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An organ system-based approach to prognosis in advanced melanoma

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1. ABSTRACT

Previous models to study the biology of melanoma have focused on individual factors, such as proliferative and invasive capacity, the microenvironment, angiogenesis, or systemic immune dysfunction. However, all of these factors contribute to melanoma progression in concert. One physiologic phenomenon that typifies the coordination of these processes is placental development, characterized by trophoblast proliferation, invasion into decidual tissues, angiogenesis, and transient organ system-based immune evasion. Herein, we explore expression of 34 proteins involved in placentation and determine their association with an established prognostic factor, tumor infiltrating lymphocytes (TILs), in a 118-patient tumor microarray (TMA). Melanoma expression of CD58 and galectin-9 independently predicted for a favorable prognosis. Patients could be categorized into three clusters based upon patterns of protein expression and TILs. Patients in Cluster 2 demonstrated frequent TILs and superior overall survival. Pathway enrichment using MetaCore™ from GeneGo, a Thompson Reuters company, showed that TIMP2 and CD44 were expressed more frequently within Cluster 2 patients, suggesting a potential association with TILs. A subset of melanoma patients appear to lack an organized immune response to the tumor, which portends a poor prognosis.

2. INTRODUCTION

A major challenge in developing the next generation of combination therapeutics for melanoma lies within the difficulty of assimilating divergent pathobiologic processes into one rational all-inclusive strategy. Currently, the primary approach embraced by many focuses on identifying molecular aberrations within the tumor itself and developing pharmacologic agents to specifically target that abnormality (1). A different and complementary approach focuses on understanding the natural host response to a developing tumor (2). Both approaches are indispensable for developing therapeutic strategies for the complex problem of cancer given significant patient-to-patient variation. New methods to evaluate and intervene upon host-tumor interactions within each patient's malignant molecular context need to be developed to address this challenge. Herein, we hypothesize that the biological events involved in fetomaternal dialogue during placentation can help identify prognostic patterns of host/tumor interactions in human melanoma.

Placentation and tumor progression share many biologic similarities (reviewed in Holtan *et al* (3)). For example, molecular programs important for invasion through normal tissues (4, 5) and vasculogenic mimicry (6)

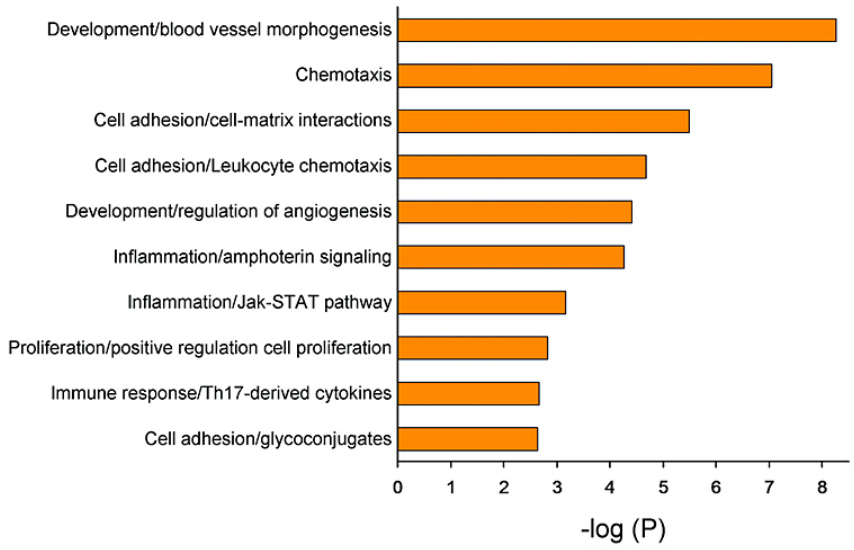

Figure 1. Functional ontology enrichment of the set of stains selected for immunohistochemistry.

