

Biobehavioral Modulation of the Exosome Transcriptome in Ovarian Carcinoma

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BACKGROUND: Social factors in the patient macroenvironment have been shown to influence molecular events in the tumor microenvironment and thereby influence cancer progression. However, biomarkers providing a window into the longitudinal effects of biobehavioral factors on tumor biology over time are lacking. Exosome analysis is a novel strategy for in vivo monitoring of dynamic changes in tumor cells. This study examined exosomal profiles from patients with low or high levels of social support for epithelial-mesenchymal transition (EMT) polarization and gene expression related to inflammation and β -adrenergic signaling. **METHODS:** Exosomes were isolated from plasma sampled from a series of 40 women before primary surgical resection of advanced-stage, high-grade ovarian carcinoma. Samples were selected for analysis on the basis of extremes of low and high levels of social support. After exosomal isolation and RNA extraction, a microarray analysis of the transcriptome was performed. **RESULTS:** Primary analyses identified significant upregulation of 67 mesenchymal-characteristic gene transcripts and downregulation of 63 epithelial-characteristic transcripts in patients with low social support; this demonstrated increased EMT polarization ($P = .0002$). Secondary analyses using promoter sequence bioinformatics supported a priori hypotheses linking low social support to 1) increased activity of cyclic adenosine monophosphate response element binding protein (CREB)/activating transcription factor (ATF) family transcription factors that mediate the β -adrenergic response to catecholamines via the cyclic adenosine monophosphate/protein kinase A signaling pathway (mean fold change for CREB: 2.24 ± 0.65 ; $P = .0019$; mean fold change for ATF: 2.00 ± 0.55 ; $P = .0049$) and 2) increased activity of the proinflammatory nuclear factor κ B/Rel family of transcription factors (mean fold change: 2.10 ± 0.70 ; $P = .0109$). **CONCLUSIONS:** These findings suggest the possibility of leveraging exosomes as a noninvasive assessment of biobehavioral factors to help to direct personalized treatment approaches. *Cancer* 2017;000:000-000. © 2017 American Cancer Society.

KEYWORDS: biobehavioral, epithelial-mesenchymal transition, exosomes, ovarian cancer, social support, transcriptome.

INTRODUCTION

Biobehavioral influences on the macroenvironment of the cancer patient's body have been shown to influence molecular events in the tumor microenvironment and thereby modulate cancer progression.¹ Perceptions of threats or challenges mediated by the central nervous system activate multiple downstream pathways, including the sympathetic nervous system and the hypothalamic-pituitary-adrenal axis.^{2,3} Preclinical evidence has demonstrated direct effects of β -adrenergic signaling and neuroendocrine hormones on key molecular processes promoting the metastasis of solid tumors, including angiogenesis, epithelial-mesenchymal transition (EMT), invasion, chemoresistance, and downregulation of cellular immunity and anoikis.⁴⁻⁶ One biobehavioral factor that has shown consistent links to cancer-related clinical outcomes is social support,⁷⁻¹² which can be defined as an individual's degree of perceived satisfaction with social relationships.^{13,14} Women with ovarian cancer reporting lower levels of emotional social support have demonstrated poorer innate immunity¹⁵ and higher levels of disease-related biomarkers linked to angiogenesis, inflammation, and invasion¹⁶⁻¹⁸ in the tumor microenvironment. Moreover, ovarian cancer patients with poorer emotional social support have shown a median survival at least 1.5 years shorter than that of patients with higher levels of social support, even after adjustments for clinical covariates.¹⁹

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It has been difficult to assess the longitudinal impact of biobehavioral factors such as social support on tumor dynamics because of the lack of reliable circulating blood biomarkers of tumor biology. The use of exosomal profiling represents a novel strategy for in vivo monitoring of tumor biology and for the assessment of dynamic changes occurring in tumor cells in the context of biobehavioral factors.²⁰ Exosomes are intact cell-derived vesicles shed from both normal and tumor cells that are involved in intercellular signaling.²¹ Tumors are the primary source of circulating exosomes in cancer patients, who have high levels of exosome shedding.²² Tumor-derived exosomes are representative of the cells from which they are derived. They have similar molecular compositions²³ and express a variety of tumor antigens that are characteristic of the originating tumor cells.²² Recent studies have demonstrated the validity of these plasma-derived cell fragments as “non-invasive liquid biopsies.”^{22,24} Exosomal profiling is starting to be used in the development of biomarkers for early detection, diagnosis, and therapy in cancer.²⁵ To date, however, this technology has not been harnessed to examine the effects of biobehavioral factors on cancer biology.

