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Depression, social support, and beta-adrenergic transcription control in human ovarian cancer

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ABSTRACT

Motivated by previous indications that beta-adrenergic signaling can regulate tumor cell gene expression in model systems, we sought to determine whether similar dynamics occur in primary human ovarian cancer. DNA microarray analyses of 10 ovarian carcinomas identified 266 human transcripts that were differentially expressed in tumors from patients with elevated biobehavioral risk factors (high depressive symptoms and low social support) relative to grade- and stage-matched tumors from low-risk patients. Promoter-based bioinformatic analyses indicated increased activity of several beta-adrenergically-linked transcription control pathways, including CREB/ATF, NF-κB/Rel, STAT, and Ets family transcription factors. Consistent with increased beta-adrenergic signaling, high biobehavioral risk patients also showed increased intra-tumor concentrations of norepinephrine (but no difference in plasma norepinephrine). These data show that genome-wide transcriptional profiles are significantly altered in tumors from patients with high behavioral risk profiles, and they identify beta-adrenergic signal transduction as a likely mediator of those effects.

1. Introduction

Biobehavioral factors such as stress, depression, and social support have long been suspected to influence cancer onset and disease progression, but the molecular mechanisms of these effects are just beginning to be elucidated (Antoni et al., 2006). Recent findings from laboratory models suggest that biobehavioral processes can influence cancer biology via direct effects of the neuroendocrine system on the functional activity of tumor cells (Thaker et al., 2006). Many solid epithelial tumors express receptors for neuroendocrine mediators from the sympathetic nervous system (SNS) (the catecholamines, epinephrine and norepinephrine) (Mitra and Carraway, 1999; Lutgendorf et al., 2003; Nagmani et al., 2003; Palm et al., 2006; Thaker et al., 2006; Ramos-Jimenez et al., 2007; Sastry et al., 2007) or the hypothalamus–pituitary–adrenal (HPA) axis (the glucocorticoid, cortisol) (Wu et al., 2004). Analyses carried out *in vitro* and in mouse xenograft models have shown that neuroendocrine receptors can regulate several biological processes involved in cancer metastasis and disease progression, including angiogenesis (Lutgendorf et al., 2003; Thaker et al., 2006), tissue invasion (Sood et al., 2006; Yang et al., 2006), cell motility (Palm et al., 2006), and programmed cell death (Wu et al., 2004; Sastry et al., 2007). Analyses of mouse xenograft models also show that experimentally imposed behavioral stress can increase the growth and metastatic activity of implanted human

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carcinoma cells via direct neuroendocrine regulation of tumor cell biology (Thaker et al., 2006). However, it is unclear whether such dynamics occur in human clinical cancer because no studies have directly examined the biological activity of primary human tumors in relationship to biobehavioral characteristics.

In model systems, neuroendocrine factors regulate cancer cell biology in large part by altering gene expression. For example, catecholamines can enhance the expression of several genes involved in angiogenesis (e.g., *VEGF, IL6*) and tissue invasion (*MMP2*, *MMP9*) in ovarian and nasopharyngeal carcinoma cells (Lutgendorf et al., 2003; Sood et al., 2006; Yang et al., 2006; Nilsson et al., 2007). These effects are mediated by beta-adrenergic receptors, and subsequent activation of the cyclic 3′,5′-adenosine monophosphate/protein kinase A (cAMP/PKA) signaling pathway. PKA regulates gene expression by phosphorylating multiple transcription factors, including members of the cAMP response element binding protein/activating transcription factor (CREB/ATF) family (Montminy, 1997). Under certain circumstances, PKA can also cross-regulate activity of the pro-inflammatory NF-κB/Rel family of transcription factors (Shirakawa and Mizel, 1989; Lahdenpohja et al., 1996; Zhong et al., 1998; Bierhaus et al., 2003; Richlin et al., 2004), the pro-inflammatory STAT family of transcription factors (Landen et al., 2007), and the growth-promoting Ets transcription factors (Janknecht et al., 1996; Vossler et al., 1997; Luttrell et al., 1999; Wu et al., 2002), providing multiple signaling pathways for functional genomic regulation by catecholamines.

In the present study, we sought to determine whether beta-adrenergic signaling might play a role in mediating the relationship between behavioral risk factors and gene expression in primary ovarian carcinomas. This hypothesis was motivated by previous observations that, (1) the catecholamine ligands of beta-adrenergic receptors are increased in people with biobehavioral risk factors such as depression or low social support (Esler et al., 1982; Seeman et al., 1994; Veith et al., 1994; Light et al., 1998; Miller et al., 1999; Hughes et al., 2004; Hamer et al., 2007), (2) ovarian cancer patients with those risk factors show elevated circulating levels of IL-6 and VEGF (Lutgendorf et al., 2002; Costanzo et al., 2005), and, (3) beta-adrenergic signaling can enhance the expression of both *IL6* and *VEGFA* genes in model systems (Lutgendorf et al., 2003; Thaker et al., 2006; Nilsson et al., 2007). To determine whether beta-adrenergic regulation of gene expression also occurs in primary ovarian cancer, we assessed genome-wide transcriptional profiles in tumor tissues from patients with high biobehavioral risk profiles (high depressive symptoms and low social support) vs. low risk profiles (low depression and high social support), and used a hypothesis-testing bioinformatic strategy to assess CREB/ATF transcriptional signaling (Cole et al., 2005). We also tested secondary hypotheses that other transcription control pathways known to be activated by beta-adrenergic signaling, such as NF-κB/Rel, STAT, and Ets transcription factors, might also show increased activity in tumors from patients with high biobehavioral risk profiles.

