

Treatment-Related Protein Biomarker Expression Differs between Primary and Recurrent Ovarian Carcinomas

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Abstract

The molecular characteristics of recurrent ovarian cancers following chemotherapy treatment have been poorly characterized. Such knowledge could impact salvage therapy selection. Since 2008, we have profiled 168 patients' ovarian cancers to determine the expression of proteins that may predict chemotherapy response or are targets for drugs that are in clinical trials for ovarian cancer treatment. Expression of epidermal growth factor receptor (EGFR), HER2, VEGF, ER, c-Met, IGF1R, Ki67, COX2, PGP/MDR1, BCRP, MRP1, excision repair complementation group 1 (ERCC1), MGMT, TS, RRM1, TOPO1, TOP2A, and SPARC was measured by immunohistochemical analyses at Clinical Laboratory Improvement Amendments–certified laboratories. Our univariate analysis of 56 primary and 50 recurrent tumors from patients with advanced stage ovarian serous carcinoma revealed that PGP and ERCC1 were significantly upregulated in recurrent lesions ($P < 0.05$). To determine whether these or any of the other markers were differentially expressed in specimens obtained from the same individual at diagnosis and at recurrence, we analyzed 43 matched tumor specimens from 19 advanced stage ovarian carcinoma patients. We confirmed the expression differences in PGP and ERCC1 that were observed in the cohort analysis but discovered that the expression levels of BCRP, RRM1, and COX2 were also discordant in more than 40% of the matched tumor specimens. These results may have implications both for the use of biomarkers in therapy selection as well as for their discovery and validation. Expression of these and other candidate response biomarkers must be evaluated in much larger studies and, if confirmed, support the need for profiling of recurrent tumor specimens in future clinical trials. *Mol Cancer Ther*; 11(2); 492–502. ©2011 AACR.

Introduction

Ovarian cancer is the most lethal gynecologic malignancy. Worldwide, 224,747 women were diagnosed with ovarian cancer in 2008 and 140,163 died from this disease (1). Most patients diagnosed with advanced stage ovarian cancer respond to first-line platinum–taxane therapy (2), but more than 75% of these patients will ultimately recur. Thus, despite the improvement in survival time with the addition of taxanes to platinum-based chemotherapy regimens, most patients with advanced stage disease (i.e., mostly high-grade serous carcinomas) have recurrences and undergo multiple subsequent regimens of chemotherapy that are mostly palliative. Only 28% of patients with stage III/IV cancer survive 5 years after diagnosis (3).

Genomic profiling analyses have revealed that ovarian carcinomas are molecularly complex and heterogeneous.

In addition to differences between tumors with different histologies (e.g., serous, endometrioid, mucinous, and clear cell; ref. 4), genotypic and phenotypic heterogeneity is also observed in tumors of the same histologic type and grade. Indeed, gene expression analyses of high-grade serous carcinomas obtained from patients before treatment classify them into at least 3 subgroups, which may correlate with different prognostic outcomes (5, 6). It is likely that molecular characteristics of a patient's tumor will be correlated with response to therapy, analogous to what has already been observed in cancers of the breast [e.g., tamoxifen/aromatase inhibitors with expression of the estrogen receptor (ER); trastuzumab with HER2 expression/gene amplification], colon [e.g., epidermal growth factor receptor (EGFR) inhibitors with KRAS wild-type genotype] and, most recently, lung (e.g., crizotinib with Alk rearrangements or mutations). These biomarkers are currently used as companion diagnostics to identify patients who are most likely to respond to these drugs. Interestingly, despite the clinical use of these drugs in breast cancer treatment for more than a decade (trastuzumab) or for more than 30 years (tamoxifen), it has only been recently recognized that expression of these key therapy selection markers may vary significantly in metastases compared with the corresponding primary tumors. In a recent large breast cancer study, 15%, 30%,

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and 5% of the tumors showed discordant expression of ER, progesterone receptor, and HER2 in metastatic versus primary specimens (7), thereby supporting previous observations that *HER2* amplification could be gained during breast cancer progression (8). Such studies have raised questions about the need to evaluate temporally proximal tumor samples for diagnostic markers before selecting therapy. In ovarian cancer, surgical debulking procedures are medically indicated for approximately 30% of patients who relapse, but the other patients do not routinely undergo surgical biopsies that would provide specimens for diagnostic tests and may not have lesions that are easily biopsied. Therefore, studies to address the necessity of such procedures are clearly needed.

There are currently no targeted biological therapies approved for treatment of ovarian carcinoma and no clinically validated molecular markers for the chemotherapeutic drugs that are used. However, there are molecular markers that have been associated in retrospective clinical research studies with responsiveness to the chemotherapies commonly used in ovarian cancer treatment (i.e., platinum, taxanes, pegylated liposomal doxorubicin, gemcitabine, and topotecan). There are multiple reports that correlate marker expression with response to these therapies in ovarian and other cancers (DAZ, BYK, and LKS, review manuscript in preparation), but no clinical

trials have been carried out to prospectively validate these markers. Furthermore, only a few studies have asked whether expression of these and other biomarkers differs in recurrent lesions compared with primary samples. Among these are the reports of elevated P-glycoprotein (PGP) levels in some recurrent ovarian tumors (9–11). However, direct comparisons of the expression of these markers in ovarian tumors present at relapse following chemotherapy treatment and in the primary tumor from the same patient are rare and have typically measured fewer than 3 proteins [i.e., PGP (12), MT (13), or TOPO1, HER2, and Ki67 (14)]. Results from such matched pair analyses of a panel of clinically relevant biomarkers would show whether primary specimens provide the same information as recurrent specimens, or whether biopsies should be taken at recurrence for molecular profiling analysis to inform selection of salvage therapies. Such data could also inform the design of clinical studies that seek to identify and validate companion diagnostics for cancer therapies that are typically evaluated in patients who have been exposed to multiple therapies.

