In Vitro Chemoresponse in Metachronous Pairs of Ovarian Cancers

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Abstract. *Background/Aim: An in vitro chemoresponse assay may aid effective therapy selection in epithelial ovarian cancer (EOC). This study explores changes in chemoresponse between paired primary and recurrent EOC tumors. Patients and Methods: Results from metachronous tumors were examined in 242 patients. Changes in in vitro chemoresponse, measured by the area under the dose response curve (AUC) between paired tumors were assessed. Results: A significant increase in AUC was identified in most first-line therapies over time. No significant difference was observed in most recurrent therapies. When the elapsed time between occurrences was <17 months, no difference was observed for any recurrent therapies, and half of first-line therapies exhibited significant increases in AUC. When ≥17 months, all 7 therapies showed significant increases. Conclusion: These results suggest an increase in chemoresistance over time, which is more pronounced for first-line therapies. This is consistent with clinical observations and suggests the biologic concordance between assay results and response to chemotherapy.*

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Epithelial ovarian cancer (EOC) is the most lethal and second most common gynecological malignancy in the United States with an estimated 21,980 new cases and 14,270 deaths expected in 2014 (1). Most patients present with advanced disease and the current standard-of-care in the primary setting includes surgical debulking followed by platinum-based chemotherapy (2). The majority of patients eventually recur at least once (3-5) and many recurrent patients undergo secondary surgical cytoreductions. Nearly all recurrent patients are treated with one of multiple guideline-recommended chemotherapies, such as pegylated liposomal doxorubicin, topotecan and gemcitabine (2). Clinical outcomes have been demonstrated to be largely equivalent across these various chemotherapies in clinical studies and, as such, second-line therapy is generally empirically chosen by treating physicians, primarily based on clinical-pathologic and patient-related factors (2, 6-9).

First progression (or recurrence) typically occurs within 16-18 months of primary occurrence (6-9). Tumors recurring within 12 months of initiation of primary therapy are classified clinically as "platinum-resistant" and are generally not treated with platinum-based therapies upon recurrence. Tumors recurring greater than 12 months following initiation of primary therapy are clinically classified as "platinum-sensitive" and physicians generally treat these patients with platinum-based therapy upon recurrence (3, 10). Despite their classification as platinum-sensitive, a large number of these patients do not respond to platinum-based treatment at recurrence. For example, in one report, 41% of EOC patients with a platinum-free interval of over 24 months did not responsed to secondary platinum-based therapy (11).

Patients' tumors are generally thought to demonstrate increased resistance to chemotherapy over time and/or exhibit a multi-drug resistance phenotype. Chemoresistance may be inherent in sub-populations of heterogeneous cancer cells that continue to proliferate after primary chemotherapy successfully decreased the chemosensitive subpopulations. Resistance may also be acquired in tumor cells, as a response to chemotherapy exposure. For example, platinum agents cause crosslinking of DNA, which ultimately triggers apoptosis in tumor cells. Resistance to these agents may result from drug-induced activation of DNA repair systems, such as mutation of cell cycle proteins like p53. Another important mechanism of increased resistance may be the action of a group of membrane proteins, such as multidrug resistance gene-1 (MDR1), which extrude cytotoxic molecules, keeping intracellular drug concentration below a cytotoxic threshold (12, 13). Clearly, drug resistance is a complex process with many explanations.

Increased resistance to chemotherapy is noted through diminishing survival at each successive recurrence in ovarian cancer. The general response rate to primary (platinum/taxane) therapy is noted to be 60-80%, with median progression-free survival (PFS) of 16-18 months and median overall survival (OS) of 44 months (6-9). However, the average response rate at any recurrence is significantly reduced to approximately 25% with a median PFS and OS for the platinum-resistant patients of only 4 months and 10 months, respectively (14) and median PFS and OS for platinum-sensitive patients of 9 months and 18 months, respectively (5, 15). In addition, a recent study found that, after relapse, chemotherapy was only beneficial for a maximum of 3 subsequent treatments with PFS decreasing drastically with each successive recurrence (16).

