

ORIGINAL ARTICLE

Biobehavioral factors predict an exosome biomarker of ovarian carcinoma disease progression

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Abstract

Background: Biobehavioral factors such as social isolation and depression have been associated with disease progression in ovarian and other cancers. Here, the authors developed a noninvasive, exosomal RNA profile for predicting ovarian cancer disease progression and subsequently tested whether it increased in association with biobehavioral risk factors.

Methods: Exosomes were isolated from plasma samples from 100 women taken before primary surgical resection or neoadjuvant (NACT) treatment of ovarian carcinoma and 6 and 12 months later. Biobehavioral measures were sampled at all time points. Plasma from 76 patients was allocated to discovery analyses in which morning presurgical/NACT exosomal RNA profiles were analyzed by elastic net machine learning to identify a biomarker predicting rapid (≤ 6 months) versus more extended disease-free intervals following initial treatment. Samples from a second subgroup of 24 patients were analyzed by mixed-effects linear models to determine whether the progression-predictive biomarker varied longitudinally as a function of biobehavioral risk factors (social isolation and depressive symptoms).

Results: An RNA-based molecular signature was identified that discriminated between individuals who had disease progression in ≤ 6 months versus >6 months,

independent of clinical variables (age, disease stage, and grade). In a second group of patients analyzed longitudinally, social isolation and depressive symptoms were associated with upregulated expression of the disease progression propensity biomarker, adjusting for covariates.

Conclusion: These data identified a novel exosome-derived biomarker indicating propensity of ovarian cancer progression that is sensitive to biobehavioral variables. This derived biomarker may be potentially useful for risk assessment, intervention targeting, and treatment monitoring.

KEYWORDS

biobehavioral, exosome, ovarian cancer, progression, social support, transcriptome

INTRODUCTION

Ovarian cancer is most commonly diagnosed at an advanced stage, and most patients show clinical disease progression within 5 years despite primary cytotoxic chemotherapy.¹ In addition to clinical and molecular aspects of the tumor,² patient biobehavioral characteristics such as depression and social isolation have been linked to differential disease progression.³⁻⁵ Preclinical studies have identified multiple biological pathways by which biobehavioral characteristics influence key mechanisms that promote tumor growth and progression including neuroendocrine-mediated effects on angiogenesis, inflammation, invasive capacity, and epithelial-mesenchymal polarization.⁶⁻¹¹ However, the translation of these mechanistic observations to clinical settings has been hampered by lack of access to residual tumor tissues and their microenvironmental context following primary surgical resection. To facilitate investigation of how biobehavioral factors may influence postsurgical tumor progression, we sought to develop an easily accessible biomarker that will permit assessment of changes in the tumor over time without the invasiveness of repeat biopsies.

Biological profiling of plasma exosomes is an emerging technology for in vivo monitoring of tumor biology and response to treatment and is frequently characterized as a noninvasive liquid biopsy.^{12,13} Exosomes are nano-sized cell-derived vesicles with genomic properties of their parent cells that are released into the extracellular environment and can propagate signaling both locally and at a distance.¹² Examination of the molecular content of plasma-derived exosomes provides a promising strategy for development of a progression-related biomarker to probe the biology of cancer as it continues to unfold posttreatment.¹⁴ In the context of biobehavioral influences, we previously found that plasma exosomes from socially isolated ovarian cancer patients showed greater mesenchymal-characteristic RNA profiles,¹⁵ suggestive of epithelial-mesenchymal transition (EMT), and paralleling results from analyses of resected tumor RNA profiles.¹⁶ The ready availability of plasma samples provides an opportunity for longitudinal analyses to clarify how changes in biobehavioral processes (e.g., resulting from interventions) might change ongoing biological risk processes in the aftermath of primary

surgery. However, such analyses would require a validated exosomal RNA biomarker of disease progression risk.

Here, we developed a molecular biomarker to predict the future progression of ovarian cancer based on exosomal RNA in a subset of patients with available progression data. Then, in a separate subset of patients we tested whether changes in social isolation and/or depressive mood were associated with changes in this disease progression propensity biomarker.