To begin to address this question, we evaluated the expression of protein markers associated with responsiveness to targeted therapies as well as chemotherapeutic agents (Table 1) in an unselected, motivated patient cohort as well as in matched paired primary and recurrent specimens from individual patients. We identified biomarkers

Table 1. Biomarkers: drug targets and drug response correlations

Abbreviations	Biomarker	Drug target ^a	Drug response ^b	Drug(s)
BCRP	Breast cancer resistance protein		Resistance	Topotecan, mitoxanthrone
COX-2	Cyclooxygenase-2	X		Cox-2 Inhibitors
EGFR	Epidermal growth factor receptor	X	Sensitivity	EGFR inhibitors
ER	Estrogen receptor	X	Sensitivity	Antiestrogens, aromatase inhibitors
ERCC1	Excision repair complementation group 1		Resistance	Platinum
HER2	Human epidermal growth factor receptor 2	X	Sensitivity	Trastuzumab
IGF1R	Insulin-like growth factor 1 receptor	X		IGF1R inhibitors
Ki67	Proliferation antigen		Sensitivity	Chemotherapy
c-MET	Met proto-oncogene (hepatocyte growth factor receptor)	X	Sensitivity	c-Met inhibitors
MRP1	Multidrug resistant protein 1		Resistance	Anthracyclines, taxanes, etoposide
MGMT	O-6-Methyl guanine DNA methyltransferase		Resistance	Temozolamide
PGP	P-Glycoprotein, multidrug resistance –1		Resistance	Anthracyclines, taxanes
RRM1	Ribonucleotide reductase regulatory subunit M1	X	Resistance	Gemcitabine
SPARC	Secreted protein, acidic, cysteine rich		Sensitivity	Nab-paclitaxel
TOPO1	Topoisomerase 1	X	Sensitivity	Topoisomerase 1 inhibitors
TOP2A	Topoisomerase 2A	X	Sensitivity	Topoisomerase 2 inhibitors
TS	Thymidylate synthase	X	Resistance	Fluoropyrimidines, anti-folates
VEGF	Vascular endothelial growth factor	X		Angiogenesis inhibitors

NOTE: References for biomarker-response correlations: <http://www.clearityfoundation.org/drugs-and-biomarkers.aspx>.

^aX indicates that biomarker is primary molecular target for indicated drug.

^bDrug response associated with high levels of biomarker protein and/or gene amplification.

that were differentially expressed in primary versus recurrent specimens from 96 patients with advanced stage serous ovarian carcinoma and found that many recurrent lesions differed in their expression of several of these markers relative to primary specimens or previous recurrences.

Materials and Methods

Patients and tumor samples

The patients were an unselected population, who sought molecular profiling assistance from The Clarity Foundation between October, 2008 and August, 2011. Formalin-fixed, paraffin-embedded tissue specimens and patient treatment histories (from medical records or patient-reported information) were obtained after written informed consent. Specimens procured during primary surgical procedures were from ovary and fallopian tube (O) or the peritoneal cavity (P; biopsies from omentum, diaphragm, peritoneum, colon, appendix, cul de sac, side wall). Recurrent cancer specimens were from the peritoneal cavity (M), lymph nodes (LN), or distant organs (MD; lung, liver, and breast). For the cohort analysis, 56 primary tumors (34 ovarian and 22 peritoneal samples) and 50 recurrent tumors (32 peritoneal, 8 distant, and 10 lymph node specimens) from 96 patients with predominantly high grade, advanced serous carcinoma [90 serous and 6 mixed serous-other histology tumors; 6 stage II (IIA-1, IIB-2, and IIC-2), 80 stage III (IIIA-2, IIIB-9, and IIIC-64), 6 stage IV, and 4 unknown] were profiled. All of the patients were treated with standard of care platinum-taxane therapy, and neoadjuvant platinum-taxane therapy was administered to 4 patients. For patients from whom recurrent specimens were procured, the most frequently received chemotherapies were carboplatin, paclitaxel, bevacizumab, cisplatin, liposomal doxorubicin, docetaxel, and gemcitabine. The patient-matched primary and recurrent specimens included tumors of serous, endometrioid, and clear cell histology and adenocarcinoma (details in Table 2).

Immunohistochemistry

Analyses were conducted by 2 Clinical Laboratory Improvement Amendments (CLIA)-certified laboratories (Clariant, Inc. and Caris Life Sciences) to ensure full validation of the protocols and reagents used as well as high reproducibility of the test results over the period of the study. The tests carried out by Clariant, and the corresponding antibodies/sources were EGFR (31G7), VEGF (polyclonal), and COX2 (COX229) from Invitrogen Life Sciences and Ki67 (MIB1) from Dako. The tests carried out by Caris Life Sciences were part of the Target Now molecular profiling system: BCRP (6D171) from Santa Cruz Biotechnology, Inc.; HER2/Neu (4B5), ER (SP1), and IGF1R (G11) from Ventana; excision repair complementation group 1 (ERCC1; 8F1) from Abcam; c-Met (8F11), MRP1 (33A6), TOPO1(1D6), and TOP2A (3F6) from Leica Microsystems; MGMT (MT21.2) and

PGP (C494) from Invitrogen; and RRM1 (polyclonal) from Proteintech Group), SPARC (12251) R&D Systems or (polyclonal) from Exalalpha (with highest scoring results reported), and TS (TS106, Dako). Both laboratories used staining protocols that used either the Ventana Medical Systems, Inc. or the Dako automated staining systems. Following heat-induced epitope retrieval, antibody incubation was for 20 to 40 minutes (antibody specific), and visualization procedure was based on the staining system, that is, Ultraview or Vision Biosystem Novolink Poly-HRP for Ventana; Biocare envision plus horseradish peroxidase Polymer Detection System for Dako. Appropriate positive and negative control specimens and slides were included for all of the proteins tested. All slides were scored manually by board-certified pathologists, and results were reported as percentage of tumor cells that stained positive and intensity of staining (0, 1+, 2+, and 3+). Expression data are represented as histoscores (i.e., product of percentage positive tumor cells and intensity).