appear to be shared between cells of the developing placenta (trophoblasts) and melanoma cells. Moreover, the maternal immunologic reaction to invading trophoblast cells and the cancer patient's immunologic reaction to invading melanoma cells share common features of inflammation, counter-regulation, and angiogenesis (7). Since all these diverse but overlapping processes are critical for both placental biology and melanoma progression, we sought to apply a systems approach to identify prognostic patterns of host/tumor interaction biomarkers in melanoma. In doing so, we tested each biomarker's prognostic importance individually as well as in the context of the other processes in multivariate, unsupervised analyses. Specifically, in a 118-patient metastatic melanoma tissue microarray, we assessed expression of 34 proteins well-described for their importance in trophoblast biology and the maternal decidual tissue response by immunohistochemistry: (1) cell adhesion and invasion (aV integrin, CD44, CD56, CD58, CD133 CXCR4, protease-activated receptor 1 (PAR1), carcinoembryonic antigen-related cell adhesion molecule 1 (CEACAM1), matrix metalloproteinase 2 (MMP2), tissue inhibitor of metalloproteinases 2 (TIMP2), vascular cell adhesion molecule 1 (VCAM1)); (2) angiogenesis (galectin-3, hypoxia-inducible factor 1-alpha (HIF1-alpha), neuropilin 1(NRP1), neuropilin 2 (NRP2), placental growth factor (PGF), vascular endothelial growth factor (VEGF)); (3) and immune response (B7H1, CCL2, CD200, CXCL12, galectin-1, galectin-9, human leukocyte antigen G (HLA-G), insulin-like growth factor binding proteins 1, and 3 (IGFBP-1 and IGFBP-3), indoleamine 2, 3 dioxygenase (IDO), leukemia inhibitory factor (LIF), macrophage migration inhibitory factor (MIF), pentraxin 3 (PTX3), osteopontin (OPN), and toll-like receptors 2, 3, and 4 (TLR2, TLR3, TLR4)). To gauge the diversity of functions represented by our selected biomarker set, we performed functional ontology enrichment on the 34 proteins with the MetaCore™ (GeneGo, a Thompson Reuters company) software suite. The histogram in Figure 1 shows the top ten GeneGo biologic process networks most enriched within the biomarker set. The process networks reflect that the stains selected are representative of the diverse biologic processes involved in placentation not biased toward immune responses or other single processes.

3. MATERIALS AND METHODS

3.1. Ethics statement

This research involving archived human tumor samples was approved by the Mayo Clinic Institutional Review Board and conducted in accordance with principles outlined in the Declaration of Helsinki. TMA specimens were given a deindentified code, and all other clinical information was deidentified upon recording for statistical analysis. Since this protocol utilized existing pathological specimens and historical clinical data, patients were not consented for participation in this exempt research activity.

3.2. Tumor microarray construction and immunohistochemistry scoring

Core tumor samples were retrieved from 118 patients with resected metastatic melanoma lesions as a part of routine clinical care from 1993 to 2004. These cores were obtained from formalin-fixed paraffin embedded macroscopic tumor specimens and arranged in at least triplicate into a recipient paraffin block. Benign lymph nodes, liver, and placental biopsies were included as non-neoplastic control tissues. Primary antibodies were diluted according to the manufacturer's instructions. Streptavidin-horseradish peroxidase (BD Biosciences, San Jose, CA) was used according to manufacturer's instructions for labeling biotinylated primary antibodies for IHC. All slides were counterstained with hematoxylin. Negative controls slides were created as above with no primary antibody. Slide images were digitized using the Olympus BLISS HD

Table 1. Patient characteristics

Median age (y, range)	59 (21-90)
Male	89 (75.4%)
Stage III	53 (44.9%)
Stage IV, M1a	44 (37.3%)
Stage IV, M1b	7 (5.9%)
Stage IV, M1c	8 (6.8%)
Stage IV, M1c, incompletely resected	6 (5.1%)

Virtual Microscopy System™ (Olympus, Center Valley, Pennsylvania) and cores linked to a Microsoft Access™ database to allow for rapid scoring and data analysis.

Although some stromal elements were visible on most cores, the immunohistochemical stains were scored for their staining characteristics on tumor cells only, with the exception of the stains chosen to identify TILs. Each core was evaluated for staining intensity (0 through 3+), location of staining (cytoplasmic, membrane, nuclear, or a combination thereof) and extent of staining (percent of positively staining tumor cells in 5% increments). This allowed for generation of a semiquantitative staining index per stain per patient (the sum of intensity multiplied by the extent of staining for each of a patient's cores, divided by the number of evaluable cores per patient). Cores were evaluable if at least 10% viable non-necrotic tumor cells without significant edge artifact were identified within the core. TMA slides were scored by one observer once and then evaluated a second time at least one week later by the same observer in conjunction with a second observer to assure consistency in assignment of core scores. Even though cores were placed in at least triplicate and at random across the TMA, staining characteristics were consistent across cores from the same patient (not shown). A key linking the arrangement of samples and survival data was maintained to allow for survival analyses as described below.

3.3. Statistical analyses

Overall survival was defined as the time of surgical resection of metastatic lesions for the TMA to death from any cause. In order to determine impact of positive staining of our selected biomarkers on survival using Kaplan-Meier estimates , we defined a positive stain as at least 1+ staining in at least 5% of viable tumor cells (equivalent to a staining index of 5 divided by a maximum of 3 evaluable cores = 1.67). Statistically significant differences between Kaplan-Meier curves were determined using log rank analysis. Survival analyses were additionally performed with the staining indices themselves as a continuous variable using Cox proportional hazards. P values of less than 0.05 were considered statistically significant. Multivariate analyses were performed using Cox proportional hazards and backward selection using all variables with a $p < 0.1$ in the model. Associations between variables were assessed by Spearman's rho. Unsupervised hierarchical clustering was performed according to Ward's method. Differences in the frequency of positive versus negative immunohistochemical scores for the biomarkers between patient clusters were determined by Fisher's exact test.

