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A unique gene expression signature is significantly differentially expressed in tumor positive or negative sentinel lymph nodes in patients with melanoma

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Abstract

Purpose—Molecular characterization through gene expression profiling of node positive and node negative sentinel lymph nodes (SLNs) in patients with clinical stage I–II melanoma may improve the understanding of mechanisms of metastasis and identify gene signatures for SLN +/SLN– that correlate with diagnosis or clinical outcome.

Methods—Gene expression profiling was performed on SLN biopsies of 48 (24 SLN+, 24 SLN–) patients (T3a/b, T4a/b) who underwent SLN for staging using transcriptome profiling analysis on 5µ sections of fresh SLN. U133A 2.0 Affymetrix gene chips were used. Significance Analysis of Microarrays (SAM) was used to test the association between gene expression level and SLN status. Genes with fold change >1.5 and q value <0.05 were considered differentially expressed. Pathway analysis was performed using Ingenuity Pathway Analysis. Benjamini and Hochberg method was used to adjust for multiple testing in pathway analysis.

Results—We identified 89 probe sets significantly differentially expressed (1.5–27 fold; q value <0.05). Upon pathway analysis, 25 genes were common among the most significant and biologically relevant canonical pathways. The molecules and pathways that achieved differential expression of highest statistical significance were notably related to melanoma and its microenvironment and signaling pathways implicated in immunosuppression and development of cancer.

Conclusions—A 25 gene signature is significantly differentially expressed between SLN+ and SLN– and is related to melanoma oncogenesis and immunosuppression. The identified expression profile provides a signature of melanoma nodal involvement. These findings warrant further

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investigation in relation to the mechanisms of metastasis, melanoma metastasis diagnosis and prediction of outcome.

Keywords

melanoma; sentinel lymph node; gene expression

Introduction

Global incidence of cutaneous melanoma is constantly rising and in the United States alone, 76,380 persons were estimated to be diagnosed with this disease in 2016 with 10,130 estimated to succumb to it^[1]. The American Joint Committee on Cancer (AJCC) classifies patients with melanoma into four stages. Stages IA–IIC include patients with thinner or thicker melanoma confined to the skin, stage III patients have regional disease spread defined by lymphatic node or other lymphatic metastasis (satellite lesions, in transit metastasis) and stage IV patients presenting with disseminated disease^[2]. Primary tumor thickness (T) is strongly associated with the risk of disease relapse and 10 year survival ranges from 92% in T1 (1.00mm) melanomas to 80% in T2 (1.01–2.00mm), 63% in T3 (2.01–4.00mm) and 50% in T4 patients with melanomas thicker than 4.00mm. Sentinel lymph node (SLN) mapping is commonly offered to patients with T2–T4 localized tumors and some “higher-risk” T1 melanomas (with adverse clinical features as ulceration or high mitotic rate) who bare a risk of relapse that exceeds 10%. The SLN status is the most important prognostic factor for patients with localized melanoma according to the extent and number of nodes involved. In microscopic (stage IIIA–IIIB) or gross (stage IIIB–IIIC) melanoma the risk of disease recurrence ranges from around 35% to more than 89% and the consecutive 5 year survival drops from 75% to around 50%^[2,3].

The SLN is defined as the first lymph node in the regional lymphatic basin which receives drainage from the primary tumor potentially being the first site of malignant cells metastasis (2 or more SLNs can be present depending on the site of the primary tumor). The technique of SLN mapping was best described by Morton et al.^[4] who led the field in its application to melanoma staging. There are at least two benefits to identify the SLN: 1) improved accuracy of nodal staging by examining multiple sections of the SLN for micrometastases using immunohistochemistry (IHC for S100, MART-1, HMB45, tyrosinase) in addition to routine hematoxylin and eosin (H&E) staining^[5] and 2) avoidance of the requirement of a complete node dissection for staging purposes in patients who do not have clinically regional nodal involvement and a non-involved SLN is identified. Nevertheless, in an updated analysis of MSLT-I, the biggest clinical trial comparing SLN mapping to observation, although SLN biopsy dropped the 10-year hazard ratio (HR) for disease free survival especially in intermediate-thickness melanomas (defined as Breslow 1.2–3.5mm vs. >3.5mm for thick melanomas), there was no impact on 10-year melanoma specific survival rate either for intermediate (HR 0.84; 95% CI, 0.64–1.09; p=0.18) or thick melanomas (HR 1.12; 95% CI, 0.76–1.67; p=0.56) respectively^[6].

At the molecular level, a common pattern in cancer progression is regional lymph node metastatic involvement^[7] potentially serving as a bridgehead for further metastatic spread^[7].

Among patients with SLN positive and negative melanoma, prognosis varies and benefits of adjuvant interferon (IFN)- α 2b, pegylated IFN and ipilimumab are confined to a subset^[8,9]. The SLN can be invaluable for understanding the host-tumor interaction. The known down-regulation of immune activity in lymph nodes proximal to the primary tumor indicates that the SLN is likely to be immunologically modulated to a greater extent than remote regional lymph nodes^[10]. Molecular characterization of positive and negative SLN through gene expression profiling may improve the understanding of mechanisms of metastasis and immune evasion and identify gene signatures of SLN⁺/SLN⁻ that correlate with diagnosis and clinical outcome.

In this prospective study, we molecularly characterized the regional nodal status of positive and negative SLNs in patients with a recent diagnosis of cutaneous melanoma. The main goal of our analysis was to search for significantly differentially expressed genes that are associated with sentinel lymph node status. Our analysis identified a 25 gene signature, consistent with metastatic melanoma and reflective of its microenvironment at the SLN level.