Statistical analysis

Comparisons of marker expression in primary and recurrent specimens were carried out with the unpaired Students *t* test with Welch's correction for any groups with unequal variance (GraphPad; Prism 5 software). $P < 0.05$ was considered significant in the univariate analyses. Multiple testing analyses were conducted with the method of Benjamini and Hochberg (15).

Results

Since the end of 2008, we have profiled tumor samples obtained from 168 ovarian cancer patients and maintained a privacy protected database of biomarker expression results with associated treatment histories. The measured biomarkers include therapeutic drug targets as well as proteins correlated with response to chemotherapeutic drugs used in ovarian cancer treatment (Table 1). Protein expression was measured by immunohistochemistry (IHC) because such analysis provides information about the cells within heterogeneous tumor tissue that are the source of expression and also enables protein expression to be evaluated in small quantities of tumor tissue (e.g., core biopsies). The analyses were conducted by CLIA-certified laboratories that have validated the assays and showed the intra- and interday reproducibility of the assay results according to guidelines for clinically used diagnostic tests. For each assay, the pathologic assessment of staining intensity and the percentage of tumor cells with positive staining were represented as an H score.

Our analysis of data from 56 primary and 50 recurrent ovarian serous carcinoma samples revealed significant differences in the expression of ERCC1 and PGP (MDR1) in these 2 groups (Fig. 1A). After correcting for multiple testing with the method of Benjamini and Hochberg (15), the significant differences in ERCC1 ($P = 0.006$) and PGP