MATERIALS AND METHODS

Participants

Women with suspected ovarian cancer were recruited from three University Medical Centers, two in the Midwest and one in Florida, as part of a larger study examining biobehavioral factors and tumor progression in ovarian cancer. Inclusion criteria included primary invasive epithelial ovarian, peritoneal or fallopian tube carcinomas, or carcinosarcoma. Histology was confirmed by pathology review. Patients were excluded who were under 18 years of age, had a history of previous cancer within the last 5 years, a comorbid condition with known immune effects, current pregnancy, regular use of systemic steroid medication in the last month, metastases to the ovaries from other organs, or inability to answer questions; these exclusion criteria were established for the larger study. Patients recruited before 2013 were excluded for a history of cancer; after that date, patients were only excluded for cancer in the last 5 years. Data from a subgroup of 90 women were used in discovery analyses to establish a molecular measure predicting progression-free survival. Of these 90 exosome samples, 78 yielded valid RNA sequencing data (12 had either insufficient RNA or RNA that was too severely degraded to yield useful results) and two had recurrence data that was nonevaluable, yielding a final sample of 76 for the first subgroup. Data from a second subgroup of 31 women were used to test whether this molecular signature showed longitudinal elevation as a function of biobehavioral risk factors. Several individual samples failed to yield valid RNA data, but RNA

data were available on at least one of the time points for all 31 individuals. However, some individuals were missing data on other measures that were the targets of analysis (social support, depression) or covariates that reduced the analyzable number of cases to 24 in the case of social support and 22 in the case of depression. Therefore, the final sample was 24 for the second subgroup. For the first subgroup of patients, the earliest date of treatment initiation was in December 2004, and all progression information was censored on December 31, 2016 or last contact before that time, and primary treatment began at least 3 years before the date of censoring. Treatment for the second subgroup was initiated between April 15, 2016 and September 25, 2017. All procedures were approved by institutional review boards at the clinical sites, and all patients provided signed informed consent.

Procedures

Participants were recruited at an initial clinic visit and completed psychosocial assessment and demographic information at home within approximately 2 weeks before surgical resection or initiation of neoadjuvant therapy (NACT). Blood sampling was done the morning of surgery or NACT and during the patient's 6-month and 12-month clinic follow-ups. Follow-up psychosocial surveys were administered in conjunction with the 6- and 12-month visits. Social support was assessed using the attachment subscale of the Social Provisions Scale (SPS-Attachment).¹⁷ This subscale measures perceived emotional connection with others and has been the measure most closely associated with disease-related biomarkers in our previous research. Based on prior studies,^{3,18-20} we defined poor social support as an SPS-Attachment score of less than 15 (the median value). Depressive symptoms were assessed by the Center for Epidemiological Studies Depression Scale (CES-D), a 20-item self-report scale measuring frequency of depressive symptoms over the last week.²¹ The CES-D is considered a valid and reliable assessment for depressive symptoms in cancer patients.²² Scores 16 or higher are considered to be consistent with clinical depression²³; therefore patients were categorized as having high/low depressive symptoms based on this cutoff.

Clinical disease characteristics (e.g., grade, stage, histology, body mass index [BMI], and date of progression) were extracted from medical records. Demographic and clinical characteristics (age, race, alcohol use, smoking, and BMI) were assessed as potential confounding factors. Alcoholic beverages were coded as ≤ 2 drinks/day (0) versus ≥ 3 drinks/day (1); smoking was coded as never (0) versus ever (1). Progression-free survival (PFS) was calculated between date of tumor resection or initiation of neoadjuvant chemotherapy and date of the first recurrence or progression, or last recorded contact before the date of censoring when the patient had not progressed, or date of death.²⁴ Progression was documented by computed tomography, initiation of new therapy, or clinical evidence of progression. The 6-month cutoff was defined as 190 days or less (see Figure S1 for a summary of study time points).

Exosome RNA profiling analyses

Detailed information about exosome data extraction and RNA profiling is provided in the Supporting Methods. For the discovery-based biomarker development analyses for study 1, normalized transcript abundance values were screened to identify all gene transcripts showing $r > 0.40$ association with disease progression class (1 = PFS ≤ 6 months; 0 otherwise) using SAS PROC CORR. The resulting 30 transcripts served as predictor features for the elastic net machine learning algorithm implemented in SAS PROC GLMSELECT, using progression class as the criterion with predictor selection by random 5-fold external cross-validation, 40 selection cycles, and L2 tuning by log grid search over the interval 0 to 1. The elastic net is a commonly used method for deriving predictors when the number of candidate variables greatly exceeds the number of cases.²⁵ The resulting composite biomarker (continuous value) was a linear combination of 22 elastic net selected genes; this was analyzed for point biserial correlation with disease progression class using SAS PROC CORR, and dichotomized at a cost-weighted value of 0.25 (i.e., assuming it was three times as important to identify a high-risk case as to misidentify a low-risk case) to quantify association with disease progression class using SAS PROC FREQ. SAS PROC GLM was used for linear model analyses relating biomarker values to disease progression class while controlling for covariates. PROC MIXED was used for mixed effect linear model analyses quantifying longitudinal change in biomarker values from presurgical baseline to 6- and 12-months postsurgery while controlling for nonindependence of repeated measures using a fully saturated (unstructured) variance-covariance matrix on residuals.