Recent clinical validations in both primary and recurrent EOC demonstrate the prognostic and predictive utility of a chemoresponse assay, ChemoFx® (17-19). For example, results of a prospective study in recurrent EOC patients demonstrated that patients receiving assay-designated sensitive treatments lived an average of 14 months longer than those receiving non-sensitive treatments (17). The survival benefit of the assay is not limited to platinum-sensitive EOC but also extends to platinum-resistant EOC (17). The assay also identified primary ovarian patients that are likely to recur early (*i.e.* experience platinum resistance) when treated clinically with carboplatin/paclitaxel (18). Both studies demonstrated that the majority of patients had at least one effective (sensitive) therapy option identified by the assay, different than the treatment administered clinically. Furthermore, a clinical utility analysis demonstrated that, on average, patients treated by physicians with access to assay results lived 10% longer than patients treated by physicians without access. The study also demonstrated that patients lived 65% (72 *vs.* 44 months) longer if physicians administered treatments deemed sensitive by the assay and OS was reduced by 36% (28 *vs.* 44 months) if physicians administered treatments deemed resistant by the assay (19).

While ChemoFx® is technologically advanced compared to historical chemosensitivity and resistance assays by utilizing state-of-the-art laboratory automation, microscopy and informatics technologies, it can also be successfully executed on small-sized tissue samples, such as core needle biopsies and ascites. Still, it is not always possible to obtain a tumor sample at the time of recurrence for ChemoFx® testing (*e.g.* surgery contraindicated; biopsy, second look laparoscope or ascites unavailable). The aim of this study is to investigate whether chemoresponse results from a primary EOC occurrence has utility in informing effective treatment decisions at the time of recurrence, when a tumor sample is unobtainable for chemoresponse analysis.

Patients and Methods

Study population. EOC patients with paired tumors (primary occurrence and recurrence), received between March 1, 2010 and July 1, 2013 for chemoresponse testing, were included in the study (n=242). Patients were de-identified and exempt from Informed Consent Forms (20). Among the patients with available pathological information, histological subtypes include serous (87%), clear cell (5%), endometrioid (3%), mixed (2%), mucinous (1%) and transition cell (1%).

Chemoresponse assay. Fresh tissue or ascites samples were collected from each patient at the time of primary occurrence and again at recurrence for *in vitro* chemoresponse testing (ChemoFx®, Precision Therapeutics, Inc., Pittsburgh, PA, USA). Details regarding the assay procedure have been described previously (21). In brief, primary cultures were initiated by mincing each tissue sample into 1 mm^3 explants, which were then seeded into culture flasks. Upon near confluency, primary cultures were trypsinized and seeded into microtiter plates (Corning, Lowell, MA, USA) and incubated with a panel of guideline-recommended chemotherapeutic therapies as requested by the ordering physicians. Multiple, increasing concentrations were tested for each therapy in triplicate. After 72 h of incubation with treatment, surviving adherent cells were stained with DAPI (Molecular Probes, Carlsbad, CA, USA)

Table I. *Comparison of average AUCs between metachronous pairs of primary and recurrent EOC.*

Chemotherapeutic agents	Guideline-recommended for primary EOC	Guideline-recommended for recurrent EOC	N	Average $AUC_{primary}$	Average $AUC_{recurrent}$	ΔAUC ($AUC_{recurrent}$ – $AUC_{primary}$)	*p*-Value
Carboplatin	√	√	201	4.53	4.64	0.11	0.105
Cisplatin	√	√	172	3.65	3.86	0.21	0.012
Paclitaxel	√	√	194	5.06	5.30	0.24	<0.001
Docetaxel	√	√	189	5.23	5.50	0.27	<0.001
Doxorubicin		√	223	3.85	4.13	0.28	<0.001
Topotecan		√	227	5.24	5.26	0.02	0.770
Gemcitabine		√	206	5.82	5.82	0.00	0.946