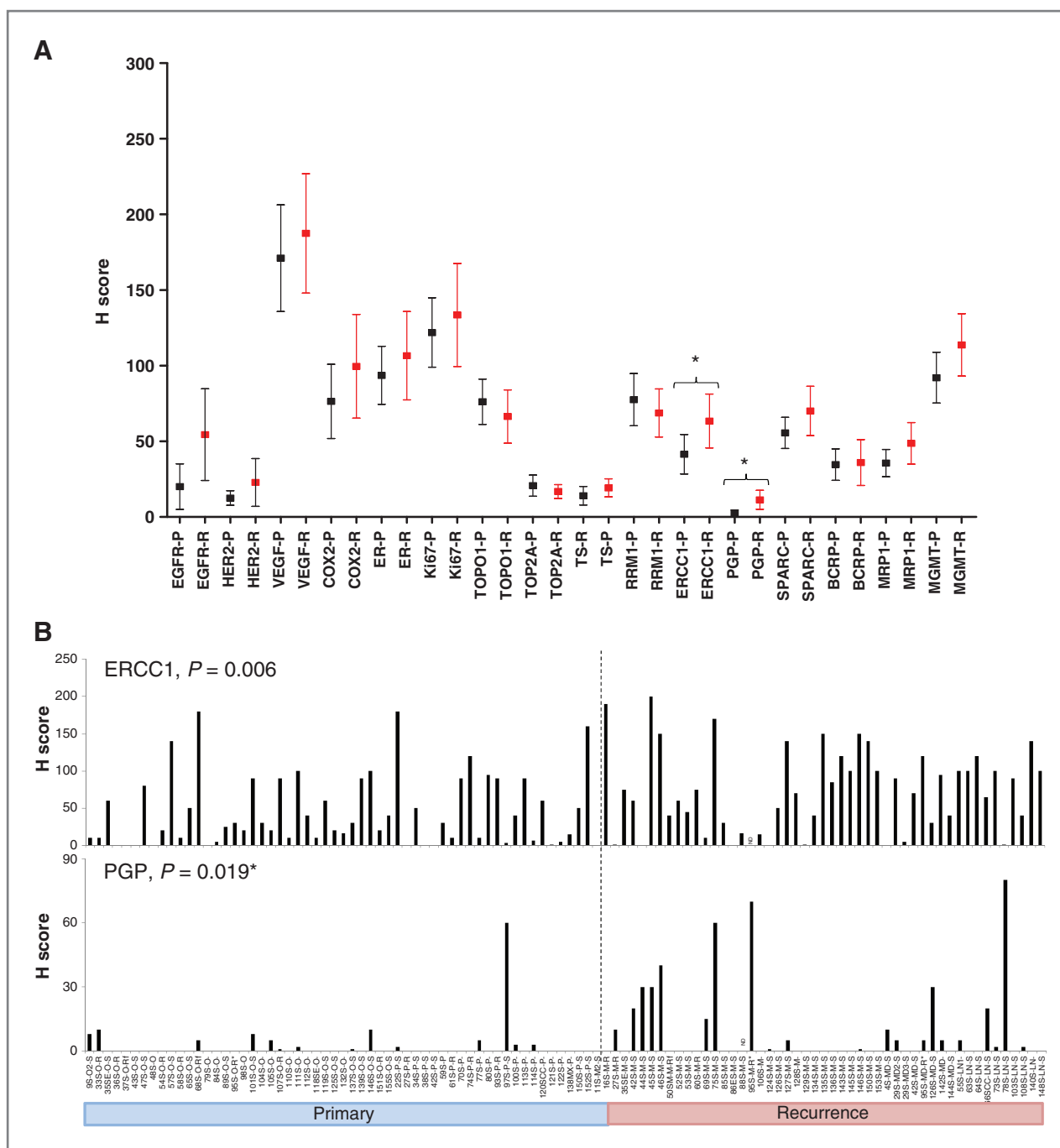


Figure 1. Biomarker expression in primary and recurrent ovarian serous carcinoma specimens. A, expression data for the indicated proteins in 56 primary (black) and 50 recurrent (red) tumor specimens are graphed as H scores. Squares, mean; bars, 95% CI. *, $P < 0.05$, t test (see Materials and Methods). B, levels for the indicated proteins in individual primary [ovary (O) and peritoneal (P)] or recurrent (M or MD) specimens. ND, not determined. *, t test with Welch's correction for unequal variances. Naming convention for samples X-Y-Z, where X is ID# and histotype (S, serous; E, endometrioid; CC, clear cell; M, mucinous; MX, mixed); Y, specimen type; Z, platinum response (S, sensitive; R, resistant; Rf, refractory).

($P = 0.019$) had only a 10% false discovery rate. PGP was detectably expressed in only 25% (14 of 56) of the primary tumors and in 43% (21 of 49) of the recurrent specimens (Fig. 1B). In contrast, ERCC1 was expressed in most of the tumors but the levels were significantly greater in recur-

rences. For PGP, the elevated H scores reflected an increased percentage of tumor cells in which PGP expression was detected, whereas both increased intensity and percentage of positive tumor cells contributed to the increased H scores observed for ERCC1 (data not shown).

Table 2. Characteristics of patients and tumors in analyses of patient-matched specimens

ID No.	Histology	Stage	Platinum response	Specimen tissue site (acquisition date)		Therapies before recurrence ^a
				Primary	Recurrences	
10	Serous	IIIB	Sensitive	Ovary (2006)	Pleural fluid (2008)	None (2008)
11	Serous	III	Sensitive	Ovary/fallopian tube (2006)	Omentum (2008)	UNK (2008)
18	Clear cell	IIIC	Refractory	Ovary/fallopian tube (2007)	Abdomen (2009)	Tam (2009)
26	Serous	IV	Sensitive	Ovary (2005)	Liver (2006); liver (2008)	XL999 → Far → C/T/Far (2008)
27	Serous	IIIC	Resistant	Omentum (2008)	Peritoneal fluid (2010)	C/Dox → Nab/Bev → Top → T/MLN8237 (2010)
29	Serous	IIIC	Sensitive		Lymph node (2005); pleural fluid (2009); pleural fluid (2010)	(Dx 2000) T-M (2005); C/T → C → C/T → Dox (2009); D/G/Bev → Tam (2010)
35	Serous-endometrioid	IIIC	Sensitive	Ovary (2007)	Connective and soft tissue (2011)	T-M (2011)
42	Serous	IIIB	Sensitive	Omentum (2005)	Omentum (1/2010); breast (5/2010)	T-M → C/T → C/T/Bev → Bev-M → Dox (1/2010); G (5/2010)
45	Serous	IIIC	Sensitive	Cul-de-Sac (2007)	Side wall (2009)	C/G/Bev → Bev-M (2009)
51	Clear cell	IIIC	Resistant	Ovary (2009)	Retroperitoneum (2011)	AMG386/Top (2011)
72	Adenocarcinoma	IIIC	Sensitive		Connective and soft tissue (1/2010); connective and soft tissue (12/2010)	(Dx 1997) Ana → T-M → G/Alt/Tam → T/Dox/Bev → Top → Rx → C/Ola (Jan/2010); Bev/Vel/Cx (Dec/2010)
73	Serous	IIIC	Sensitive	Ovary (2004)	Lymph node (2009)	UNK
75	Serous	III	Sensitive	Ovary (2003)	Peritoneum (2010)	D/T → UNK (2010)
95	Serous	IIIC	Resistant	Ovary/fallopian tube (2009)	Pelvis (3/2010); pleural fluid (2011)	None (3/2010) Dox → Bev/Pem (11/2010) → Nab (2011)
96	Endometrioid	IC	Sensitive	Ovary (2003)	Colon (2006); pelvis (2011)	None (2006); C/T → Tax/Afli → Ana (2011)
116	Endometrioid	IIIC	Sensitive		Peritoneum (2009); pelvis (2011)	(Dx 2002) C/Tax → Dox → Let-M → C/G → Let-M (2009); C → Let-M (2011)
126	Serous	II	Sensitive		Colon (2007); chest wall (2010)	(Dx 1994); UNK → (2007); C/T → Rx (2010)
146	Serous	IIIC	Sensitive	Ovary (2008)	Peritoneal fluid (2011)	None (2011)
150	Serous	IIIC	Sensitive	Omentum (2009)	Peritoneum (2011)	Dox → C/Nab (2011)

NOTE: Stage, FIGO; platinum response: refractory, no response or progression; resistant, recurrence less than 6 months; sensitive, recurrence more than 6 months after cessation of initial platinum-taxane therapy.

Abbreviations: C, carboplatin; D, cisplatin; T, paclitaxel; Tax, docetaxel; Nab, nab-paclitaxel; Dox, liposomal doxorubicin; G, gemcitabine; Top, topotecan; Cx, cyclophosphamide; Pem, pemetrexed; Far, farletuzumab; Bev, bevacizumab; Afli, aflibercept; Let, letrozole; Ana, anastrozole; Tam, tamoxifen; Alt, altretamine; Ola, olaparib; Vel, veliparib; M, maintenance; UNK, unknown; Dx, year of diagnosis provided for patients without primary specimens; Rx, radiation treatment.