4. RESULTS

4.1. Survival analyses

Patient characteristics are described in table 1. Nearly all (95.8%) of the patients underwent complete resection of all known metastatic melanoma at the time of surgery. In six patients, metastasectomy was for palliative purposes only. 91 patients have died with a median overall survival for the entire cohort of 21.2 months. Median follow up for this cohort is 6.6 years for the living. Table 2 details the univariate and multivariate results of our 34 immunohistochemical biomarker set as well as TILs in the TMA. Neither age, sex, nor stage influenced survival (not shown), the latter likely due to the TMA being enriched with tumor from patients with completely resected stage III or M1a disease. Univariate analysis revealed tumor cell expression of the following as positive prognostic factors: B7H1, CD56, CD58, galectin-9, IDO, IGFBP-1, and MIF. Infiltrating CD138+ plasma cells, CD20+ B cells, and CD3+ T cells were each associated with a favorable prognosis. Analysis of TMA results using the staining index as a continuous variable was consistent with the analysis of stains as dichotomous (positive/negative) variables with one notable exception: higher intensity osteopontin expression was associated with a worse prognosis ($p=0.03$, not shown). Too few patients with unresectable disease had samples placed in the TMA to draw any reliable conclusions regarding differences between stage and immunohistochemical results.

When factors that demonstrated a $p<0.1$ on univariate analysis (Table 2, bold) were included in a multivariate model, tumor expression of CD58, galectin-9, and infiltrating B cells retained independent favorable prognostic significance. Since we assessed many biomarkers with this approach, we recognized the possibility of significant associations between the stains. Moderate but significant interactions within the TILs (CD3, CD20, and CD138 positive cells) were apparent (not shown). A lack of B7H1 association with other immunologic biomarkers was noteworthy even though it did not retain independent prognostic significance within the multivariate model. Representative examples of TMA staining for B7H1, CD44, CD58, galectin-9, and TIMP2 are shown for illustration in Figure 2.

4.2. Unsupervised hierarchical clustering of immunohistochemical staining patterns

Next, to assess whether any inherent patterns of immunomodulatory protein expression and TILs had impact on patient outcomes, we performed unsupervised hierarchical clustering on the TMA results (Figure 3a). Virtually all samples stained positively for galectin 1,

Table 2. Results of the melanoma tumor microarray immunohistochemical staining. Stains highlighted in bold were $p < 0.1$ and therefore included in multivariate analysis. Additional clinical factors including age, sex, and stage did not have an impact on survival in this analysis. Overall survival (OS) is listed in months.

IHC stain	Percent positive	Median OS if positive	Median OS if negative	p-value	Multivariate RR	95% CI	p-value
aV integrin	73.7%	22.1	13.2	0.12			
B7H1	82.7%	22.1	10.2	0.007			
CCL2	86.7%	22.1	13.2	0.22			
CD133	86.1%	21.2	19.4	0.5			
CD138+ TIL	47.4%	21.7	18	0.05			
CD20+ TIL	42.1%	26.5	16.3	0.006	0.42	0.24 - 0.75	0.005
CD200	45.1%	21.6	18.1	0.8			
CD3 +TIL	50.9%	22.5	18.8	0.04			
CD44	21.1%	23.1	18.8	0.36			
CD56	49.6%	23.3	13.8	0.05			
CD58	81.7%	22.8	10.8	0.008	0.52	0.33 - 0.81	0.004
CEACAM-1	78.8%	18.8	22.8	0.96			
CXCL12	10.6%	21.2	20.1	0.81			
CXCR4	28.6%	16.3	22.8	0.7			
Galectin 1	99.1%	21.2	17.8	0.97			
Galectin 3	96.4%	21.2	30.2	0.61			
Galectin 9	50.5%	24.1	14.3	0.02	0.63	0.41 - 0.98	0.04
HIF1a	95.6%	21.6	10.5	0.15			
HLA-G	45.1%	24.3	14.1	0.06			
IDO	79.2%	22.1	7.5	0.01			
IGFBP-1	95.6%	21.6	5.7	0.04			
IGFBP-3	47.3%	20.1	22.5	0.68			
LIF	38.9%	22.3	15.6	0.2			
MIF	91.2%	22.1	8.8	0.02			
MMP2	60.2%	22.4	15.6	0.39			
NRP1	58.1%	21.2	19.3	0.83			
NRP2	63.5%	21.6	14.3	0.11			
OPN	91.8%	18.8	43.7	0.23			
PAR1	64.6%	21.2	19.4	0.53			
PGF	86.4%	21.5	30.2	0.57			
PTX3	7.7%	22.7	14.1	0.15			
TIMP2	68.5%	22.4	13.7	0.16			
TLR2	88.7%	22.1	22.3	0.88			
TLR3	9.4%	38.3	21.6	0.18			
TLR4	52.4%	20.1	22.4	0.38			
VCAM1	41.5%	21.2	23.3	0.37			
VEGF	51.4%	20.1	23.5	0.66			