2. Materials and methods

2.1. Participants

Tumor tissue and psychosocial data were collected from 20 patients undergoing primary surgical resection of ovarian carcinoma. These patients were sampled from a larger series of 60 adult women who were diagnosed with ovarian epithelial cancer, peritoneal cancer, or cancer of the fallopian tube, and were undergoing treatment at the University of Iowa ($n = 53$) or the University of Miami ($n = 7$). Patient samples were selected for analysis based on high vs. low biobehavioral risk profiles defined by depressive symptoms and social support (as detailed below). The only exclusion criteria were the presence of non-epithelial ovarian tumors, metastases to the ovaries from other organs, previous cancer diagnosis, regular use of systemic steroid medication in the last month, presence of a co-morbid condition with known effects on the immune system (e.g., autoimmune diseases), or inability to accurately answer questions (e.g., dementia).

2.2. Procedures

Participants completed psychosocial and background measures one to seven days prior to surgery. Resected tumor tissues were acquired for microarray gene expression analysis as described below, and each tumor was classified by pathology according to grade, stage (FIGO classification), and histologic subtype (serous/mucinous, papillary/endometroid, carcinoma/adenocarcinoma). Individual tumor tissues from high- vs. low-risk patients were matched based on stage, grade, and histological subtype prior to genome-wide expression analysis. All procedures were approved by Institutional Review Boards at the University of Iowa, University of Miami, and University of California at Los Angeles.

2.3. Depression, social support, and background characteristics

Depressive symptoms were assessed by the Center for Epidemiologic Studies Depression Scale (CESD) (Radloff, 1977). Social support was assessed by the Attachment subscale of the Social Provisions Scale (SPS-Attachment) (Cutrona and Russell, 1987). Based on an *a priori* hypothesis derived from previous studies of biobehavioral risk factors in ovarian cancer (Costanzo et al., 2005; Lutgendorf et al., 2005), high psychosocial risk was defined by the presence of a CESD score $\geqslant 16$ and SPS-Attachment score $\leqslant 15$ (the median value). The Beck Depression Inventory (BDI) was also collected for comparison with other published studies, but the CESD served as the primary measure of depression. Background demographic characteristics (age, ethnicity, and socio-economic status) were assessed as potential confounders.

2.4. Tumor tissue gene expression

Tumor fragments (.1 g) were excised from tissue samples maintained at −70 °C, homogenized in 600 μL of RNA-stabilizing lysis buffer (RNeasy RLT; Qiagen, Valencia CA), and supplemented with 400 μL of RNAse-free water (Qiagen) to produce a 1 ml nucleic acid lysate. One milliliter of Qiazol reagent (Qiagen) and 300 μL chloroform were added, and lysates were centrifuged for 5 min at 1500 rpm in a 15 mL centrifuge tube maintained at 4 °C. Aqueous phase products were mixed with 1 volume of 70% EtOH and applied to a RNeasy Mini spin column (Qiagen). Total RNA was extracted and treated with RNAse-free DNAse (Qiagen) following the manufacturer's protocol (supplemented by one additional wash in RW1 reagent and one additional wash in RPE reagent to remove excess salt). RNA purity and integrity were assessed using an Agilent 2100 bioanalyzer (Agilent Technologies, Santa Clara CA), and genome-wide transcription profiling was carried out using Affymetrix U133A high density oligonucleotide arrays (Affymetrix, Santa Clara CA) in the UCLA DNA Microarray Core, as previously described (Cole et al., 2003, 2005). Low level gene expression was quantified by Robust Multiarray Averaging (Bolstad et al., 2003), and differentially expressed genes were identified by a $\geqslant 2$-fold difference in mean expression level (corresponding to a 5% False Discovery Rate) (Benjamini and Hochberg, 1995; Cole et al., 2003) in paired comparisons of grade- and stage-matched tumors from high vs. low biobehavioral risk patients. Gene expression data are deposited in the National Center for Biotechnology Information Gene Expression Omnibus (GEO Series GSE9116).

2.5. RT-PCR verification

Differential gene expression was confirmed by quantitative real-time RT-PCR using commercial TaqMan Gene Expression Assays (Applied Biosystems, Foster City CA) and Quantitect Probe RT-PCR enzymes (Qiagen, Valencia CA), in 25 μL reactions carried out according to the manufacturer's specified 1-step thermal cycling protocol (60 °C annealing) on an iCycler instrument (Bio-Rad, Hercules CA). All reactions were carried out in triplicate, with analyte threshold cycle numbers normalized to GAPDH threshold cycle numbers prior to analysis.

