Mann-Whitney test
‡ Chi-squared test

Table 3 Comparison of clinical responses and survival between patients with carboplatin-sensitive and -resistant tumors in the HDRA

	Carboplatin-Sensitive ($n = 22$)	Carboplatin-Resistant ($n = 15$)	p value
Clinical Response			0.651†
Complete remission	17	13	
Partial response	3	1	
Stable disease	1	1	
Progressive disease	1	0	
Carboplatin-sensitivity in the clinical outcome			0.157†
Sensitive	20 (90.9%)	11 (73.3%)	
Resistant	2 (9.1%)	4 (26.7%)	
Disease free interval (months)*	23.2 (6.3–55.3)	13.8 (4.9–35.6)	<0.05‡
Overall survival (months)*	60.4 (6.3–64.7)	37.3 (7.7–79.5)	0.621‡

† Chi-squared test
‡ Kaplan-Meier test
* Median (range)

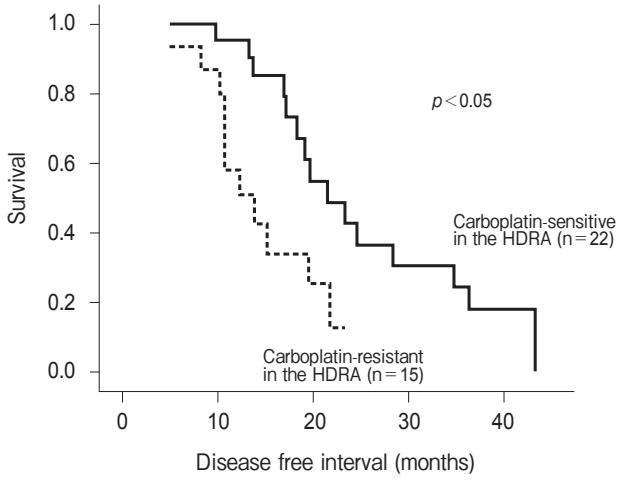

Fig. 2 Kaplan-Meier survival curves showing disease-free intervals (DFI) of patients sensitive (n = 22) and resistant (n = 15) to carboplatin in the HDRA.

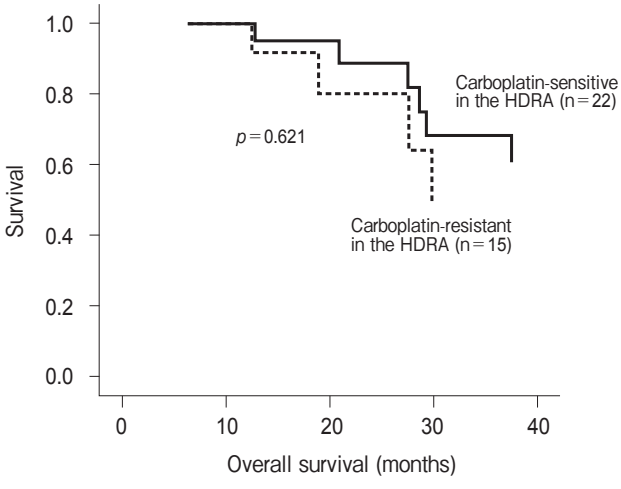

Fig. 3 Kaplan-Meier survival curves showing overall survival (OS) of patients sensitive (n = 22) and resistant (n = 15) to carboplatin in the HDRA (n = 15).

survival of these 2 groups, however, did not differ significantly (60.4 vs. 37.3 months, $p = 0.621$) (Fig. 3). Clinical outcomes were also not dependent on the use of other chemotherapeutic agents, including paclitaxel.

Discussion

Chemosensitivity tests can be performed *in vivo* using animals and *in vitro* using histoculture or cell culture techniques. Several *in vitro* and *in vivo* chemosensitivity assays have been developed to predict tumor responsiveness to a clinically administered drug and to improve the effectiveness of treatment and prognosis. Currently available, clinically applicable chemosensitivity tests include the MTT assay [17], the 3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium inner salt (MTS) assay [18], the HDRA [4–8], and the collagen gel droplet-embedded culture drug sensitivity test [19, 20].