^aTherapies received after standard first-line platinum-taxanes.

Because these biomarkers are associated with response to taxanes/anthracyclines and platinum, respectively, a change in their expression levels in tumors present at relapse could impact clinical responses and selection of second line and subsequent salvage therapies. Importantly, these data suggest that the primary tumor molecular profile may not be representative of the tumors present at relapse.

Expression of a subset of biomarkers is frequently altered in patient-matched recurrent versus primary tumors

We asked whether these or other differences between recurrent and primary lesions would be observed if

the corresponding specimens from the same patient were compared. We evaluated expression of these biomarkers in 34 matched primary and recurrent specimens from 15 ovarian cancer patients and 9 matched recurrences from 4 additional patients. Most of these patients were diagnosed with stage IIIC serous histology ovarian carcinoma and were initially sensitive to platinum-based chemotherapy (Table 2). Ovarian tissue was the source of most of the primary specimens, whereas the recurrent tumor samples were primarily within the omentum, peritoneum, connective tissue, or colon, but a few of the specimens were lymph nodes, liver, breast, or peritoneal/pleural fluids (Table 2). The expression levels for each marker were determined by IHC analysis for each

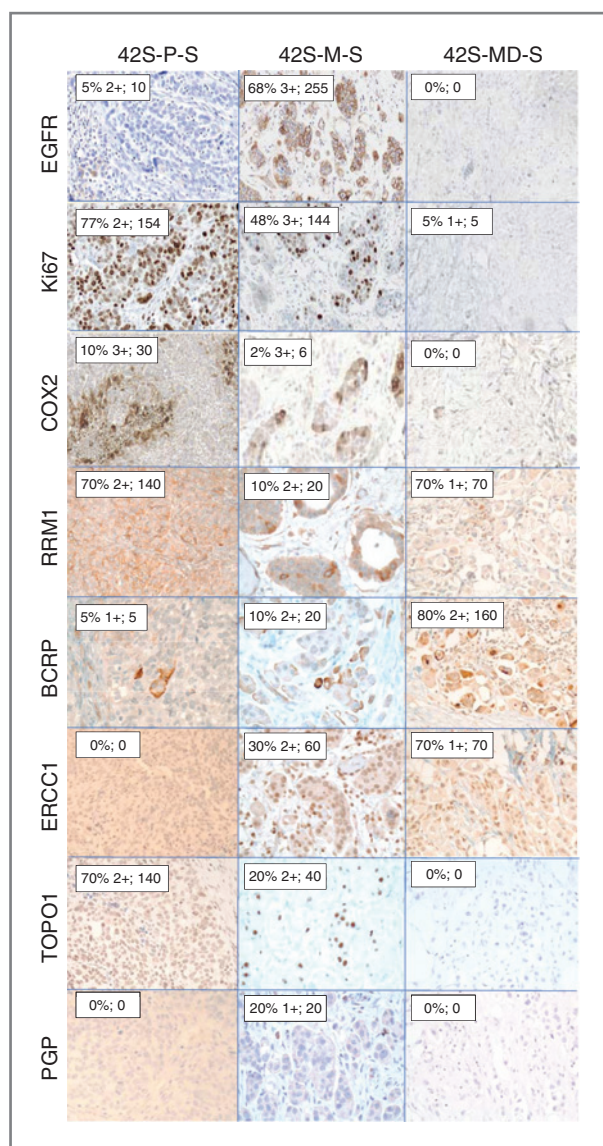


Figure 2. Immunohistochemical detection of biomarkers evaluated in this study. Representative photomicrographs from IHC staining for the indicated markers in specimens obtained from the primary peritoneal specimen (42S-P-S) and 2 recurrences in the omentum (42S-M-S) and breast (42S-MD-S) of patient 42S. Inset boxes, pathology assessment: percentage of positive tumor cells and intensity of staining (0–3+); histoscore (0–300).

specimen (see example in Fig. 2), results compared for each patient-matched pair of specimens, and the relative similarity of expression was assessed by determining the frequency with which the H scores for each pair differed by more than 4-fold. In cases in which only recurrent lesions were examined, the similarity of those specimens to each other was evaluated. The expression of some of the markers was relatively constant in the paired samples whereas expression of others was highly variable (Fig. 3). Differences in MGMT, HER2, and ER levels of more than 4-fold were observed in only rare cases, whereas ERCC1,

PGP, BCRP, and COX2 expression differed by more than 4-fold in more than 45% of the paired specimens. Importantly, the increased ERCC1 and PGP levels that were detected in the cohort analyses of primary versus recurrent serous ovarian tumors (Fig. 1) were also observed in many of the patient-matched tumors (Figs. 3 and 4), even though some of these tumors were from patients with endometrioid and clear cell ovarian carcinoma. However, although ERCC1 and PGP levels increased in multiple recurrent specimens compared with the corresponding primary specimens, expression of these proteins was surprisingly decreased in recurrent tumors in some patients relative to the expression observed in either the primary or in earlier recurrences (Figs. 3 and 4: 42S, 96E). It is also noteworthy that the specimens with the highest levels of EGFR were from recurrent tumors (Figs. 3 and 4: 27S, 42S, 95S). Elevated EGFR levels were also found in recurrent tumors in the cohort analysis (Fig. 1) but that observation did not reach statistical significance ($P = 0.058$; Student's t test with Welch's correction).