2.6. Transcription control pathway analysis

The primary hypothesis regarding CREB/ATF transcription factors and secondary hypotheses regarding NF-κB/Rel, STAT, and Ets family transcription factors were tested using a 2-sample variant of the Transcription Element Listening System (TELiS) (Cole, 2005; Cole et al., 2005). TELiS contains data on the prevalence of 192 vertebrate transcription factor-binding motifs (TFBMs) from the TRANSFAC database (Wingender et al., 1996) in the promoters of all genes assayed by the Affymetrix U133A high-density oligonucleotide array. TELiS differential expression analyses monitor the functional activity of the transcription factors binding to each TFBM by defining putative transcription factor-responsive genes (TFRGs) *a priori* based on promoter DNA sequence information, and then testing for empirical differential expression of those TFRGs using established statistical approaches (Cole et al., 2005). In the present study, activation of the CREB/ATF transcription control pathway was assessed by genes bearing the V$CREBP1_Q2 motif, activation of the NF-κB/Rel factors cRel and RelA were assessed by V$CREL_01 and V$NFKAPPAB65_01 motifs, activation of STAT family transcription factors was assessed by V$STAT_01, and activation of the prototypic Ets family transcription factor ELK1 was assessed by V$ELK1_01. Primary analyses utilized default motif detection parameters (Cole

et al., 2005), and sensitivity analyses examined parametric variations of promoter length (−300 bp relative to RefSeq gene transcription start site, −600 bp, and −1000 bp to +200) and TFBM match stringency (MatSim = .80, .90, .95) (Cole et al., 2005).

2.7. Norepinephrine concentration

Norepinephrine levels in tumor and plasma extracts were determined by high performance liquid chromatography with electrochemical detection (HPLC-ED) as previously described (Hoffman et al., 2002). Pre-surgical blood samples were collected between 6:30 and 11:30 AM (average 8:20 AM, an average 3 h before tumor tissue capture), drawn into chilled EDTA Vacutainers (BD Biosciences, Franklin Lakes, NJ), and maintained on ice prior to centrifugation, separation of the plasma, and storage at −80 °C until the time of extraction and assay. Plasma catecholamines were absorbed onto activated alumina at pH 8.6, washed, and eluted with dilute acid prior to injection onto a reverse-phase column to separate the individual catecholamines. Detection was accomplished by the use of a Coulochem II Dual Potentiostat Electrochemical Detector (ESA, Chelmsford, MA.). A calibration curve using "blank" human plasma (dialyzed to remove endogenous catecholamines) and linear regression analysis was used to determine sample plasma norepinephrine concentration. The calibration curve used the internal standard (DHBA, 3,4 dihyroxybenylamine) method which calculates an average response factor using peak areas and concentrations of added catecholamine in relation to the peak area of the internal standard. The interassay and intra assay coefficients of variation were 3.4% and 3.1%, respectively, and the lower limit of detection was 25 pg/ml.

Dissected 2–3 mm tumor tissue samples were snap frozen in liquid nitrogen, pulverized using liquid nitrogen, and held on dry ice. The tissue powder was weighed and briefly homogenized (Fisher PowerGen 500 and sawtooth generator) in 5 ml 0.4 M perchloric acid containing 6 mM glutathione. Following centrifugation of the homogenate (1000*g*, 30 min), the supernatant was neutralized with 5 ml of TRIS buffer (1.5 M, pH 8.5) and catecholamines were absorbed onto acid washed alumina (100 mg). The alumina was washed twice with water and catecholamines were eluted with 1 ml 0.05 M perchloric acid containing 0.1 mM sodium metabisulfite. After microfiltration, the eluate was diluted in 4% acetic acid, and catecholamines were resolved using an Aquasil C18 4 μm (100 × 4.6 mm) HPLC column (Thermo Electron Corp., Bellefonte, PA), followed by electrochemical detection as described above. Chromatograph peak areas for norepinephrine and epinephrine were compared to the average peak areas determined from the injection of 100 pg pure standard, and corrected for extract dilutions and tissue wet weights.

2.8. Statistical analyses

Group differences in demographic and clinical parameters were tested using χ^2 analysis (categorical variables) or paired *t*-tests (continuous variables) (Miller, 1986). Analyses of promoter TFBM distributions tested for differential expression using an independent sample *t*-test with Welch's correction for heteroscedasticity (Miller, 1986). To confirm the statistical significance of differential TFBM distributions, we carried out randomization tests as previously described (Cole et al., 2003, 2007). Briefly, the total set of 10 samples was randomly partitioned into two groups of 5 samples, differentially expressed genes were identified based on ⩾2-fold difference in mean expression level, and the resulting difference in the frequency of target TFBMs was quantified. This process was repeated 10,000 times to estimate the sampling distribution of differential TFBM distribution under the null hypothesis of random population sampling. The observed asymmetry in TFBM distributions in low vs. high biobehavioral risk patients was compared to this null hypothesis sampling distribution to estimate the tail probability of Type I (false-positive) errors (Bratley et al., 1983).