The monolayer culture MTT assay is based on the reduction of MTT by mitochondrial succinic dehydrogenase in living cells; this reaction yields the colored compound formazan, which is measured optically using a plate reader. This enzymatic receptor assay can evaluate drug sensitivity rapidly and simply, and is accurate in more than 90% of samples [13]. The MTT assay has been used in the screening of chemotherapeutic agents at the National Cancer Institute since 1991. The MTS assay is a modified, improved version of the MTT assay. However, monolayer culture MTT and MTS assays eliminate cell-cell contact because the cancer cells are assessed as single-cell suspensions [7]; thus, these tests are significantly influenced by the presence of contaminating fibroblasts, and the viability of tumor cells decreases during culture under control conditions. Chemosensitivity assays using three-dimensional HDRA, which utilize a soft agar substrate, may more accurately mimic the *in vivo* response. The HDRA is a robust assay for a number of cancers, with an evaluation rate of 98.8%, a mean sensitivity of nearly 100%, a specificity of 70–90%, and a clinical correlation of 80–90% [8, 12]. These high rates are due, at least in part, to the maintenance of the three-dimensional tissue architecture in the HDRA. Correlations between survival and *in vitro* chemosensitivity have been observed in patients with gastrointestinal cancer and in those with ovarian cancer [5, 21]. We previously reported that the HDRA may provide information useful for designing individualized treatments for patients with uterine cervical cancer [22]. To date, however, only a few retrospective studies have shown correlations between survival benefits in cancer patients and chemosensitivity assays. This is especially true for ovarian cancer, for which there have been few reports on chemosensitivity using MTT or MTS assays.

The extreme drug resistance (EDR) assay may also

be used to guide therapeutic strategies. For example, in 79 patients with advanced ovarian cancer, the median progression-free survival was 6 months for patients with EDR to platinum, compared with 24 months for patients sensitive to platinum (RR 3.78, CI 1.82–7.83) in multivariate analysis [23]. The estimated overall 5-year survival rates of these 2 groups were 19% and 68%, respectively (RR 2.32, CI 1.06–5.07). Moreover, *in vitro* drug resistance at recurrence was not influenced significantly by therapy [24], suggesting that assay results at diagnosis may be used to guide treatment modalities at recurrence.

The combination of paclitaxel and a platinum compound (usually carboplatin or cisplatin) has been preferred in the primary treatment of women with advanced epithelial ovarian cancer, with response rates as high as 75% [25, 26]. However, although chemotherapy has increased survival duration, most women suffer from recurrent disease, and only 20% to 25% remain alive at 5 years. The preferred agents for treating tumor recurrences include carboplatin, cisplatin, paclitaxel, gemcitabine, liposomal doxorubicin and topotecan. Among the cytotoxic alternatives are altretamine, capecitabine, cyclophosphamide, docetaxel, etoposide, ifosfamide, irinotecan, melphalan, oxaliplatin, and vinorelbine. Except for cisplatin or carboplatin, as monotherapy or combined with paclitaxel, most regimens lack a uniform National Comprehensive Cancer Network consensus based on primary evidence, including clinical experience. We found that the IRs of topotecan and belotecan were higher than that of cisplatin, and that the IR of the cisplatin and paclitaxel combination was somewhat lower than expected. Similarly, MTT assays of 32 clinical samples of tumor and ascites cells showed that these cells were most sensitive to topotecan [27].

Chemosensitivity testing in patients with epithelial ovarian cancer should be performed to determine patient prognosis and to select proper chemotherapeutic agents. When ovarian cancer samples were assayed *in vitro* for their sensitivity to cisplatin using the HDRA and compared with clinical response and survival, using a cut-off value set at a 50% inhibitory concentration of 25μg/mL, the 5-year survival rate was found to be significantly higher in patients with chemosensitive tumors than in those with chemoresistant tumors, and the accuracy of the assay was 82.8% (24/29) [28]. As chemosensitivity to cisplatin increased, the number of apoptotic cells also increased. In comparison, we found that when we assessed 37 patients with FIGO stage Ⅲ/Ⅳ serous adenocarcinoma who had received at least 3 cycles of chemotherapy with carboplatin plus paclitaxel, the median disease-free interval was significantly higher in patients with carboplatin-sensitive samples than in those with carboplatin-resistant samples (23.2 vs. 13.8 months, $p < 0.05$), although their response rates and overall survival did not differ significantly. This result shows the critical role of HDRA to reflect *in vivo* chemosensitivity to carboplatin, since the time of relapse after chemotherapy with carboplatin plus paclitaxel was later in the carboplatin-sensitive group as determined by HDRA. Furthermore, although overall survival was not different according to the HDRA result, a relation between HDRA and survival might be revealed if the period of observation is extended.

Ideally, chemotherapy should be based on individual cellular and genetic differences. Chemosensitivity tests, including the HDRA, enable chemotherapy regimens to be individualized. These tests, however, are not widely accepted in the treatment of ovarian cancer because the available clinical trial data are insufficient, although some *in vitro* chemosensitivity assays are restricted for use in the United States, Germany and Italy. The HDRA can analyze chemosensitivity and provide information that can guide primary treatment and therapeutic strategies at relapse in patients with ovarian cancer. HDRA results can help select the appropriate chemotherapeutic agents to improve clinical outcomes for individual patients. Large, randomized, controlled trials are required, however, to determine the clinical benefits of the HDRA in patients with epithelial ovarian cancer.

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