Unique marker profiles in primary and metastatic tumors from the same individual

The protein expression profiles for each of the patient-matched samples were also compared with each other to determine whether marker expression variability was more frequently observed in some patients than in others and whether some markers were coordinately regulated. For the majority of the patients, expression of most of the proteins in the profile was consistent between the matched-paired tumor samples (Fig. 4 and data not shown). There was a range of marker expression variability across the tumors that were profiled. For tumors from some patients (e.g., 35SE and 116E), we observed differences of more than 4-fold in only one or two markers, whereas for other patient tumors (e.g., 27S and 42S; Fig. 4), multiple markers met those variability criteria. Both increases and decreases in marker protein expression were observed in most of the matched tumor pairs, and there was no evidence of marker coregulation in this small sample cohort.

Despite the altered expression of some markers, the protein expression pattern for individual tumors most closely resembled the pattern of protein expression in other tumors from the same patient. For example, there was remarkable similarity in the profiles of the primary tumor and 2 recurrences that were obtained from patient 96E 3 and 5 years after her diagnosis with stage 1C endometrioid ovarian cancer (96E; Fig. 4). In contrast, profiles were more discordant for the primary and 2 recurrences obtained within 4 months of each other from patient 42S (Figs. 2 and 4) who was diagnosed with stage IIIB serous carcinoma disease 5 years earlier. For patient 96E, the recurrent specimens were obtained at first relapse and after 2 cycles of taxane-based therapy, an antiangiogenic drug, and an aromatase inhibitor, whereas for patient 42S, both recurrent specimens analyzed were obtained after multiple rounds of platinum–taxane, an

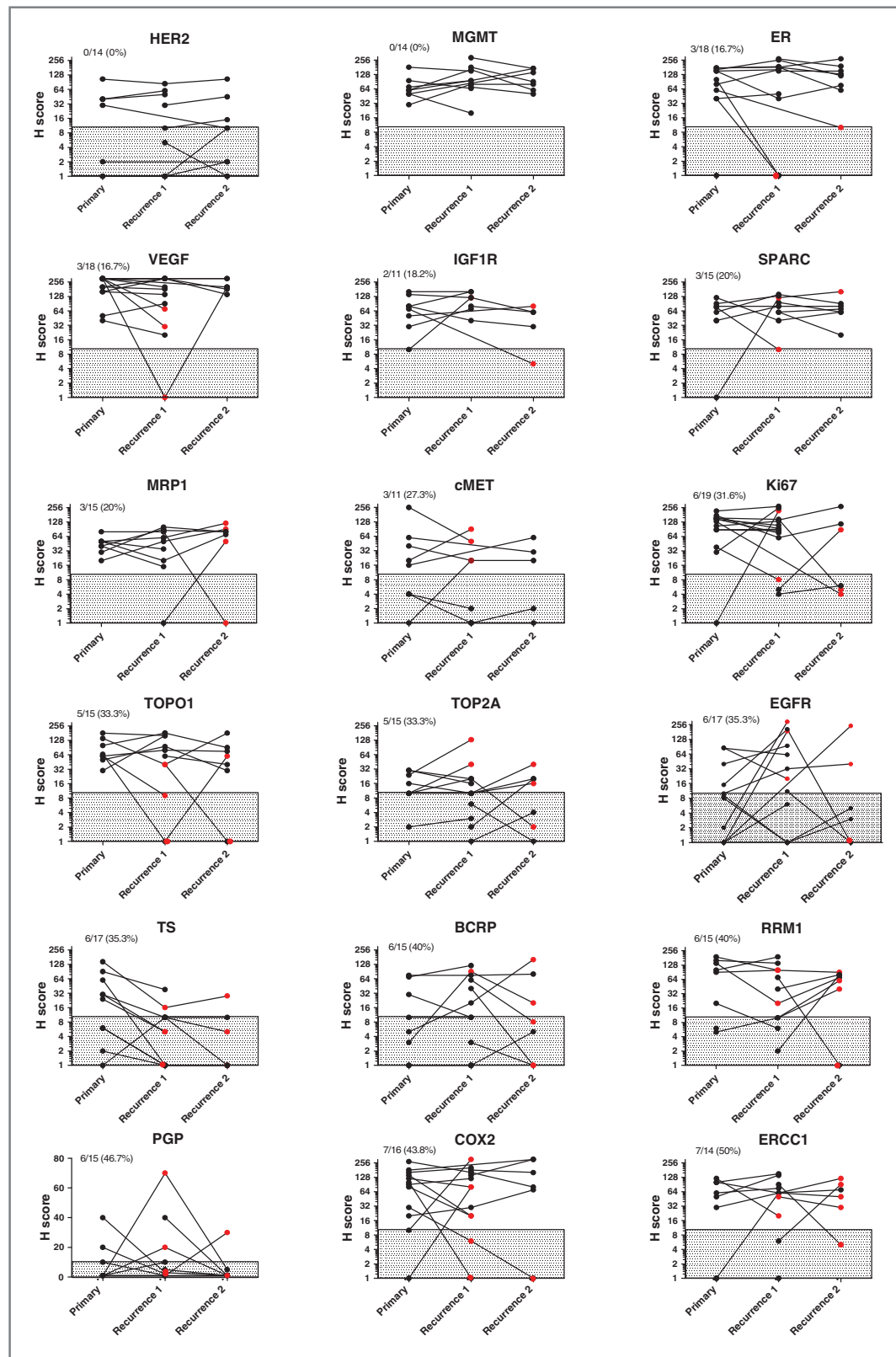


Figure 3. Expression of biomarker panel in matched primary and recurrent tumor specimens. Protein expression levels (H scores) for primary and matched recurrences (1 and 2) ordered by specimen acquisition date are connected by lines. Red circles, recurrent specimens with protein expression level that differ by at least 4-fold from primary (or previous recurrence); fraction, number of specimens with 4-fold or more variation in expression from matched sample/total; paired samples with H scores of 10 or less (within range of hatched box) were not considered in this measurement.