3. Results

3.1. Tumor characteristics

Twenty ovarian carcinoma tissues were sampled from a series of 60 women undergoing primary surgical resection of Stage IA–IIIC ovarian cancer at two gynecologic oncology practices (Table 1). The majority of analyzed patients were diagnosed with advanced disease (55% stage III, 10% stage II, 35% stage I) and poorly differentiated tumor histology (55% grade 3, 15% grade 2, 25% grade 1). Participants ranged in age from 35 to 87 years (median = 61), 95% were white (1 African American), and they reported household incomes ranging from <$5000 per year to >$80,000 per year (median = $30,000–$40,000). Depressive symptoms and social support were measured at pre-surgical clinic visits occurring 1–7 days prior to tumor resection. These variables showed a strong inverse correlation ($r = -.43$, $p = .0015$ in the total sample of 60; $r = -.91$, $p < .0001$ in the 20 individuals analyzed), and were therefore treated as two indicators of a common underlying biobehavioral risk cluster. Using established threshold values of CESD ⩾16 and SPS-Attachment ⩽15, 11 participants (42%) were identified by pre-surgical psychometric assessments as showing high levels of biobehavioral risk factors. Across these high- and low-risk strata, 5 pairs of tumors could be matched on common grade, stage, and histologic subtype, and were thus selected for microarray gene expression analysis (Table 1). These 10 tissue samples came from patients who were representative of the total sample on all assessed demographic and disease parameters (difference from sample-wide average, all $p > .50$). All analyzed tumors were grade 3 serous carcinomas/adenocarcinomas, recovered at stages ranging from IA to IIIC (80% IIIC). The selected high- and low-risk

Table 1
Patient characteristics

Sample ID	Match Pair[a]	Risk Group[b]	CESD	SPS	BDI	Age	Income[d]	Grade	Stage	Diagnosis
Iowa 1	A1	High	34	10	30	49	50–60	3	IIIC	Serous cystadenocarcinoma
Iowa 2	B1	High	47	9	30	66	20–30	3	IIIC	Papillary serous adenocarcinoma
Iowa 3	—	Low	7	16	5	78	20–30	1	IA	Endometrioid adenocarcinoma
Iowa 4	C1	High	47	9	30	47	< 5	—[c]	IIIC	Papillary serous adenocarcinoma
Iowa 5	D2	Low	—[c]	12	2	87	10–20	3	IIIC	Papillary serous adenocarcinoma
Iowa 6	—	High	27	11	20	80	DTS	2	IIIC	Serous and endometrioid adenocarcinoma
Iowa 7	—	Low	11	16	—[c]	54	DTS	1	IA	Serous adenocarcinoma
Iowa 8	D1	High	29	13	13	56	30–40	3	IIIC	Serous carcinoma
Iowa 9	A2	Low	2	16	3	63	30–40	3	IIIC	Serous carcinoma
Iowa 10	—	High	21	11	15	74	10–20	3	IIIC	Serous adenocarcinoma
Iowa 11	C2	Low	5	16	11	61	30–40	3	IIIC	Papillary serous adenocarcinoma
Iowa 12	E2	Low	7	16	7	64	60–80	3	IC	Serous papillary and endometrioid carcinoma
Iowa 13	—	Low	8	16	9	65	10–20	1	IB	Serous adenocarcinoma
Iowa 14	—	High	33	13	13	39	DTS	1	IA	Mucinous cystadenocarcinoma
Iowa 15	—	High	20	11	23	74	DTS	3	IIIC	Serous carcinoma
Miami 1	—	Low	6	16	2	52	> 80	1	IC	Serous carcinoma
Miami 2	—	High	21	13	10	69	5–10	2	IIA	Fallopian tube [ovarian carcinoma]
Miami 3	—	High	33	12	16	51	10–20	2	IB	Adenocarcinoma
Miami 4	E1	High	19	14	15	49	> 80	3	IIC	Serous adenocarcinoma
Miami 5	B2	Low	6	16	2	35	20–30	3	IIIC	Papillary serous carcinoma

[a] Grade/stage-matched high- and low-biobehavioral risk samples are labeled A1 and A2, etc.
[b] Biobehavioral risk defined by CESD ⩾ 16 and SPS-Attachment ⩽ 15.
[c] "—" = data not available.
[d] ×$1000 per year (DTS = decline to state).

patients did not differ on any of the demographic dimensions assessed, or in tumor grade or stage (all $p > .30$). The only measured properties that differed significantly across groups were levels of depression (CESD, $p = .0017$; BDI, $p = .0026$) and social support (SPS-Attachment, $p = .0129$), as induced by the biobehavioral risk stratification process.

Affymetrix U133A high-density oligonucleotide arrays were used to survey the expression of 22,283 human transcripts in ovarian tumor samples. Expression of 266 transcripts differed by more than twofold across high- vs. low-risk groups, including 220 transcripts up-regulated by at least 100% in tissues from high-risk patients, and 46 transcripts down-regulated by more than 50% in tissues from high-risk patients (Fig. 1). Differentially expressed genes are listed in Supplementary data (Table S1) and available on-line at http://colelab.med.ucla.edu/OvarianCancer/PrimaryTumors/Genes.htm. Twenty-five transcripts identified as differentially expressed in microarray analyses were confirmed by quantitative RT-PCR (Table S2), or on-line at http://colelab.med.ucla.edu/OvarianCancer/PrimaryTumors/ConfirmatoryRTPCR.htm). Significant differential expression was confirmed for 23 of the 25 assayed transcripts (92%), with the magnitude of difference averaging 9.6-fold across the entire sample of 25, and p-values averaging .014. Because our primary interest focuses on testing the *a priori* hypothesis that up-stream beta-adrenoreceptor-linked transcription control pathways drive the observed patterns of differential gene expression (rather than on any *a priori* hypothesis about the specific nature of differentially expressed genes), no descriptive analysis of gene functional characteristics was conducted.