IGFBP-1, HIF1a, galectin-3, MIF, and osteopontin. Virtually none of the samples stained positively for TLR3 and PTX3. Overall, three distinct clusters of patients were identified with clustering analysis. Patients in Cluster 2 demonstrated superior long-term survival compared with Clusters 1 and 3 (Figure 3b). Patients in cluster 1 had significantly less frequent expression of the following compared with those in clusters 2 and 3: aV integrin, B7H1, CCL2, CD200, CD44, CD56, CD58, HLA-G, IDO, IGFBP3, LIF, MIF, MMP2, NRP1, NRP2, PAR1, PGF, CXCL12, TIMP2, TLR2, TLR3, TLR4, VCAM1, and VEGF. Additionally, significantly fewer patients within cluster 1 had tumor-infiltrating B cells, T cells, and plasma cells. The principal difference accounting for the survival difference between cluster 2 and the others appeared to be the presence of TILs (white box).

4.3. Exploratory pathway enrichment

To determine whether any pattern of protein expression within the biomarkers analyzed in our TMA might have been associated with the presence of TILs, we performed an exploratory pathway enrichment analysis of patterns of protein expression within the three clusters using MetaCore™. One benefit of this type of analysis is the direct visualization of experimental data in the context of networks and functional processes. This approach is most commonly applied to gene expression data, but the manually created database can also be interrogated for protein interactions. To begin this analysis, we identified the median staining index value of each immunohistochemical stain within each of the three clusters (Table 3) and uploaded these values as separate experiments into MetaCore™. We then performed pathway enrichment based upon this data to allow visual distinction of the clusters in the context of biologic pathways.

Two canonical pathway maps showing a statistically significant difference between the clusters are shown in Figures 4a and 4b. Cluster 1 compared with clusters 2 and 3 demonstrated significantly less PGF, VEGF, neuropilin 1, and neuropilin 2 staining, suggesting that the aggressive phenotype of this cohort may be due to factors other than angiogenesis factor-driven cell survival (Figure 4a). In Figure 4b, the group of patients with the best overall survival, cluster 2, has evident expression of CD44, a leukocyte cell adhesion molecule, and increased expression ofTIMP2 relative to clusters 1 and 3. Upon closer inspection of the unsupervised clustering analysis, CD44 is situated adjacent to the TILs (column immediately