Study 2 analyses (biobehavioral prediction analyses) quantified associations between longitudinal variation in progression biomarker values and biobehavioral risk factors in a separate data set. Gene expression data were derived as described above for study 1, with the multi-gene progression biomarker scored using SAS PROC GLMSELECT to apply the algorithm developed in study 1. SAS PROC MIXED was then used for mixed effect linear model analyses examining the association between biobehavioral risk factors (social isolation and depressive symptoms) over time and longitudinal variation in biomarker values from presurgical baseline to 6- and 12-months post-surgery while controlling for clinical characteristics (stage, grade, and histological subtype) and patient demographics (age, BMI, race, and smoking history) and accounting for nonindependence of repeated measures using a fully saturated (unstructured) variance-covariance matrix on residuals.

RESULTS

As shown in Table 1, most patients had advanced-stage (83%), high-grade (91%), serous (79%) cancer. The average age was 62 (± 10 , range 27–85) years and 95% of patients were White non-Hispanic (two African American and three White Hispanic). The average BMI was 27.8 (± 6.2 ; range, 17.7–50.3). Average social support was below

TABLE 1 Demographics and clinical characteristics

Characteristic	Mean (SD) or n (%)
Age, mean (SD) years (n = 100)	61.7 (\pm 10.4) years; range, 27–85 years
Race	
White	98 (98.0%)
Black	2 (2.0%)
Ethnicity: Hispanic	3 (3.0%)
Marital status	
Married/with partner	70 (70.0%)
Education (n = 99)	
High school or less	40 (40.0%)
Trade school/some college	31 (31.0%)
College grad/postgraduate	28 (28.0%)
Body mass index	27.74 (\pm 6.18); range, 17.72–50.26
Ever smoked	25 (25.0%)
Alcohol use (>2 drinks) (n = 98)	0 (0.0%)
Stage	
I	3 (3.0%)
II	5 (5.0%)
III	83 (83.0%)
IV	9 (9.0%)
Grade	
High	91 (91.0%)
Serous	79 (79.0%)
Cytoreduction	
Suboptimal	18 (18.8%)
Neoadjuvant therapy	3 (3.0%)
Time to progression (n = 76) ^a	
\leq 6 months (\leq 190 days)	13 (17.1%)
>6 months (236 days–10.47 years)	63 (82.9%)
Mean time to progression (n = 76) ^a	2.61 (\pm 2.66) years (range, 30 days–10.47 years), 0.95 years median
Social support ^b	
Baseline (n = 24)	14.54 (\pm 1.89)
6 months (n = 23)	13.96 (\pm 2.62)
12 months (n = 22)	14.45 (\pm 2.40)
Depression ^b	
Baseline (n = 25)	17.16 (\pm 12.67)
6 months (n = 23)	11.70 (\pm 8.54)
12 months (n = 22)	10.73 (\pm 9.66)

^aStudy 1 subgroup only.^bStudy 2 subgroup only.

the median and remained relatively constant over time, with at least 34% of participants showing substantial social isolation (SPS-Attachment scores <15) at each time point. Average depressive symptoms were in the mildly depressive range, 15.4 (± 10.4) at study entry and remained at a mild level over time.

Study 1: discovery analysis identifying an exosome RNA biomarker of ovarian cancer progression