3.2. Primary hypothesis: CREB/ATF-mediated transcription

TELiS bioinformatics analysis was applied to test this study's central hypothesis that genes up-regulated in ovarian cancers from women with high biobehavioral risk factors would show over-representation of targets of the CREB/ATF transcription control pathway, which mediates beta-adrenergic response to catecholamines through the cAMP/PKA signaling pathway. Results showed a >3-fold elevation in the prevalence of CREB/ATF transcription factor-binding motifs (TFBMs) within promoters of genes up-regulated in tumors from patients with a high-risk biobehvioral profile (TRANSFAC V$CREBP1_Q2 motif: average .000 ± standard error .000 sites/promoter for genes over-expressed in tumors from low-risk individuals vs. .070 ± .026 in genes over-expressed in high-risk tumors; difference $p = .0071$ by t-test) (Fig. 2). Sensitivitiy analyses utilizing parametric variations of promoter length (−300, −600, −1000 to +200 bp) and TFBM match stringency (MatSim = .80, .90, .95) yielded similar results, with an average 2.35-fold (± .38) difference across all 9 parametric combinations ($p = .0003$).

3.3. Secondary hypotheses: NF-κB/Rel-, STAT-, and Ets-mediated transcription

Studies in *in vitro* model systems suggest that beta-adrenergic signaling can also activate NF-κB/Rel-family transcription factors, although these effects are variable across cell type and experimental conditions (Shirakawa and Mizel, 1989; Lahdenpohja et al., 1996; Zhong et al., 1998; Bierhaus et al., 2003; Richlin et al., 2004). In sec-

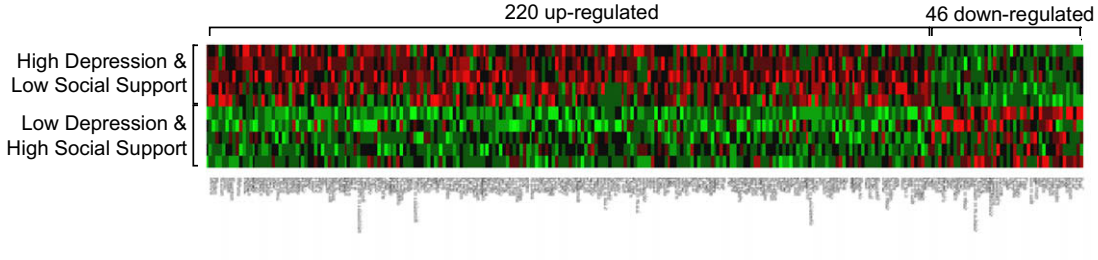

Fig. 1. Differentially expressed genes in ovarian cancers from patients with high vs. low biobehavioral risk profiles. Genome-wide transcriptional profiles were assessed in ovarian carcinoma RNA samples collected from patients showing high depressive symptoms and low social support (high risk) vs. low depressive symptoms and high social support (low risk). Analysis by Affymetrix U133A high-density oligonucleotide arrays identified 266 transcripts showing >2-fold difference in mean expression level across group (red = over-expression in high-risk, green = under-expression in high-risk).

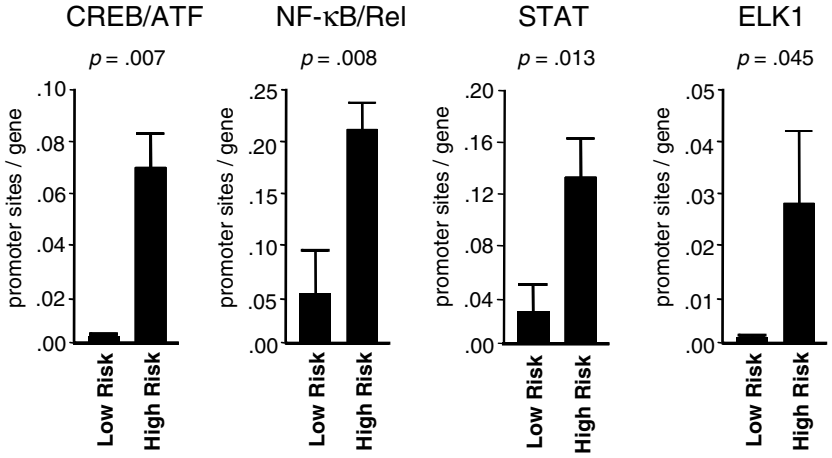

Fig. 2. Activity of beta-adrenoreceptor-linked transcription control pathways. Promoter-based TELiS bioinformatics assessed differential activity of CREB/ATF, NF-κB/Rel, STAT, and ELK1 transcription factors in promoters of 266 genes differentially expressed in ovarian tumors from patients with high vs. low biobehavioral risk profiles (shown in Fig. 1).