and counted using proprietary, automated, computer-assisted microscopy (Precision Therapeutics, Inc., Pittsburgh, PA, USA) (22). The survival fraction (SF) of tumor cells at each concentration was calculated as compared to control (no treatment). The summation of SF values over concentrations 1 through 7 was computed as the drug response score, which represents the area under the dose response curve (defined as AUC score hereafter). A smaller AUC score indicates that a tumor is more sensitive to a treatment *in vitro*; a larger score indicates greater resistance to a treatment. For each treatment, *in vitro* tumor response was classified into one of three categories according to the AUC score: sensitive (S), intermediate (I) or resistant (R). The cut-point thresholds for the classifications were previously and independently established based on the 25th and 75th AUC percentiles in external and independent referent specimens. Agents included in the analysis are commonly used in primary first-line treatment (carboplatin (n=201), cisplatin (n=172), paclitaxel (n=194), docetaxel (n=189)), as well as in treatment of recurrent EOC (doxorubicin (n=223), gemcitabine (n=206) and topotecan (n=227)), where "n" indicates the number of patients with both primary and recurrent chemoresponse results for that treatment.

Statistical analysis. Assay results were compared for each patient's metachronous tumor samples (*i.e*. primary *vs*. recurrent) for each available treatment. Average AUC scores of each treatment were tabulated for both primary (average $AUC_{primary}$) and recurrent (average $AUC_{recurrent}$) EOC patients. Further, the change in AUC (ΔAUC=average $AUC_{recurrent}$ – average $AUC_{primary}$) for each treatment was calculated and the statistical significance was assessed by a paired *t*-test. An increase in the AUC (positive ΔAUC value) indicates an increase in resistance. In addition, longitudinal, time-based analyses were carried out to investigate the impact of the passage of time on chemoresponse profiles between primary and recurrent cancer occurrences. As the median PFS for primary EOC is 16-18 months, the midpoint of 17 months was used as the demarcation in longitudinal analyses.

Results

Drug resistance to different chemotherapy agents in metachronous pairs. Recurrent tumor assay response profiles across 7 commonly-utilized, guideline-recommended EOC treatments were generally more resistant than their paired primary (metachronous) tumors from the same patients. In recurrent tumors (compared to their matched primary tumor), a significant increase in AUC, indicating increased resistance, was observed in 3 of 4 first-line therapies (cisplatin (p=0.012), paclitaxel (p<0.001), docetaxel (p<0.001)) and trended for carboplatin (p=0.105). However, only 1 of 3 recurrent therapies (doxorubicin (p<0.001)) demonstrated an increase in resistance, while the remaining recurrent therapies, gemcitabine and topotecan, showed little to no change (Table I). Further, the degree of increased resistance across metachronous tumor samples was generally more evident for first-line treatments *vs*. recurrent treatments.

Drug resistance in metachronous pairs: effect of elapsed time. Additional analyses investigated the impact of the passage of time on chemoresponse profiles of metachronous pairs of EOC tumors. The results indicate that the increased passage of time corresponds to increased tumor resistance, as designated by the assay. Specifically, when the elapsed time between occurrences was <17 months (median PFS for EOC), no difference was observed for any recurrent therapies and a significant increase in AUC (increased resistance) was observed in 2 of the 4 front-line therapies (paclitaxel (p=0.04) and docetaxel (p=0.02), with an average ΔAUC of 0.18). Comparatively, when the elapsed time was ≥17 months, a significant increase in resistance was observed for all 7 treatments with an average ΔAUC across all treatments of 0.40 (Table II).

Increased resistance to treatments between metachronous pairs of EOC was also evident in dose-response curves (DRCs). Consistent with the results presented in Tables I and II, the shift toward resistance was more evident (*i.e*. a larger differential response in DRCs between primary occurrence and recurrence) in common first-line treatments (*e.g*. paclitaxel), as opposed to those typically administered upon recurrence (*e.g*. topotecan) (Figure 1). The differential response was further increased with elapsed time between occurrences.