To define a “liquid biopsy” measure of disease progression propensity in ovarian cancer, we conducted machine learning analyses of genome-wide messenger RNA (mRNA) profiles in plasma exosome samples collected pretreatment from 76 women with known disease progression times. Cases were classified as short PFS (≤ 6 months; $n = 13$, 17%) versus longer PFS (>6 months; $n = 63$, 83%) and the elastic net machine learning algorithm was applied to identify a composite biomarker based on pre-surgical exosome RNA profiles that optimally predicted subsequent postsurgical progression class (i.e., short PFS vs. longer PFS). Results of this analysis identified a 22-gene predictive biomarker that was strongly correlated with progression class ($r = 0.95$; $p < .0001$) and correctly classified 98.6% of cases (75 of 76) with good sensitivity (92%) and specificity (100%). In multi-variable linear model analyses, the biomarker’s predictive accuracy emerged above and beyond the effects of disease grade and histological subtype (serous vs. non-serous) as well as age, BMI, race, and smoking history (adjusted $r = 0.95$, $p < .0001$). The 22-gene composite biomarker was approximately 2.4-fold greater in cases with a short PFS compared to those with a longer PFS (difference: 1.25 log₂ mRNA abundance units \pm SE 0.07, $t(65) = 17.95$, $p < .0001$) (Figure 1). As would be expected for a valid measure of disease progression, additional plasma samples collected longitudinally from the same patients at 6 and 12 months postsurgery showed elevated biomarker levels compared to pretreatment baseline ($F [2|122] = 132.65$, $p < .0001$) with a modest increase over the first 6 months (difference: 0.07 ± 0.06 log₂ mRNA abundance, $t(122) = 1.19$, $p = .2346$) and a more substantial increase by 12 months (difference: 1.37 ± 0.09 , $t(122) = 15.08$, $p < .0001$). The overall association of progression biomarker levels with time since surgery was strong, $r = 0.70$ ($p < .0001$).

Study 2: biobehavioral influences on exosome progression biomarker

After deriving a “liquid biopsy” measure of disease progression biology in study 1, we next sought to use that measure in longitudinal analyses assessing how biobehavioral risk factors (social isolation and depressive symptoms) might affect disease progression biology in the interval between surgery/neoadjuvant treatment and subsequent clinical progression. To ensure that these analyses did not capitalize on chance by using the same data set to derive the biomarker

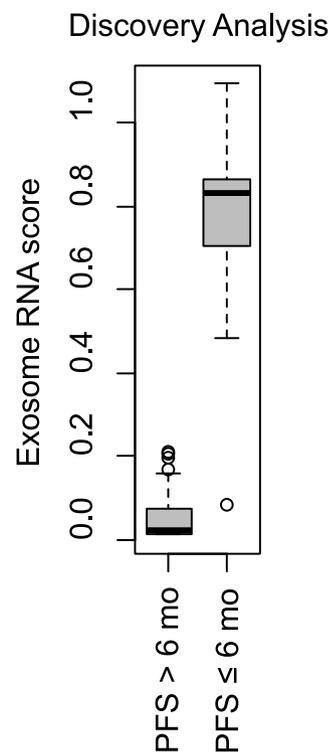


FIGURE 1 Discovery analysis: exosome RNA composite score for ovarian cancer patients who progressed in ≤ 6 months versus >6 months

outcome and to test its association with other potential predictors, these analyses were conducted in a second subgroup of 31 cases that were collected from the same clinical context as in study 1 but for whom clinical disease and survival end points were not yet available. In these analyses, mixed effect linear models analyzed changes in exosome RNA biomarker activity from pre-surgical baseline to 6- and 12-months post-surgery, and additionally tested whether social isolation or depressive symptoms might be associated with higher levels of the biomarker while controlling for potentially confounding effects of disease stage, tumor grade, tumor histological subtype, age, BMI, race, and smoking history. In analyses of 62 longitudinal observations from 24 individuals for whom social isolation data were available, results showed significantly elevated levels of the exosome RNA progression biomarker over time in those who were socially isolated ($b = 0.174$, $t(16) = 4.26$, $p = .0006$) (Figure 2A). In analyses of 58 longitudinal observations from 22 individuals for whom depressive symptom data were available, results also showed significantly elevated levels of the exosomal RNA progression biomarker over time in those with high levels of depressive symptoms ($b = 0.145$, $t(14) = 2.83$, $p = .0134$) (Figure 2B). In multi-variable analyses examining both depression and social isolation simultaneously, social isolation continued to be associated with significantly elevated progression biomarker values while controlling for depressive symptoms ($b = 0.233$, $t(13) = 4.66$, $p = .0004$), whereas depressive symptoms were not significantly associated with the biomarker values after controlling for social isolation ($b = 0.092$, $t(13) = 1.81$, $p = .0941$).