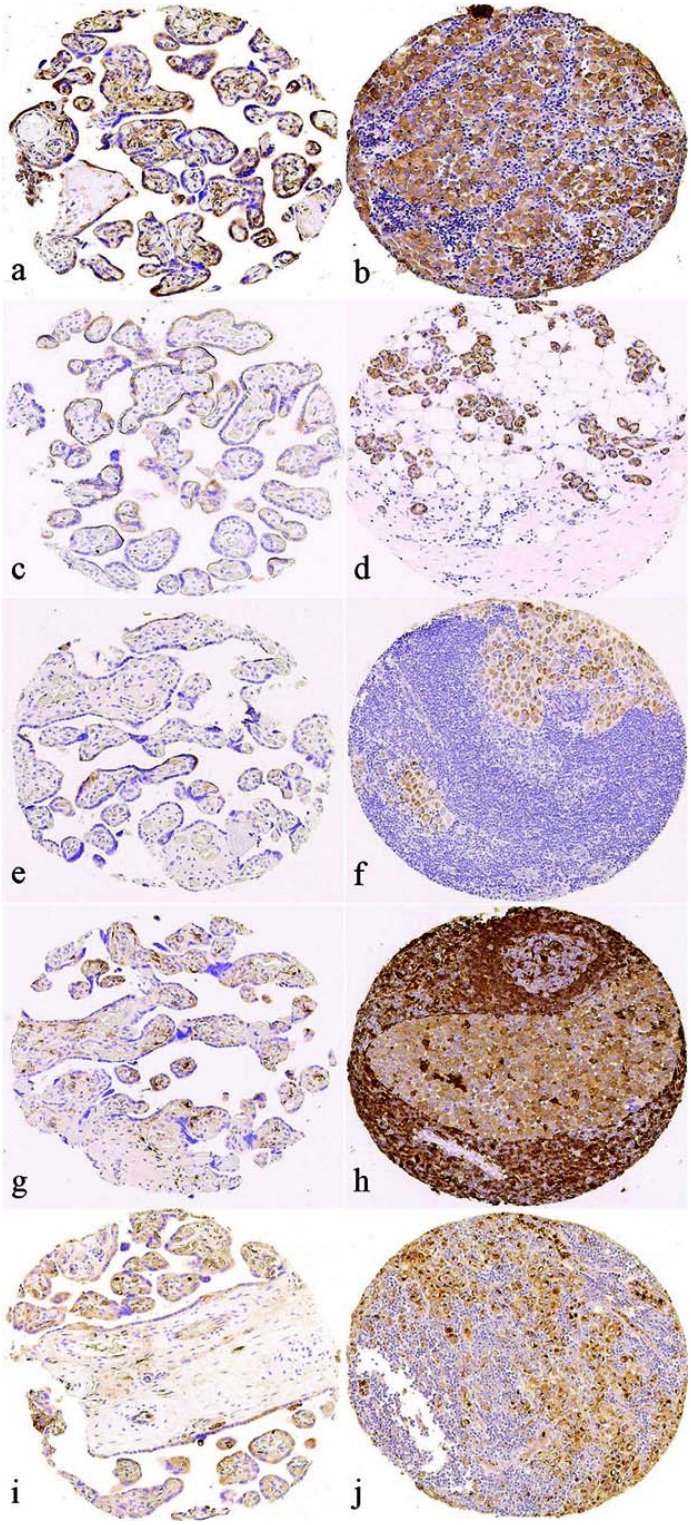

Figure 2. Expression of (a, b) B7H1, (c, d) CD44, (e, f) CD58, (g, h) galectin 9, (i, j) and TIMP2 in placenta and melanoma metastases from representative samples in the tumor microarray (10X). Positive staining is indicated by brown coloration. All slides were counterstained with hematoxylin (blue). Galectin 9, g and h, also stains endothelial cells visible in the placental core and lymphocytes surrounding metastatic melanoma cluster present in the lymph node core. Cells within the lymph node germinal center are largely negative for galectin 9.