ondary analyses examining the distribution of cRel (cRel/p50 heterodimer) and RelA (p65/p50 heterodimer) promoter motifs, the prevalence of V$CREL_01 TFBMs was 3.7-fold higher in genes over-expressed in tumors from high-risk patients relative to low-risk patients (mean = .057 ± .040 in low risk vs. .210 ± .040 in high risk; difference, $p = .0076$) (Fig. 2). Similar results emerged across parametric variations of promoter length and scan stringency (mean = 2.80-fold ± .58 across all 9 combinations; $p = .0014$). For the RelA variant of NF-κB, TRANSFAC V$NFKAPPAB65_01 TFBMs were found at a 3.18-fold heigher prevalence in promoters of genes over-expressed in tumors from high-risk patients vs. low-risk patients (mean = .091 ± .026 in high risk vs. .029 ± .029 in low risk). This difference failed to reach statistical significance in primary analyses ($p = .1103$), but it was significant on average across all 9 parametric combinations of promoter length and TFBM match stringency (mean difference = 4.65-fold ± 1.73; $p = .0275$).

Analyses of ovarian carcinoma cells *in vitro* also indicate that beta-adrenergic receptors can activate the pro-inflammatory transcription factor STAT3 (Landen et al., 2007). TELiS analyses confirmed increased STAT transcripitonal activity in primary ovarian carcinoma cells by revealing a 4.7-fold higher prevalence of the V$STAT_01 TFBMs in promoters of genes up-regulated in tumors from high-risk patients (mean = .029 ± .029 in low risk vs. .133 ± .030 in high risk; difference, $p = .0134$) (Fig. 2). Parametric sensitivitiy analyses yielded consistent results, with an average 2.20-fold (± .38) increase in STAT activity across all 9 parameter combinations ($p = .0141$).

Data from *in vitro* models suggest that beta-adrenergic receptors can also activate the Ras/MAP kinase signaling pathway (Luttrell et al., 1999), and we therefore assessed activity of Ets family transcription factors that mediate Ras/MAP kinase-driven gene transcription. Analyses of the prototypic Ets factor, ELK1, revealed a significantly higher prevalence of the V$ELK1_01 TFBM in promoters of genes up-regulated in tumors from high-risk patients (mean = .000 ± .000 in low risk vs. .028 ± .014 in high risk; difference, $p = .0451$) (Fig. 2). Due to the high intrinsic stringency of the V$ELK1_01 nucleotide motif, this TFBM yielded non-zero prevalence frequencies for only 6 of the 9 total parametric combinations of promoter length and TFBM match stringency. However, among the 6 evaluable datasets, ELK1 TFBMs were approximately threefold more prevalent among the promoters of genes up-regulated in high-risk tumors relative to those in low-risk tumors (mean difference = .023 ± .007, $p = .0081$).

3.4. Joint relationships of beta-adrenergic transcription control pathways

To verify the accuracy of univariate statistical analyses and assess the combined significance of multiple beta-adrenergic transcription control pathways, we carried out randomization tests gauging the likelihood of the observed effects under the null hypothesis of random sampling (Bratley et al., 1983; Cole et al., 2003, 2007). The probability of observing 266 genes showing ⩾2-fold differential expression was quite low under random sampling ($p = .0119$), as were the probabilities of the observed differences in distributions of CREB/ATF ($p = .0287$), NF-κB/Rel ($p = .0092$), and STAT family transcription factors ($p = .0213$). Differential distribution of Ets family TFBMs approached statistical significance ($p = .0821$). Results indicated that simultaneous alteration in all 4 of these beta-adrenergically-linked transcription control pathways was exceedingly unlikely under random sampling ($p < .0001$).

3.5. Norepineprhine levels

To identify the basis for differential activation of beta-adrenergically responsive transcription control pathways in tumors from patients with high vs. low biobehavioral risk profiles, we quantified intra-tumor concentrations of the beta-adrenergic ligand, norepinephrine, in the 10 samples analyzed by DNA microarray. Tissues from patients with a low-risk profile showed minimal intra-tumor norepinephrine (all samples ⩽0.1 pg/mg tissue assay lower limit of detection), whereas tissues from high-risk patients showed significantly greater norepinephrine concentrations (mean = 19.5 ± 6.9 pg/mg tissue, difference from low-risk, $p = .0482$) (Fig. 3). Parallel analyses of plasma collected an average 2 hrs prior to tumor resection failed to indicate any significant difference in circulating norepinephrine levels (low risk = 735.3 ± 60.5 pg/ml; high risk = 667.0 ± 13.5 pg/ml; difference, $p = .1068$) (Fig. 3). Plasma norepinephrine levels immediately prior to surgery were not significantly correlated with intra-tumor norepineprhine ($r = -.18$, $p = .4362$). Tumor norepinephrine concentrations did show moderate magnitudes of correlation with continuous scores on the CESD ($r = .73$, $p = .1253$) and SPS-Attachment scale ($r = -.50$, $p = .2302$), but those correlations failed to reach statistical significance due to the limited sample size. Plasma norepinephrine levels were not substantially correlated with either continuous measure of biobehavioral risk (CESD: $r = -.32$, $p = .3814$; SPS-Attachment: $r = .22$, $p = .4233$).