Figure 4. Protein expression profiles for selected patient-matched tumor specimens. Expression (histoscore) of each protein is graphically represented for each of the tumors profiled. Arrows, markers with more than 4-fold increased (red) or decreased (green) expression relative to the primary tumor or prior recurrence.

Sample ID	Specimen source	EGFR	HER2	IGF1Rb	c-MET	VEGF	COX-2	ER	Ki-67	TOP1	TOP2A	TS	RRM1	ERC1	PGP	SPARC	BCRP	MRP1	MGMT
27S-P-R	Omentum (2008)	0	40	ND	ND	300	100	0	52	60	30	24	160	0	0	75	30	40	180
27S-M-R	Peritoneal fluid (2010)	↑188	50	ND	ND	200	0	0	122	9	16	5	140	1	10	↓10	10	15	150
42S-P-S	Omentum (2005)	15	ND	ND	ND	300	30	160	154	140	10	6	140	0	0	0	0	20	60
42S-M-S	Omentum (1/2010)	↑204	5	ND	ND	0	6	255	144	40	20	0	20	60	↑0	↑120	↑20	50	160
42S-MD-S	Breast (5/2010)	0	0	ND	ND	200	0	120	5	0	0	0	70	↑70	↑160	↑160	↑90	30	30
95S-O-R	Ovary/FT (2009)	0	0	50	16	300	180	60	130	65	10	6	5	↑30	0	95	3	30	50
95S-MD-R	Pleural fluid (3/2011)	↑240	2	30	30	200	300	10	4	30	20	0	30	↑120	5	90	↑20	50	140
146S-O-S	Ovary (2008)	ND	2	160	4	ND	ND	100	ND	100	24	160	100	100	10	90	10	50	95
146S-M-S	Peritoneal fluid (2011)	ND	2	160	1	ND	ND	0	ND	180	130	1	190	150	1	130	35	65	65
150S-P-S	Omentum (2009)	0	30	30	0	300	160	40	30	30	10	0	6	50	0	80	0	80	30
150S-M-S	Peritoneum (2011)	0	10	70	20	300	200	50	249	160	40	10	100	↑141	0	80	0	80	80
126S-M-S	Colon (2007)	11	30	80	0	300	160	180	4	60	1	1	2	05	0	40	0	0	180
126S-MD-S	Chest wall (2010)	0	45	60	2	300	80	270	6	40	4	0	30	↑06	↑06	30	5	50	30
35SE-O-S	Ovary (2007)	8	0	80	20	50	120	150	88	60	2	2	20	60	0	70	0	40	70
35SE-M-S	Connective/soft tissue (2011)	1	0	160	90	90	80	160	90	80	3	1	9	57	0	120	0	90	95
96E-O-S	Ovary (2003)	ND	105	70	60	ND	ND	150	ND	50	16	30	90	100	40	40	75	50	60
96E-M-S	Colon (2006)	0	84	ND	ND	300	180	40	5	95	10	10	99	09	3	80	0	20	95
96E-M2-S	Pelvis (2011)	5	105	5	30	300	160	75	88	30	16	5	96	05	0	80	80	70	170
116E-M-S	Peritoneum (2009)	0	1	120	1	300	140	150	140	80	10	10	0	06	0	60	3	60	80
116E-M2-S	Pelvis (2011)	3	10	60	0	140	300	150	267	75	40	10	40	5	0	70	0	120	30
51CC-O-R	Ovary (2009)	0	40	ND	ND	160	270	40	144	180	30	30	191	120	20	120	70	50	50
51CC-M-R	Retroperitoneum (2011)	6	60	ND	ND	300	160	0	108	160	20	5	100	↓02	2	90	120	60	20

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antiangiogenic agent, and an anthracycline (Table 2). Therefore, both time after initial diagnosis and the number of chemotherapeutic modalities are likely to impact the extent of marker expression variation in recurrent specimens.

Discussion

In this study, we have evaluated the expression of a panel of drug targets and candidate drug response biomarkers in primary and recurrent ovarian cancer specimens using both a population-averaging and an individual-centric approach. Our cohort analysis of 96 primary and recurrent specimens obtained from mostly advanced stage serous ovarian cancer patients revealed significant differences in the expression of 2 key biomarkers that are associated with treatment responses to some of the therapies currently used in ovarian cancer treatment. The individual-driven approach that evaluated biomarker expression in primary tumors and recurrences from the same patient confirmed these differences. However, differences in expression of other markers in the panel were revealed by analysis of patient-matched specimens but not in the cohort study, suggesting limitations of population-averaging approaches for biomarker evaluation.

The levels of PGP and ERCC1 proteins were significantly increased in recurrent tumors from many serous ovarian cancer patients. These observations are particularly relevant because these biomarkers are associated with resistance to chemotherapies [i.e., PGP for taxanes and anthracyclines (10) and ERCC1 for platinum (16)] used in the treatment of ovarian cancer. Previous investigators have also reported increased expression of PGP in recurrent ovarian cancers (9, 10), although other studies did not show elevated levels in recurrent tumors (12, 17, 18). The reasons for these disparate results may be due to differences in the patient disease characteristics, sample size, assay type, and conditions (e.g., mRNA vs. protein, antibody, and methodology), cut points defined for high PGP expression, or type of prior chemotherapy. Indeed, induction of PGP expression in tumors following taxane (19) or doxorubicin (11) treatment has been documented. All of the patients in our cohort received standard of care platinum–taxane therapy at diagnosis, but many of the tumor specimens were not first recurrences, so effects of subsequent rounds of chemotherapy on marker expression cannot be excluded. The observation that some recurrent tumors have a higher percentage of PGP-expressing tumor cells than primary tumors is also consistent with a selection and proliferation of rare PGP-positive tumor cells from the primary lesions appearing in the recurrent setting. In this regard, it is noteworthy that increased levels of PGP were found in the side population (i.e., putative cancer stem cells) isolated from platinum-resistant ovarian tumors, although no correlation with taxane response was shown in that study. High levels of ERCC1 protein and mRNA have been correlated with resistance to platinum-based chemotherapies in ovarian and other