In the current study, we sought to determine whether transcriptional profiling of plasma-derived exosomes from women with invasive epithelial ovarian cancer could be used to assess transcriptional alterations associated with the biobehavioral risk factor of low social support. To do this, we assessed genome-wide transcriptional profiles in exosomes derived from plasma of ovarian cancer patients with low or high levels of social support. Next, we used a targeted hypothesis-testing bioinformatics strategy to assess EMT polarization and transcription factor activation.²⁶ On the basis of recent observations showing that β -adrenergic signaling polarizes ovarian tumor cells from an epithelial phenotype to a more mesenchymal phenotype (S.W.C., S.K.L., and A.K.S., unpublished data, 2017), we hypothesized that exosomal profiles from patients with low versus high levels of social support would demonstrate greater polarization of EMT-related gene transcripts. Furthermore, on the basis of findings from multiple studies indicating that psychosocial factors such as social support and/or stress reduction are related to the expression of genes regulating β -adrenergic and inflammatory signaling,²⁷⁻³⁰ we hypothesized that exosomes from patients with low social support versus high social support would show upregulated expression of genes related to inflammation (ie, genes regulated by nuclear factor κ B [NF- κ B]/Rel transcription factors) and β -adrenergic signaling (genes regulated by cyclic

adenosine monophosphate response element binding protein [CREB]/activating transcription factor [ATF] factors). Based on related data,^{27,29,31} exploratory analyses also examined whether patients with low social support would have downregulated expression of genes related to the cellular immune response and to glucocorticoid receptors.

MATERIALS AND METHODS

Participants

Exosome samples were isolated from plasma sampled from a series of 40 women before primary surgical resection of stage III and IV ovarian cancer at 3 clinical sites: the University of Iowa (n = 33), the University of Miami (n = 1), and Washington University (n = 6). These patients were sampled from a larger series of 240 adult women diagnosed with advanced-stage, high-grade serous epithelial ovarian, peritoneal, or fallopian tube cancer. Patient samples were selected for analysis on the basis of extremes of low or high levels of social support, as described later. The exclusion criteria were the presence of nonepithelial ovarian tumors, metastases to the ovaries from other organs, a previous cancer diagnosis, regular use of systemic steroid medication in the last month, the presence of a comorbid condition with known effects on the immune system (eg, autoimmune diseases), and an inability to accurately answer questions (eg, dementia). 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; they completed a psychosocial assessment and provided demographic information at home approximately 1 to 14 days before their surgical resection. Blood sampling was performed the morning of the surgery. Social support was assessed with the Attachment subscale of the Social Provisions Scale.³² According to a priori hypotheses derived from previous studies of biobehavioral risk factors in ovarian cancer,^{17,19,33,34} high psychosocial risk was defined by a Social Provisions Scale Attachment score lower than 15 (the median value). Background demographic characteristics (age, ethnicity, and socioeconomic status) were assessed as potential confounders. Ancillary analyses assessed social support as a continuous variable.

Whole blood collected in EDTA sodium tubes was centrifuged at room temperature for 15 minutes at 2200 rpm. Plasma was collected, aliquoted into 1-mL cryovials, and frozen immediately at -80°C . Heparin-treated plasma (1 mL) was spun down at 10,000 rpm for

10 minutes. Exosomes were then isolated from the supernatant with the ExoQuick reagent (System Biosciences, Mountain View, California). Exosomal RNA extraction was performed with the SeraMiR Exosome RNA Purification Kit (System Biosciences). The quantity and quality of the RNA were determined with an Agilent Bioanalyzer 2100 and an RNA 6000 Pico Kit (Agilent Technologies, Santa Clara, California). Exosomal RNA is stable and does not change significantly with storage²²; samples were stored at -70°C until the analysis. One sample had insufficient RNA yield thus final analyses were based on tissue from 39 patients.

Transcriptome Profiling

Exosomal RNA samples were converted into complementary DNA (NuGEN Pico WTA System V2) and hybridized to Illumina HT-12 v4 bead arrays at the Neuroscience Genomics Core Laboratory (University of California Los Angeles) according to the manufacturer's standard protocol. Resulting data were tested for valid probe fluorescence distributions (this resulted in the rejection of 1 sample that yielded insufficient complementary DNA), quantile-normalized, and \log_2 -transformed for analysis.