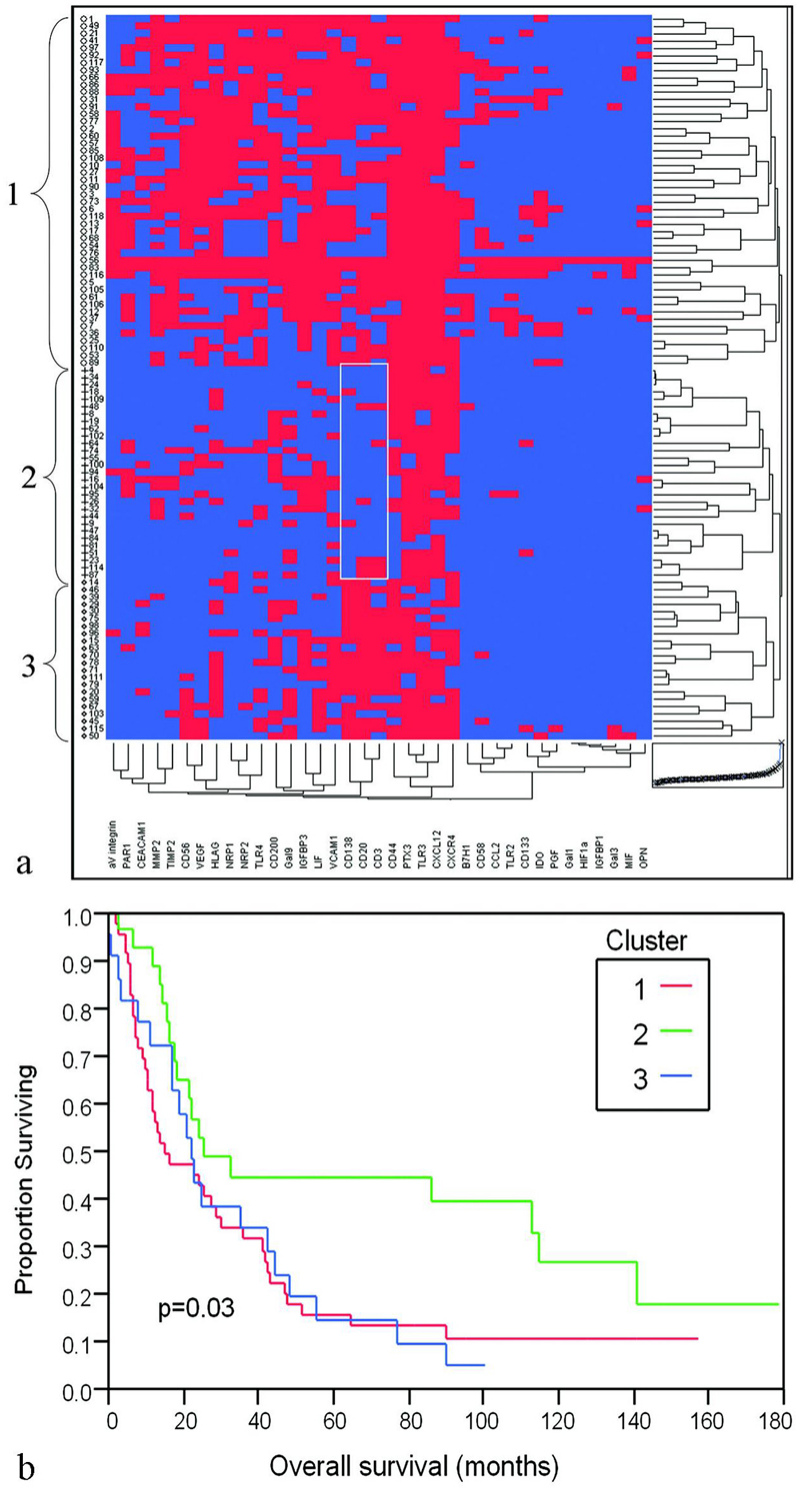

Figure 3. a, Unsupervised hierarchical clustering based upon staining patterns in the tumor microarray demonstrates three distinct groups of patients (rows) with differing patterns of protein expression by immunohistochemistry (columns). Blue indicates that the stain for a given patient was positive; red indicates that the stain was negative. b, Estimates of overall survival by the Kaplan-Meier method indicate that those in cluster 2 exhibit long-term survival compared with the other two clusters, and the predominant difference between the clusters is the presence of tumor-infiltrating lymphocytes (a, white box).

Table 3. Median staining indices by cluster

	Cluster 1	Cluster 2	Cluster 3
aV integrin	1.7	6.7	11.7
B7H1	7.5	27.5	18.3
CCL2	6.7	29.4	28.3
CD133	22.5	61.1	75.8
CD138	0.0	5.0	0.0
CD20	0.0	9.0	0.0
CD3	0.0	31.7	0.0
CD44	0.0	0.6	0.0
CD56	0.0	3.3	2.9
CD58	3.2	21.7	17.5
CEACAM1	42.5	23.3	38.3
CXCL12	0.0	0.0	0.0
CXCR4	100.0	100.0	100.0
Galectin 1	80.0	96.7	85.0
Galectin 3	87.5	113.3	100.0
Galectin 9	0.0	8.3	2.7
HIF1a	84.2	93.3	116.7
HLAG	0.0	3.3	0.0
IDO	2.5	50.0	33.3
IGFBP-1	56.5	96.7	111.7
IGFBP-3	0.0	11.7	10.0
LIF	0.0	3.8	0.8
MIF	17.9	60.0	71.7
MMP2	0.0	5.0	5.0
NRP1	0.0	6.7	5.0
NRP2	0.0	5.0	5.0
OX2	0.0	3.3	2.5
PAR1	0.0	5.0	10.8
PGF	27.1	50.0	68.3
PTX3	0.0	0.0	0.0
OPN	17.5	66.7	70.0
TIMP2	0.0	10.0	6.3
TLR2	7.1	40.0	45.0
TLR3	0.0	0.0	0.0
TLR4	0.0	3.3	3.8
VCAM1	0.0	15.0	1.2
VEGFA	0.0	5.0	9.2

to the right of the white box), suggesting that its expression may have been closely associated with TILs. Both clusters 2 and 3 had increased expression of TIMP2 compared with cluster 1, but the intensity of expression was significantly greater in cluster 2 (median staining index 10.0 vs. 6.3, $p<0.001$). Overall, this exploratory analysis identified the cluster of patients having the best prognosis, Cluster 2, as expressing angiogenic factors and their coreceptors, CD44, and TIMP2 within the tumor tissue.

5. DISCUSSION

Our study confirms previously published data that the presence of TILs is associated with improved survival in metastatic melanoma (8, 9). However, this study uniquely identifies the fundamental importance of an organized immune response in advanced melanoma as paramount to survival, even when compared to biomarkers of seemingly very divergent biologic processes as detailed in Figure 1. Furthermore, we sought to identify a molecular profile associated with presence of an immune response, and hence an improved prognosis using proteins known to be present at the fetomaternal interface, a structure that in many ways shares common features with a tumor microenvironment (3). Using our organ system-based approach, we identified the following candidates for TIL chemoattraction and improved prognosis: melanoma expression of CD58 and galectin-9.

5.1. CD58

CD58 is a ligand for CD2 expressed on T cells (10), and *CD58* gene expression is highly upregulated in endometrium during the window of implantation (11). Soluble CD58 is elevated in healthy pregnant women compared to non-pregnant controls, and lower soluble CD58 levels in preeclamptic women are associated with more severe preeclamptic symptoms (12). Previous studies have confirmed CD58 expression on melanoma cell lines and on metastatic lesions by immunohistochemistry (10). To our knowledge, it has not previously been demonstrated to be associated with improved survival in melanoma. Additionally, we are not aware of determination of soluble CD58 levels in the setting of melanoma. Its potential indicator of active host/tumor recognition, as it appears to function in fetomaternal recognition, should be evaluated in future melanoma studies.