4. Discussion

Based on previous indications that beta-adrenergic signaling can regulate tumor cell gene transcription in model systems (Lutgendorf et al., 2003; Sood et al., 2006; Thaker et al., 2006; Landen et al., 2007; Nilsson et al., 2007), this study sought to determine whether similar dynamics occur in the context of primary human ovarian cancer. Results show that gene expression profiles in primary human tumor tissues are systematically altered in association with patient-level biobehavioral risk factors, and promoter-based bioinformatics analyses confirmed that beta-adrenergic transcription control pathways represent key mediators of those differences. TELiS analysis of 266 promoters found to be differentially active in ovarian carcinomas from patients with high depressive symptoms and low social support showed enrichment of regulatory elements for several beta-adrenergically regulated transcription factors, including CREB/ATF, NF-κB/Rel, STAT, and Ets factors. Consistent with genomic indications of increased beta-adrenergic signaling, tumors from patients with high biobehavioral risk profiles also showed elevated tissue levels of the sympathetic

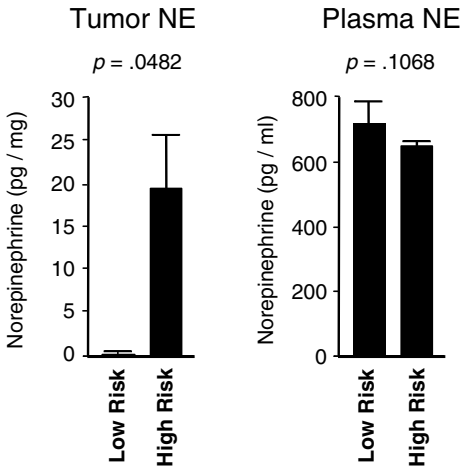

Fig. 3. Norepinephrine levels in ovarian tumors and circulating blood. Norepinephrine levels were assayed by high performance liquid chromatography in tumor parenchyma and circulating blood plasma from patients with high vs. low biobehavioral risk profiles.

neuroeffector molecule norepinephrine. Thus, the present data are consistent with findings from laboratory animal and cellular models in suggesting that increased activity of the sympathetic nervous system may constitute a primary mechanism by which biobehavioral factors impact gene expression in solid epithelial tumors.

One remarkable finding of this study involves the differential regulation of norepinephrine levels in tumor tissue vs. circulating blood. Patients with high biobehavioral risk profiles showed substantial elevations in intra-tumor norepinephrine concentrations, but no significant elevation (and, in fact, slightly lower levels) in plasma. The biological basis for this tissue-specific difference in noradrenergic signaling is not clear, but it could potentially stem from stress-induced up-regulation of tissue innervation by sympathetic neural fibers (e.g., as recently observed in lymphoid organs) (Sloan et al., 2007; 2008). One potential mechanism for such effects in the context of ovarian cancer might involve chronic elevations in circulating catecholamines during tumor development (Weiner, 1992), leading to increased tumor vascularization (Thaker et al., 2006), and a consequent densification of vasculature-associated sympathetic fibers. The lack of correlation between blood and tumor norepinephrine levels is not surprising, given that circulating catecholamine levels are highly variable (Weiner, 1992) and blood samples were collected an average of 3 h prior to tumor resection. However, volatile catecholamine levels cannot explain the emergence of systematically higher norepinephrine levels in tumors from patients with high biobehavioral risk profiles. The parallel expression of RNA transcriptional alterations, which lag receptor-mediated signal transduction by 2–4 h in ovarian carcinoma cells (Lutgendorf et al., 2003; Thaker et al., 2006; Landen et al., 2007; Nilsson et al., 2007), also implies that the observed differences are not likely to stem from any transient alteration in tissue norepinephrine levels during surgery. The gene expression profiles observed here likely reflect the combined effects of basal individual differences in the biology of the tumor microenvironment (e.g., as driven by differential innervation or angiogenesis) and any transient effects occurring 2–4 h prior to surgery (i.e., well before any transient influences on tissue norepinephrine levels at tumor resection, and contemporaneous with the plasma catecholamine determinations). The most parsimonious explanation for the observed pattern of results involves stable alterations in sympathetic signaling within the tumor microenvironment. Regardless of the mechanism of these effects, the present data suggest that biobehavioral signaling dynamics can have marked effects in solid tissue that are not observable in circulating blood.

Although the specific nature of differentially regulated genes was not a target of this study, the simultaneous up-regulation of pro-inflammatory NF-κB/Rel and STAT family transcription factors suggests that tumors from high-risk patients may be exposed to heightened inflammatory signaling in the tumor microenvironment. Inflammation is believed to play a key role in the initiation and progression of may carcinomas (Coussens and Werb, 2002; de Visser et al., 2006), including those of the ovarian epithelium (Wang et al., 2005). To the extent that increased SNS activity promotes inflammation in the tumor microenvironment, inflammatory response genes may constitute a significant molecular mediator of biobehavioral influences on cancer. Given the known link between inflammatory gene expression and biology of malignant disease (Coussens and Werb, 2002; de Visser et al., 2006), the present data suggest that patients with high depressive symptoms and low social support might show accelerated disease progression in the aftermath of surgery (e.g., shorter times to disease recurrence or mortality). That speculation would be consistent with the existing epidemiologic literature in suggesting that biobehavioral factors exert their most pronounced effects on cancer by facilitating the progression of established tumors, rather than by spurring the development of new malignancies (Antoni et al., 2006).