cancers (16). Increased ERCC1 expression has been observed in metachronous relative to either synchronous metastases or primary colorectal cancers (20), but, to our knowledge, has not yet been reported in recurrent ovarian tumors. Elevated levels of ERCC1 and PGP could play a role in the inverse relationship between response rates to subsequent platinum treatment and the length of time before progression following platinum-based therapy (21) or in the increasingly short duration of therapeutic responses observed following each subsequent recurrence.

Our comparison of biomarker expression in patient-matched primary and recurrent tumor samples revealed that the levels of some proteins (i.e., MGMT, ER, and HER2) were very similar in the matched specimens, whereas expression of ERCC1, PGP, RRM1, BCRP, and COX2 differed in more than 40% of the paired specimens. These data for altered expression of ERCC1 and PGP in matched primary versus recurrent lesions are consistent with the population-averaging analyses in the cohort study, although some of the recurrences in the paired tumor samples expressed lower levels of these proteins than the corresponding primaries. Such inconsistency in the direction of the expression change for some markers in primary versus recurrent specimens could explain the observation that BCRP and COX2 expression differences were only apparent in paired analyses. In addition to the differences noted in recurrences compared with primary specimens, we found that expression of some of the markers (i.e., PGP as well as EGFR, BCRP, RRM1, and TOPO1) also differed in recurrent specimens taken from the same patient at different times during the course of disease. These differences may reflect alterations in gene expression programs that are dependent on the site of the tumor as suggested by genomic profiling studies of primary ovarian and omental lesions (22) or the genotypic divergence observed in multiple metastases derived from matched primary tumors obtained from untreated patients (23). In our study, the recurrences tested were obtained from patients who had received 1 to 5 prior therapies, so it is also possible that the chemotherapy regimen received in the intervening time period either induced/repressed expression of these proteins or selected for a preexisting tumor cell population with an altered expression of that marker. As one example, patient 42S was treated with gemcitabine between the 2 recurrences that were profiled and her postchemotherapy specimen contained elevated levels of RRM1 relative to the pregemcitabine specimen. As this enzyme is the target for gemcitabine that inversely correlates with drug response in some cancers (24), increased RRM1 in the recurrence is consistent with increased proliferation of tumor cells resistant to gemcitabine.

Differences in the expression of response prediction biomarkers in the most proximal recurrent tumor specimens compared with prior recurrences or to primary tumor samples will only impact clinical decision making if the marker expression is at a level that results in a

change in the treatment recommendation (e.g., ER positive vs. ER-negative status would impact a decision for tamoxifen or aromatase inhibitor treatment). For the protein markers evaluated in this study, there are not yet clinically validated expression cut points that are used to assign therapy in ovarian cancer. Future studies are needed to establish the cut points for these markers and prospectively show their clinical predictive ability so that the clinical implications of the differences described here between primary and recurrent specimens can be determined.

Despite the discordant expression of certain markers in each matched tumor pair, the overall biomarker profiles for the matched patient specimens were very similar. For the few cases in which primary tumors were compared with 2 recurrences, we observed that the recurrences had distinct marker profiles that distinguished them from each other but that their profiles shared substantial similarity with the primary lesion. In one of these cases (i.e., 42S), the 2 recurrent specimens obtained within a few months of each other were markedly discordant, thereby suggesting that coexisting recurrent lesions may be molecularly different and raise critical questions about which and how many lesions should be biopsied and analyzed to establish a treatment plan.

Our observations also raise questions about approaches to drug response biomarker identification/validation that use primary tumor specimens and attempt to correlate clinical responses with those results. Given the number of prior therapies and recurrences experienced by patients who enroll in early clinical trials and our results suggesting that expression of many biomarkers may differ in recurrent versus primary lesions, it is not surprising that many biomarker correlation analyses have failed or have reached only borderline statistical significance when they have used archival specimens.

Finally, the immunohistochemical analyses used to determine the expression levels of the proteins in this panel were all carried out by CLIA-certified laboratories that have validated the individual assays and shown intra- and interday reproducibility in accordance with guidelines for clinically-used diagnostic tests. We have not, however, evaluated the potential contributions of

sample acquisition methods, preservation, processing, or storage on the reliability of these assays. These variables can impact the immunoreactivity of some antigens and could therefore lead to differences in the measured expression levels. It is likely that many of the differences are due to biological variation as there is increasing evidence for genomic heterogeneity both within tumors and in metastatic lesions. Given this heterogeneity, it is also possible that tumor sampling or the portion of the specimen evaluated could contribute to differences in the levels of antigen detected. In the future, we expect that standardized methods for specimen acquisition and processing will be universally implemented across hospitals and pathology laboratories to ensure reproducible evaluation of every biomarker that is used in therapy decision making.

In summary, recurrent lesions have different protein expression profiles of drug targets and candidate drug response markers from their corresponding primary lesions and from prior recurrences. These differences may be sufficiently large as to impact selection of therapy. These pilot data must be validated in a larger study, and if confirmed, support the need for profiling recurrent tumor specimens rather than archival blocks in future clinical trials.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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