5.2. Galectin-9

Galectins are glycan-binding proteins that function in the resolution of inflammatory reactions during health and disease. Galectin-9 is the ligand for Tim-3 expressed on Th1 cells (13) and is expressed in endometrium during mid to late secretory phases as well as decidual phases of the reproductive cycle (14). It appears to exert a regulatory role on the T cell compartment through T-cell apoptosis, although it can also induce pro-inflammatory cytokine production independent of Tim-3 (15). Galectin-9 has previously been shown to suppress melanoma metastasis in a mouse model (16), and positive galectin-9 staining of primary melanomas is associated with a reduced risk of recurrence and mortality (17). Determination of soluble galectin-9 levels has not previously been described in melanoma, although its potential as an exogenous, therapeutic regulator of immunity is being actively pursued (18).

5.3. CD44

Pathway enrichment also suggested that CD44 and TIMP2 expression might be associated with TILs and an improved prognosis. CD44 is a cell adhesion molecule expressed on activated T cells as well as several normal epithelial tissues. The antibody we used for CD44 IHC recognizes CD44v6, which has previously been demonstrated to stain extravillous trophoblast cells in hypoxic areas, with decreased staining presence and intensity in term placentae (19). Melanoma expression of CD44 associated with improved survival in our study requires validation, as previous studies suggest that it is involved in metastasis (20), tumor progression (21), and facilitation of leptomeningeal attachment (22). However, low levels of circulating CD44 can be seen in the immune dysregulation associated with chronic inflammation (23), and low levels soluble CD44 have been associated with a poor prognosis in melanoma (24). Further studies aimed at reconciling these potentially discrepant findings and understanding CD44-regulated immune responses *in vivo* will be required.