Transcription Factor Bioinformatics

The primary hypothesis regarding EMT-related gene expression was tested with a 130-gene signature previously derived to discriminate between epithelial-polarized and mesenchymal-polarized breast cancer cells.³⁵ Using this a priori defined gene set, we tested whether the average expression of the 67 mesenchymal-characteristic genes and the average expression of the 63 epithelial-characteristic genes differed significantly between patients with low high levels of social support and patients with high levels of social support; we controlled for differences in age, race, body mass index, and smoking history, which could confound the effects of social support on the outcomes of interest. Ancillary analyses also tested whether effects were independent of the disease stage (IIIA/B, IIIC, or IV), hypertension and chronic or perioperative use of β -blockers, and antidepressant use. The differential expression of each gene was estimated with a standard linear statistical model relating quantile-normalized, \log_2 -transformed transcript abundance estimates to social support (scored as 1 for low or 0 for high) while we controlled for a 1/0 indicator of white race, continuous age (years), continuous body mass index (kg/m^2), and a 1/0 indicator of smoking history. The average differential expression estimate across the a priori specified gene set

was tested for statistical significance using a standard error of the mean derived by bootstrap resampling of linear model residual vectors across genes (ie, we accounted for any potential correlation among genes).³⁶ We also examined potential differences in a 130-gene composite EMT score that weighted all mesenchymal genes as +1 and all epithelial genes as -1 when the average composite score was computed.

Secondary hypotheses concerning the role of CREB/ATF and NF- κ B/Rel transcription factors were tested with the Transcription Element Listening System (TELiS) promoter sequence-based bioinformatics analysis, as previously described.²⁶ This analysis used as input a list of all genes showing >1.25-fold differential expression between low and high social support groups, with up- and downregulated genes tested for the differential prevalence of CREB and ATF transcription factor-binding motifs (TFBMs; TRANSFAC V\$CREB_02 and V\$ATF_01 position-specific weight matrices, respectively) and NF- κ B TFBMs (V\$NFkB_C). The \log_2 -transformed ratios of TFBM prevalence in upregulated and downregulated promoters were computed for 9 combinations of 3 core promoter lengths (-300 , -600 , and -1000 to $+200$ bp with respect to the RefSeq gene transcription start site) and 3 TFBM detection stringencies (TRANSFAC MatSim values of 0.80, 0.90, and 0.96), with log ratios averaged over all 9 parametric combinations and tested for statistical significance using a standard error of the mean derived via bootstrap resampling of linear model residual vectors across genes (ie, accounting for any potential correlation among genes).³⁶ Ancillary sensitivity analyses examined the stability of results with an alternative differential expression cut point of 1.15- or 1.35-fold.

RESULTS

All patients were diagnosed with advanced-stage, high-grade serous disease (87.2% [$n = 34$] at stage III [2 at IIIA, 1 at IIIB, and 31 at IIIC] and 12.8% [$n = 5$] at stage IV). The average age was 62.9 years, and 97.4% of the patients were white non-Hispanic (1 Native American/Alaskan Native). Household incomes ranged from less than \$5000 to more than \$80,000 annually (annual median income, \$30,000-\$40,000).

Primary analyses examined whether exosome samples from patients with low social support would show greater abundance of EMT-related gene transcripts in comparison with patients with high social support; they controlled for age, race, body mass index, and smoking history. In an analysis of a single composite score defined by 130 gene transcripts previously identified as

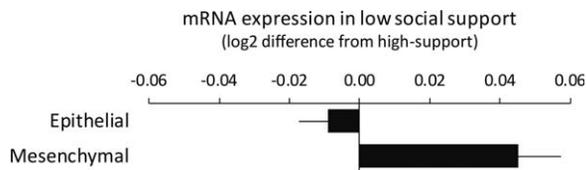


Figure 1. Differential expression of mRNA from epithelial and mesenchymal gene sets in exosome samples from patients with low levels of social support versus high levels. mRNA indicates messenger RNA.

EMT-related,³⁵ with 67 mesenchymal-characteristic transcripts weighted as +1 and 63 epithelial-characteristic transcripts weighted as -1, exosomes from patients with low social support showed increased EMT polarization (average score difference: $+0.028 \pm 0.007$ log₂ messenger RNA [mRNA] abundance; $P = .0002$). In separate analyses of the 67 mesenchymal-characteristic genes and the 63 epithelial-characteristics (Fig. 1), low-support patients showed increased expression of the mesenchymal gene composite ($+0.045 \pm 0.012$ log₂ mRNA abundance; $P = .0004$) but no difference in the expression of the epithelial gene composite (-0.009 ± 0.008 log₂ mRNA abundance; $P = .2862$).