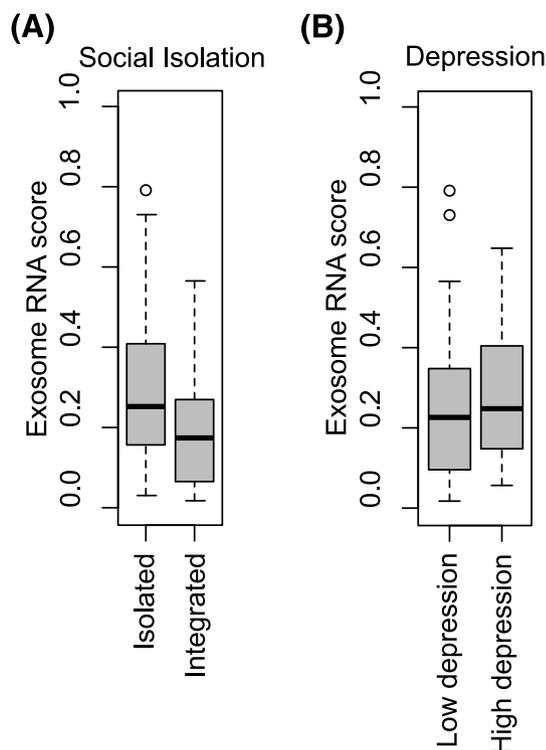


FIGURE 2 Biobehavioral study: exosome RNA composite score for socially isolated versus socially integrated ovarian cancer patients (A) and for patients with low (Center for Epidemiological Studies Depression Scale [CES-D] <16) versus elevated depressive mood (CES-D \geq 16) (B) over time

DISCUSSION

This study used machine learning discovery analyses to develop a novel multi-gene biomarker of rapid ovarian cancer progression based on exosome mRNA samples obtained at the time of primary surgery/NACT and subsequently used that newly-derived biomarker to quantify in a separate data set the impact of biobehavioral risk factors (social isolation and depressive symptoms) on progression-associated biology in the aftermath of primary surgery/NACT but before ascertainment of clinical progression. Results identified social isolation in particular as a significant risk factor for elevations in progression-associated biology in the aftermath of primary surgery/NACT and indicated that such effects emerge above and beyond the correlated effects of depressive symptoms, clinical disease, and demographic characteristics. In addition to clarifying the molecular pathways through which biobehavioral risk factors may influence the progression of ovarian cancer,^{15,16,20} these results identify a “liquid biopsy” biomarker that may be useful in other contexts such as clinical progression and treatment monitoring as well as a surrogate marker for outcomes in clinical trials.

The exosome biomarker of disease progression identified here included multiple gene transcripts that have previously been implicated in ovarian cancer pathogenesis and progression (e.g., *CD109*, *JAM3*, *MCPH1*, *PCMT1*, *PRDM2/RIZ1*, *PRSS21*, and *RHEB*). These involve a wide range of tumor-relevant biological processes including

DNA and protein repair, gene regulation, cell-cycle regulation, intercellular adhesion, angiogenesis, and chemoresistance (see Table S1 for details). Approximately half of the transcripts identified as survival-predictive had no previous studies indicating a role in ovarian cancer and may represent promising candidates for future mechanistic research (e.g., *ADGRL3*, *EXOC5*, *HECTD1*, *LPGAT1*, *LTA4H*, *NTAN1*, *SBF1*, *SCOC*, and *TMEM104*).

Biobehavioral factors such as social isolation and depressive mood have been previously associated with modulation of molecular events in the tumor microenvironment and with poorer clinical outcomes in a variety of cancers.^{11,26–31} These factors operate via neuroendocrine pathways including the sympathetic nervous system and the hypothalamic pituitary adrenal axis,³² both of which can activate transcription factors supporting tumor progression.^{7,10,33–36} Previously reported associations of social isolation with higher levels of tumor norepinephrine¹⁸ and with greater cyclic adenosine monophosphate response element-binding protein signaling^{15,20,27} are consistent with a potential role for beta-adrenergic signaling in the relationships observed here.

Prior research linked social isolation to EMT polarization in primary tumor in both ovarian and breast cancer patients^{15,16,27} but was limited by the use of indicator genes derived from breast cancer cell lines to define EMT-related profiles.³⁷ The present findings are unique in using a biomarker specifically relevant to disease progression in ovarian cancer patients and extending these findings to a longitudinal setting.