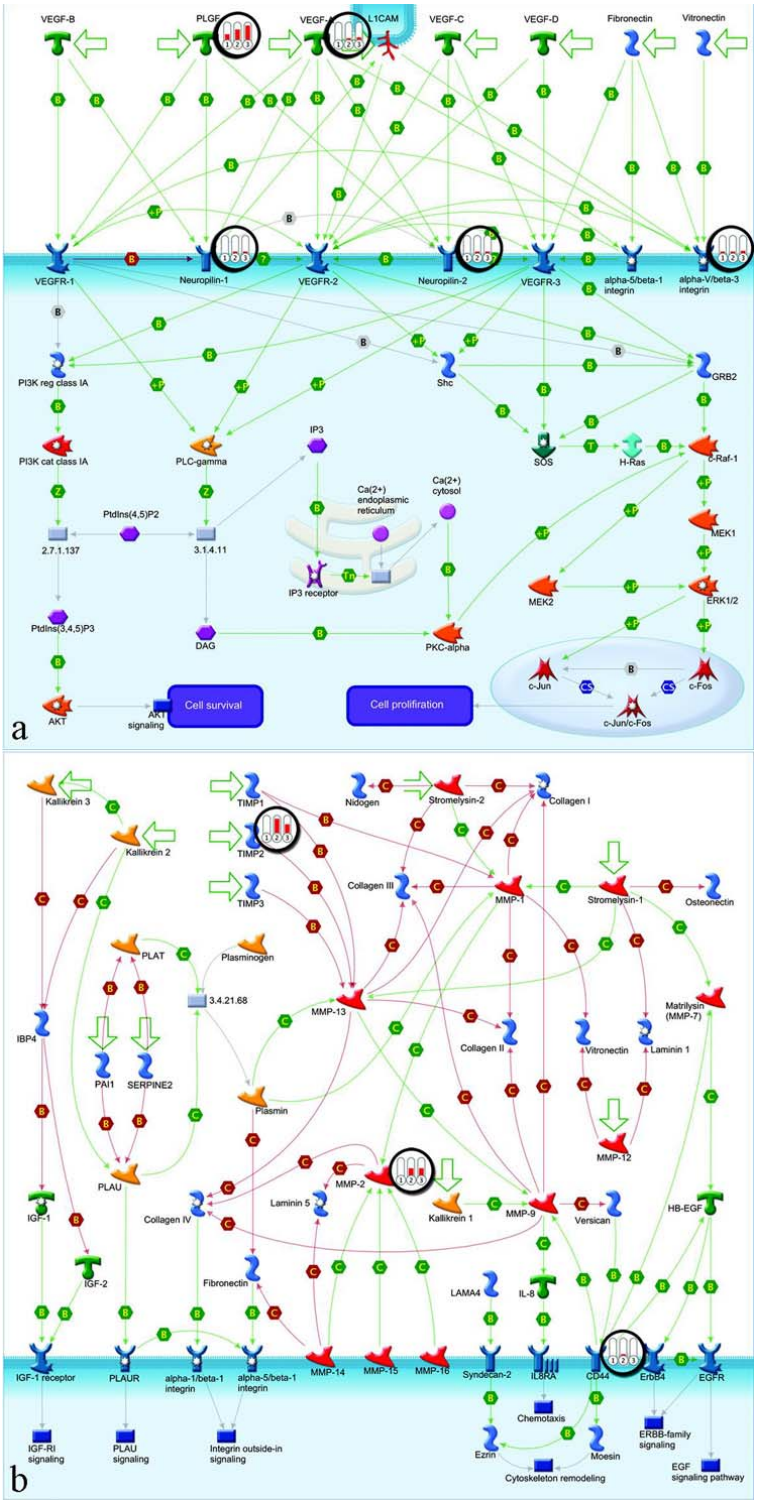

Figure 4. Canonical pathway maps based upon enrichment distribution of immunohistochemical staining profiles by patient cluster. In the images below, the relative staining intensity between the clusters is denoted by thermometers 1, 2, and 3 (circled) representing each cluster respectively. (a) A VEGF family signaling map shows relatively less intense staining of VEGF, placental growth factor, and neuropilins 1 and 2 in patient's melanoma lesions in cluster 1 compared with clusters 2 and 3. (b) An extracellular matrix remodeling map shows that CD44 and TIMP2 staining is increased in cluster 2, the patient cohort with the most frequent tumor-infiltrating lymphocytes and improved survival, relative to the other patient clusters.

5.4. TIMP2

TIMP2 is a matrix metalloproteinase inhibitor produced by many cells types including trophoblasts and decidual cells, where it is probably involved with very early fetomaternal recognition (25). TIMP2 expression on trophoblasts and decidual cells substantially increase near term pregnancy (26). Interestingly, TIMP2 has been demonstrated to have anti-angiogenic properties independent of its MMP inhibiting activity (27) and may be negatively regulated by angiogenesis (28). Since VEGF inhibitors are widely used in combination cancer therapeutics at present, further determination of TIMP2 as a biomarker of therapeutic angiogenesis inhibition is possible. In melanoma, previous studies have indicated a reduction in invasive capacity of TIMP2-positive cells, but this phenotype is also associated with increased resistance to apoptosis (29). Future studies to clarify the role of TIMP2 in the multiple processes of immunologic engagement, angiogenesis, and malignant phenotype will help clarify its potential as a prognostic and therapeutic target in melanoma.

6. CONCLUSION

Rather than focusing on isolated pathways, we used known interactions at the fetomaternal interface to identify markers of prognostic host-tumor interactions in metastatic melanoma. A limitation of type of analysis is that it yields descriptive rather than mechanistic results. However, our multidimensional approach may yield information that is more illustrative in biologic distinctions between groups of patients than a biomarker study of a smaller number of less diverse parameters. Our results show that, among several biomarkers involved in the processes of immune modulation, angiogenesis, cellular adhesion, and invasion, evidence of immunologic engagement between the host and tumor is the most important factor indicative of improved survival. Tumor expression of CD58 and galectin-9 are independently favorably prognostic. Additionally, concomitant melanoma expression of angiogenic factors and their co-receptors, CD44, and TIMP2 appear to be a feature of the group of patients with the most frequent TILs and with the best prognosis.

In a subset of melanoma patients, there appears to be a relative lack of an organized immune response to the tumor, which portends a poor prognosis. This finding does not appear to be an artifact of the biomarkers chosen since important processes for tumor progression, including cellular invasion and angiogenesis, were included in the set of immunohistochemical stains. Given both tumor and patient heterogeneity, future efforts to develop individualized therapeutic strategies for metastatic melanoma and other advanced cancers will require an integrated effort to address all facets of the disease that make it deadly. Simultaneous consideration of somatic mutations driving the malignant phenotype as well as the profile of the permissive host micro/macroenvironment is critical to improve outcomes for patients with metastatic melanoma.

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Abbreviations: CD=cluster of designation, CEACAM1= carcinoembryonic antigen-related cell adhesion molecule 1, IDO=indoleamine 2,3-dioxygenase, HIF=hypoxia inducible factor, IGFBP=insulin-like growth factor binding protein, LIF=leukemia inhibitory factor, , MIF = macrophage migration inhibitory factor, MMP=matrix metalloproteinase, NRP=neuropilin, OPN=osteopontin, OS=overall survival, PAR1= protease-activated receptor 1, PGF=placental growth factor, PTX3=pentraxin 3, TIL=tumor infiltrating lymphocyte, TIMP2=tissue inhibitor of metalloproteinase 2, TLR=toll-like receptor, VCAM1=vascular cell adhesion molecule 1, VEGF=vascular endothelial growth factor

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