Low social support was also associated with EMT polarization of exosome RNA profiles in ancillary analyses that additionally controlled for the tumor stage (IIIA/B, IIIC, or IV; $+0.029 \pm 0.006$; $P < .0001$), for hypertension and chronic or perioperative β -blocker use ($+0.027 \pm 0.006$; $P < .0001$), and for antidepressant use ($+0.028 \pm 0.007$; $P = .0002$). Similar results also emerged when social support was analyzed as a continuous variable (-0.012 ± 0.004 log₂ EMT mRNA abundance per Social Provisions Scale Attachment score standard deviation; $P = .0030$).

To test our secondary hypotheses regarding potential transcription control pathways that may mediate the observed shift in exosome mRNA abundance, we conducted a TELiS bioinformatics analysis of transcription factor-binding site prevalence in promoters of all gene transcripts that were found to differ in average expression by 1.25-fold or more across low versus high social support exosome samples (Fig. 2). The results supported the a priori hypothesis linking low social support to increased activity of CREB/ATF family transcription factors, which mediate the β -adrenergic response to catecholamines through the cyclic adenosine monophosphate/protein kinase A signaling pathway (mean fold change for CREB: 2.24 ± 0.65 ; $P = .0019$; mean fold change for ATF: 2.00 ± 0.55 ; $P = .0049$). The results also supported the a priori

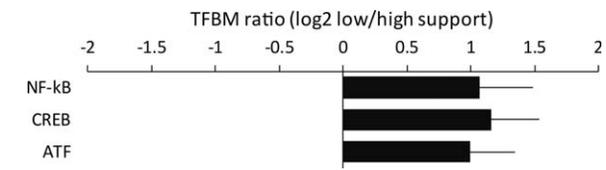


Figure 2. Transcription Element Listening System bioinformatics analysis of transcription factor-binding site prevalence in promoters of gene transcripts that differed in average expression by 1.25-fold or greater in low and high social support exosome samples. ATF indicates activating transcription factor; CREB, cyclic adenosine monophosphate response element binding protein; NF-κB, nuclear factor κB; TFBM, transcription factor-binding motif.

hypothesis linking low social support to increased activity of the proinflammatory NF-κB/Rel family of transcription factors (mean fold change: 2.10 ± 0.70 ; $P = .0109$). Similar indications of CREB and NF-κB activation emerged in sensitivity analyses that used alternative fold-difference thresholds for identifying differentially expressed genes (eg, 1.15- or 1.35-fold; all P values $< .05$).

Ancillary exploratory analyses also identified decreased activity of the glucocorticoid receptor (0.73 ± 0.11 ; $P = .0450$) as well as several findings that were not hypothesized a priori, including indications of decreased activity of C/EBP (0.70 ± 0.10 ; $P = .0181$), GATA1 (0.88 ± 0.05 ; $P = .0397$), and GATA2 (0.86 ± 0.06 ; $P = .0227$) and increased activity of aryl hydrocarbon receptors (2.03 ± 0.47 ; $P = .0009$), EVI (2.38 ± 1.08 ; $P = .0216$), MYB (1.22 ± 0.10 ; $P = .0177$), and ETS family transcription factors (1.29 ± 0.14 ; $P = .0146$) in patients with low levels of social support.

DISCUSSION

The key finding of this study is that plasma-derived exosomes from ovarian cancer patients show differential mRNA profiles related to increased EMT polarization, β -adrenergic activity, and inflammation, which were structured as a function of a biobehavioral risk factor, namely, patients' levels of social support. Specifically, exosomes from patients with low social support showed increased expression of EMT-related gene transcripts in comparison with their counterparts with high social support, and this reflected increased expression of a composite of mesenchymal genes. In addition, patients with low social support demonstrated greater activity of CREB/ATF family transcription factors and the proinflammatory NF-κB/Rel family of transcription factors. Although acute modulation of the exosome proteomic and microRNA composition in response to experimental stress has been previously demonstrated in preclinical studies,^{37,38} the

current study presents the first demonstration of the modulation of exosomal gene transcripts according to biobehavioral risk factors in a clinical population. Tumor-derived exosomes have functional properties that can support inflammation, angiogenesis, metastasis,³⁹ and down-regulation of cellular immunity,⁴⁰ and they are thus thought to play biological roles in ovarian cancer progression.^{22,40,41} Therefore, these findings are likely to have clinical significance.