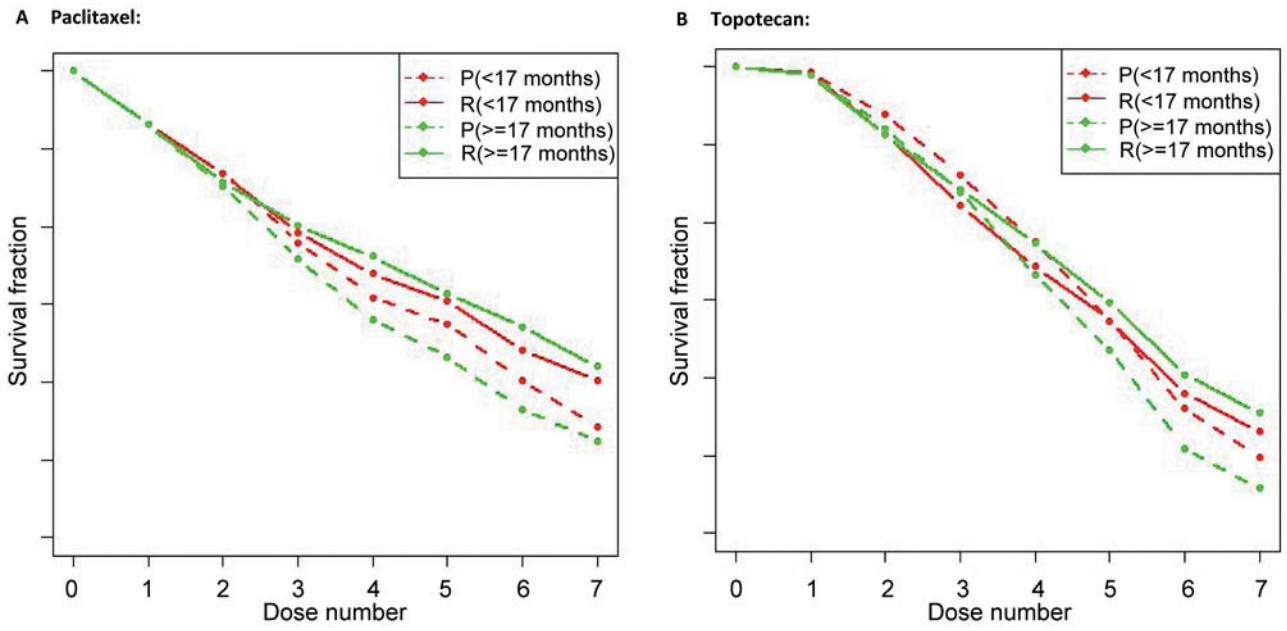

Figure 1. *Representative average dose response curves for a first-line treatment (A; paclitaxel) and a recurrent treatment (B; topotecan).*

Table II. *Longitudinal, time-based comparison of ΔAUC between metachronous pairs of primary and recurrent EOC.*

Drug	<17 Months			≥17 Months		
	N	ΔAUC	*p*-Value	N	ΔAUC	*p*-Value
Carboplatin	143	0	0.98	58	0.38	0.001
Cisplatin	124	0.10	0.31	48	0.48	<0.001
Paclitaxel	139	0.17	0.04	55	0.40	0.001
Docetaxel	135	0.19	0.02	54	0.43	<0.001
Doxorubicin	163	0.17	0.1	60	0.58	<0.001
Topotecan	165	–0.07	0.35	62	0.26	0.009
Gemcitabine	152	–0.1	0.26	54	0.26	0.02

Discussion

The present study, based on a large cohort of metachronous paired tumors, demonstrates that recurrent EOC tumors generally have a more resistant chemoresponse profile than primary tumors from the same patients, which is in concordance with clinical observations. These results further support the validity of the chemoresponse assay and indicate that assay results will have highest utility when a tumor sample is available immediately preceding a treatment decision.

When comparing assay response profiles of the therapies included in this study, the standard first-line platinum and taxane treatments generally demonstrated a more significant shift toward resistance (*i.e.* larger ΔAUC , larger magnitude DRC differences and larger proportion of resistant categorization) in the recurrent tumor sample than the typical recurrent therapy choices (doxorubicin, gemcitabine, topotecan). The mean ΔAUC among the 4 first-line therapies was more than double the ΔAUC of the 3 recurrent therapies (0.21 *vs.* 0.10), suggesting that the degree of increased resistance from primary to recurrent tumors may be more evident in the previously administered first-line therapies, as compared to recurrent therapies not yet clinically administered. This observation also matches clinical expectations, presumably because tumors may develop resistance to first-line chemotherapies as a result of treatment with them at primary occurrence (13, 23). Consistent with this hypothesis, changes in assay response to recurrent

therapies, such as topotecan and gemcitabine, between primary and recurrent tumors are small in comparison to the changes observed for first-line treatments. Furthermore, the chemo-response profiles of these recurrent therapies are similar in both primary and recurrent paired tumors. These findings suggest that, if a new tissue sample is not available at recurrence, ChemoFx® results from the previous occurrence may have utility for therapies not previously administered.