Exosomes mediate intercellular communication in the female reproductive system.¹⁴ In ovarian cancer, exosomes carry microRNAs, proteins, and other molecules that can promote chemoresistance,^{38,39} induce the EMT,^{38,39} and mediate tumor escape from the immune response.³⁹ Ovarian tumor-derived exosomes promote establishment of a pre-metastatic niche by (1) converting local fibroblasts into cancer-associated fibroblasts that enhance permeability of the basement membrane and remodel stroma to create a more favorable environment for tumor growth; (2) downregulating the local immune response, including inhibition of both natural killer cell and T-cell signaling and induction of macrophage polarization thus promoting tumor escape from the immune response; and (3) stimulating angiogenesis, invasion, and migration.⁴⁰

Previous studies have identified cellular-based biomarkers associated with ovarian cancer progression, but their use has been hampered by the inability to do longitudinal sampling to monitor changes in risk over time.^{41–46} Because exosomes are stable, plentiful, and can indicate pre-metastatic niche formation, an exosomal molecular biomarker as a “liquid biopsy” may have ready clinical utility.⁴⁰ Several approaches for exosomal profiling for ovarian cancer diagnosis or surveillance have been suggested.^{14,47} Following further validation, our derived biomarker could potentially be applied to determine risk of future cases of ovarian cancer by (1) collecting mRNA abundance data on predictor transcripts from plasma exosomes to monitor changes in risk over time, and (2) applying the predictive algorithm to assess likelihood of disease progression. This biomarker prediction can be used for potential purposes including

allocating patients to different treatments or monitoring those predicted to show rapid disease progression and rapidly assessing the impact of various treatments or other interventions by monitoring changes in biomarker predictions of disease progression, rather than waiting for clinical disease progression.

Limitations

It is possible that some of the observed transcriptional activity may have been from exosomes derived from normal cells. Previous research has indicated that in cancer patients, tumor is the primary source of circulating exosomes,⁴⁸ which have similar molecular composition as the cells from which they were derived.⁴⁹ As additional techniques are developed to isolate tumor-specific exosomes, these methods can be used to further validate these findings. In addition, exosomes originating from nontumor cells may well contribute to disease progression by several possible mechanisms, including changes in vascular biology, stromal cells, inflammation, and other changes in nontumor tissues that affect the development of metastatic niches and other aspects of disease progression.¹⁰ The present findings have focused on potential biobehavioral modulation of exosomal content and future research will be needed to assess other processes such as exosome production, release, and concentration. Although depressive symptoms and social support independently predicted the propensity biomarker, it is also possible that the reverse is true, and this is a potential limitation of the data. Additionally, the sample size was limited and additional validation in larger, more diverse independent samples is needed.

Clinical significance

The present study identifies an exosome-based biomarker of ovarian cancer progression and finds that biobehavioral factors of social isolation and depressive mood predict its variation over time. This biomarker may have additional utility as an easily accessible measure of clinical disease progression risk that can be used for intervention targeting and clinical monitoring.

AUTHOR CONTRIBUTIONS

Susan K. Lutgendorf: Conceptualization, funding acquisition, investigation, methodology, administration, data curation, formal analysis, writing–original draft, and writing–review/editing. **Premal H. Thaker:** Conceptualization, resources, investigation, supervision, and writing–review/editing. **Michael J. Goodheart:** Resources, investigation, and writing–review/editing. **Laila Dahmouh:** Investigation and writing–review/editing. **Jesusa M. G. Arevalo:** Methodology, investigation, data-curation, and writing–review/editing. **Mamur A. Chowdhury:** Investigation, data curation, and writing–review/editing. **George M. Slavich:** Writing–review/editing. **Alyssa E. Noble:** Data-curation and writing–review/editing. **Frank J. Penedo:** Resources, supervision, and writing–review/editing. **Anil K. Sood:** Conceptualization,

investigation, methodology, writing–original draft, and writing–review/editing. **Steven W. Cole:** Conceptualization, investigation, methodology, formal data analysis, writing–original draft, and writing–review/editing.

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CONFLICT OF INTEREST

Anil K. Sood reports consulting fees from Merck, Astra Zeneca, Kiyatec, Galaxo Smith Kline, Iylen, and Onxeo; research funding from M-Trap; is a Biopath shareholder; and holds a patent on EGFL6 antibody. Susan Lutgendorf reports consulting fees from the Princess Margaret Cancer Foundation; and is an AbbVie shareholder. Frank Penedo reports research funding from the National Cancer Institute; consulting fees from Blue Note Therapeutics. Premal Thaker reports consulting fees from Iovance Biotherapeutics, Celsion, AstraZeneca, Clovis Oncology, Glaxo Smith Kline, Eisai, Agenus, Immunogen, and Merck; research funding from Merck and Glaxo Smith Kline; and is a Celsion shareholder. The other authors made no disclosures.

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SUPPORTING INFORMATION

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