The current findings in exosomes are consistent with emerging data from preclinical studies showing that mesenchymal gene expression in primary ovarian tumors is stimulated by β -adrenergic activation of the SNAIL and Twist families of transcription factors (S.W.C., S.K.L., and A.K.S., unpublished data, 2017). These findings also parallel data showing that the perioperative treatment of breast cancer patients with a β -adrenergic antagonist (propranolol) and a cyclooxygenase 2 inhibitor (etodolac) decreased EMT in the primary tumor.⁴² Social support has been previously related to levels of both tumor and ascites norepinephrine in ovarian cancer patients,³³ suggesting that these results are consistent with biobehavioral modulation of β -adrenergic signaling with downstream effects on mesenchymal cell polarization.

The secondary analyses parallel previous findings from our group indicating activation of CREB/ATF and NF- κ B/Rel family TFs within promoters of upregulated genes in tumors from ovarian cancer patients with low social support and high depressive symptoms.³⁴ These findings also are consistent with lower NF- κ B/Rel family TF expression in the leukocyte transcriptome of breast cancer patients who participated in a 10-week social support and stress management intervention.²⁹ CREB/ATF mediates β -adrenergic signaling, which in turn promotes multiple pathways involved in angiogenesis, invasion, and metastasis.⁴ NF- κ B/Rel mediates inflammation and is linked to many tumor-promoting pathways as well; chronic inflammation has also been implicated in the progression and recurrence of several cancers, including breast cancer^{43,44} and ovarian cancer.⁴⁵⁻⁴⁸ Taken together with the EMT data described previously, these findings suggest that the patterns observed in the exosome transcriptome of ovarian cancer patients according to biobehavioral factors mirror the patterns observed in the primary tumor. Exosomal signaling enables long-range communication between cells within an organism, and this suggests that exosomal signaling may provide an additional pathway by which biobehavioral processes can influence a cancer's biology and treatment response.¹⁹

There is a substantial literature linking biobehavioral factors such as stress, depression, and social support to disease progression in a variety of cancers,^{19,49} and the molecular mechanisms underlying these relationships are being elucidated in both clinical^{11,30,34} and preclinical settings.^{4,50} The current data extend this work in an important new direction by suggesting that longitudinal sampling of exosomes may potentially be applied to track changes in tumor biology that stem from changing biobehavioral conditions (eg, in response to a social support intervention).

Limitations

Although most exosomes captured in the circulating blood of cancer patients are tumor-derived, it is possible that some of the transcriptional activity observed was in exosomes derived from normal cells. However, even if the exosome transcriptome described here reflects activities of various nontumor cells, these findings still demonstrate EMT polarization and modulation of β -adrenergic and inflammatory signaling pathways paralleling social environmental risk that would have downstream effects regardless of the source. Another limitation is that the specific gene expression profiles used to define epithelial and mesenchymal transcripts³⁵ are derived from breast cancer cell lines and may not fully reflect the genomic signature of EMT in epithelial ovarian cancer.

Conclusions and Clinical Significance

The current study demonstrates differential EMT polarization of plasma-derived exosomes structured according to levels of a known biobehavioral risk factor, low social support, in ovarian cancer patients. Social support and the downstream effects of β -adrenergic signaling have been previously related to a variety of molecular pathways implicated in ovarian cancer survival. The current data extend this work by supporting the possibility of using exosomes as a noninvasive liquid biopsy to assess the effects of biobehavioral factors and help to direct personalized treatment approaches for cancer that optimize patient-level physiological influences on cancer biology. As the development of exosomal-based biomarkers progresses, exosome RNA profiling could potentially be used as an indication for earlier biobehavioral or pharmacological interventions for cancer survivors.

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CONFLICT OF INTEREST DISCLOSURES

Premal H. Thaker has performed consulting for Celsion.

AUTHOR CONTRIBUTIONS

Susan K. Lutgendorf: Principal investigator, concept and design, funding acquisition, project administration, data analysis and interpretation, manuscript writing and revision, and final approval of the manuscript. **Premal H. Thaker:** Participant referrals to the trial, design, interpretation, manuscript writing and revision, and final approval of the manuscript. **Jesusa M. Arevalo:** Methodology, investigation, data interpretation, manuscript revision, and final approval of the manuscript. **Michael J. Goodheart:** Participant referrals to the trial, design, interpretation, investigation, manuscript revision, and final approval of the manuscript. **George M. Slavich:** Design, interpretation, manuscript revision for intellectual content, and final approval of the manuscript. **Anil K. Sood:** Concept and design, investigation, methodology, data interpretation, manuscript editing and revision, and final approval of the manuscript. **Steve W. Cole:** Concept and design, investigation, methodology, data analysis, data interpretation, manuscript writing and revision, and final approval of the manuscript.

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