Longitudinal, time-based analysis using a 17-month (median PFS for EOC) demarcation showed that the length of time elapsed between primary and recurrent occurrences also had an impact on chemoresponse profiles: when the time elapsed between occurrences was <17 months, no difference in AUC was observed for therapies typically used in the recurrent setting and a significant increase in resistance was observed in only 2 of the 4 first-line therapies. Comparatively, when the elapsed time was ≥17 months, there was a significant increase in resistance to all 4 first-line therapies, as well as all 3 recurrent therapies. In addition to the 17 month demarcation, two other time point demarcations were examined due to their clinical relevance (data not shown): 6 months (threshold between platinum refractory and platinum resistant categorizations) and 12 months (threshold between platinum resistant and platinum sensitive categorizations). In general, paired tumors assayed within a timepoint demarcation have more similar assay response profiles than paired tumors assayed beyond the demarcation. For example, when the time elapsed between assays of primary and recurrent tumors was less than 6 months, only one treatment had a statistically significant change in AUC between occurrences. When the time elapsed was less than 12 months, only 2 treatments demonstrated statistical significant differences. Conversely, and as described earlier, when the elapsed time was more than 17 months, paired tumors exhibited a significantly increased resistant profile for all 7 treatments. The average ΔAUCs across all 7 treatments at ≥6 months, ≥12 months and ≥17 months, were all higher than their counterparts at <6 months, <12 months and <17 months, respectively (data not shown). As biologically anticipated, the number of treatments with significantly different response profiles (increasing resistance over time), as well as the magnitude of change in resistance, increase with increased elapsed time between assaying metachronous tumor samples.

All 7 treatments were also investigated separately in the longitudinal analysis using 6 month, 12 month and 17 month demarcations. For the 3 recurrent treatments, the average AUC change between occurrences increased consistently with increasing time demarcations. Whereas, for the first-line treatments, the average increase in AUC between occurrences varied. The degree of AUC increase was similar at the 6- and 17-month demarcations but was decreased between occurrences at the 12 month boundary. Clinically, while it has been observed that recurrent disease is generally more resistant to chemotherapies than primary disease, physicians typically re-treat platinum sensitive patients with platinum-based therapies. The observed reduction in change in resistance at 12 months supports this common clinical practice.

Although the clinically administered treatments, as well as related clinical outcomes, were not obtained for the patients' tumors included in this study, the assumptions regarding first-line and recurrent treatments are supported by consistent guideline recommendations, including use of a platinum/taxane combination as part of the primary EOC treatment regimen. Furthermore, in a recent primary ovarian study conducted using the assay, 88% of first-line treatments included a platinum and taxane combination, while the remaining 12% received regimens that included at least one taxane agent or one platinum agent (24). Therefore, it is rational to assume that patients' tumors included in this study were treated with platinum and/or taxane agents at the time of primary occurrence, though regimens may have been modified prior to completion in some patients due to lack of response and/or drug toxicity. In addition, although the primary status of the first of the paired tumors received was confirmed as part of the assay protocol (*via* required pathology reports), these same procedures cannot distinguish recurrent tumors as being from first recurrence or from a subsequent recurrence. As a result, analyses related to clinical platinum sensitivity (platinum-sensitive or -resistant) could not be conducted.

Taken as a whole, and matching both clinical and biological expectations, these metachronous pair analyses demonstrate a general increased resistance to chemotherapies over time between primary and recurrent disease, especially for therapies most likely to be previously administered. The analyses also convey that a chemoresponse assay has highest utility when a tumor sample is available immediately preceding a treatment decision. However, if a tumor sample is unobtainable at recurrence, assay results obtained within the prior 17 months may provide utility in effective therapy selection, particularly for therapies not previously administered.

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