Given the limited number of tissues available for this analysis, the present findings should be regarded as a preliminary indication that laboratory-identified biobehavioral mechanisms extend into the human clinical setting. It is notable that this study identified significant differences in tumor cell transcriptional dynamics that were both predicted by laboratory models *a priori*, and sufficiently large in magnitude to reach statistical significance despite the limited statistical power available in this small sample. If larger replication studies continue to confirm a significant role for beta-adrenergic control of multiple transcription factors in primary human cancers, this pleiotropic signaling pathway could provide a novel molecular target for adjunctive therapies aimed at blocking catecholamine support for disease progression (e.g., beta-blockade) (Antoni et al., 2006).

This study's analytic strategy rules out several potential alternative explanations for the observed differences in transcriptional activity in patients showing high vs. low biobehavioral risk profiles, including differences in tumor histological characteristics (which were controlled by matching high- vs. low-risk tissues for grade, stage, and histological subtype), and patient demographic characteristics (which were uncorrelated with depression or social support). However, several limitations of this study must be considered when interpreting the present results. These data come from a small sample of tissues that emerged from a stringent matching procedure that intentionally controlled for differences in tumor phenotype (e.g., grade, stage, histologic subtype) in high vs. low-risk patients. Larger studies will be required to establish the generality of these findings, and to explore any potential relationship between biobehavioral influences and the tumor phenotypes that were held constant by this study. Larger samples will also be required to distinguish any unique biological correlates of depressive symptoms from those associated with low social support. In the sample analyzed here, those two psychosocial risk factors were so highly correlated as to be statistically indistinguishable. Their strong correlation is consistent with previous indications that psychosocial risk factors often come in clusters which may reflect the propagation of adverse conditions throughout multiple aspects of an individual's life (Williams et al., 2003; Lehman et al., 2005; Taylor et al., 2006).

An additional limitation of this study involves its cross-sectional design. Previous experimental studies have shown that beta-adrenergic signaling can alter gene expression and cellular function in ovarian carcinoma cells (Lutgendorf et al., 2003; Antoni et al., 2006; Sood et al., 2006, 2007; Thaker et al., 2006; Landen et al., 2007; Nilsson et al., 2007). However, it is conceivable that a reverse causal pathway might contribute to the cross-sectional association observed here, with spontaneous heterogeneity in tumor biology influencing behavioral characteristics (e.g., via effects of pro-inflammatory cytokines on the brain, resulting in decreased social motivation and increased depressive symptoms) (Dantzer et al., 2008). Although this hypothesis could explain an association between behavioral characteristics and pro-inflammatory transcription factors (e.g., NF-κB/Rel and STAT), it would not account for the increased CREB/ATF or Ets factor activity observed in patients with high-risk behavioral profiles. Moreover, any spontaneous mutagenic variations in the cancer genome that occur independently of biobehavioral influences would likely manifest as correlated differences in both transcriptional regimes and tumor histological phenotypes. This study's matching-based control for tumor phenotype prevents the misattribution of phenotype-related differences to biobehavioral influences, thus reducing the likelihood of reverse causality from tumor biology to behavior. Histological matching also "controls away" any biobehavioral influences that might affect tumor characteristics prior to detection (e.g., at the level of tumor initiation or promotion), and may thereby underestimate the total influence of biobehavioral factors. How-

ever, the appearance of differential gene expression profiles with a heightened inflammatory signature at the time of tumor diagnosis and resection suggests that subsequent progression of ovarian cancer may be accelerated in patients with high depressive symptoms and low social support.

The present findings are consistent with other recent data linking biobehavioral factors to gene expression differences via signaling pathways such as the inflammation-related glucocorticoid receptor, NF-κB/Rel, and JAK/STAT transcription factors, as well as the CREB/ATF and Ets factors (Irwin et al., 2006; Cole et al., 2007; Miller, in press). This study is novel in identifying genome-wide transcriptional correlates of biobehavioral risk factors specifically within diseased tissue. The close concordance between the transcriptional mediators identified here in diseased tissue and those identified in leukocyte "reporter cell" studies underscores the common role of systemic neuroendocrine activity in shaping global gene expression profiles in both healthy and diseased tissue. As new therapeutic strategies are developed to block adverse effects of biobehavioral risk factors, the transcription control "fingerprints" identified in these studies may provide novel molecular targets for intervention, as well as functional genomic biomarkers for assessing the impact of those interventioins. As such, the gene expression fingerprint of depression and low social support in primary ovarian carcinomas provides new insights into the social epidemiology of cancer (Berkman and Kawachi, 2000; Hernandez and Blazer, 2006; Weinstein et al., 2007) and suggests novel strategies for protecting cancer patients from the adverse effects of stress on the progression of malignant disease (Antoni et al., 2006; Sood et al., 2007).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbi.2008.04.155.

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