PATIENTS, MATERIALS AND METHODS

Patients

Patients were eligible if they were at least 12 years of age, had primary melanoma with Breslow thickness ≥ 2.01 mm (T3 or T4, without or with ulceration) who underwent a SLN biopsy as a standard of care staging procedure. All participants signed a written informed consent. The study was conducted at the University of Pittsburgh cancer Institute in accordance with Good Clinical Practices, local regulatory requirements, and the Declaration of Helsinki. The study and the informed consent were approved by the IRB of the University of Pittsburgh.

Laboratory and Statistical Methods

Study Endpoints—Our main goal for this study was to properly collect and process SLN material of melanoma patients to explore the potential different molecular features of the tumor microenvironment. The primary objective was gene-profiling analysis, to molecularly characterize the regional nodal status of positive and negative SLNs (SLN⁺ and SLN⁻) in patients undergoing SLN mapping and dissection for routine staging of melanoma. (Primary endpoint: mRNA expression by gene array). Secondary objectives included investigation of an association between specific gene signatures for positive and negative SLN and clinical outcomes (e.g. recurrence and survival). Because of the relatively short follow up, analysis of the secondary objectives could not be completed at this time.

SLN Tissue Procurement—Surgically resected SLN was dissected and, if sized over 8mm in its longest diameter, the peripheral parts were stained for H&E and no more than 20% of the tissue was immediately placed in RNA lysis buffer (Qiagen RNeasy kit) and frozen at -80°C for future mRNA extraction for gene expression profiling. The rest of the LN was placed in 10% formalin buffer and along with the H&E sections sent for central reviewing. SLN positivity was assessed by H&E and immunohistochemistry (MART-1/

melena-A, gp100, tyrosinase, S100 staining). Patients were pathologically characterized as SLN⁺/SLN⁻. Tissue was not used for research until the pathology report established a final diagnosis. A schematic of the study procedure can be viewed in Figure 1.

mRNA Expression Assays: Eukaryotic Target Preparation and Hybridization—

Total RNA was extracted from 24 negative and 24 positive, fresh-frozen SLNs using Qiagen miRNeasy kit (Qiagen Inc., Valencia, CA) according to the manufacturer's protocol. Only total RNA of high quality and integrity was subjected to further processing after purification as defined by an absorption ratio 260/280>1.8 by spectrophotometry on the NanoDrop 1000 (NanoDrop, Wilmington, DE) and a RIN value >8.0 via electrophoretic analysis on the Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA). In vitro transcription was performed using the Ambion MessageAmp Premier Enhanced assay protocol (Ambion Inc., Austin, TX) starting with 500ng of purified total RNA. Confirmation of cRNA diversity was obtained using the Bioanalyzer 2100 to generate an electrophoretogram for each IVT reaction regarding sample yield, integrity, and size diversity against a Universal Human Reference RNA (Stratagene, La Jolla, CA). Fifteen micrograms of purified, amplified, biotin labeled cRNA was fragmented and hybridized on to Affymetrix Human Genome HGU133A 2.0 arrays (Affymetrix Corp., Santa Clara, CA) for 18 hours. Washing, staining and scanning of arrays was performed on the Affymetrix Fluidics Station 450 and Scanner 3000 immediately after completion of hybridization.

Data Analysis—Microarray data was processed using the RMA express software^[11–13]. Gene expression level between SLN⁺ and SLN⁻ groups are compared using the Significance analysis of Microarrays (SAM) implemented in R^[14] and the false discovery rate (FDR) is controlled at 5%. We considered probe sets with fold change >1.5 and q value <0.05 as differentially expressed. Heatmap presentation of the gene expression level between the SLN⁺ and SLN⁻ groups for the differentially expressed genes were provided. Pathway analysis of the differentially expressed gene list was performed using the Ingenuity Pathways Analysis (IPA) software (Ingenuity Systems, Redwood city, CA, USA). The Benjamini and Hochberg's method was used to adjust for multiple testing in pathway analysis.

RESULTS

Patients

A total of 48 patients were enrolled between February 2009 and August 2013 and all had primary cutaneous melanoma Breslow thickness ≤ 2.01mm (T3 or T4), without (N = 19) or with (N = 29) ulceration. Half of the patients had a SLN involved by melanoma metastasis as assessed by H&E and immunohistochemistry (MART-1/melena-A, gp100, tyrosinase, S100 staining). Table 1 summarizes patient demographics and disease characteristics.

Differentiated Gene Expression Profiling between SLN⁺ and SLN⁻ patients

Markers analyzed with SAM with a fold change of ≥ 1.5 and a q value of <0.05 were considered to be differentially expressed. A total of 89 probe sets (73 genes) were identified as having significantly different expression between SLN⁺/SLN⁻. Figure 2 shows the

heatmap of the 89 probe sets for genes that were differentially expressed at significant levels (1.5–27 fold; q value <0.05). Gene enrichment analysis of top differentially expressed genes was conducted using the IPA program. Of the 73 originally identified transcripts, 25 achieved differential expression of highest statistical significance and were common among the top (most significant) pathways, allowing us to establish a gene signature consistent with melanoma and its microenvironment at the SLN. Figure 3 shows the heatmap of the 25 genes. These genes are described in Table 2. The top canonical pathways in order of statistical significance according to adjusted p value were associated with the mitotic roles of the Polo Like Kinases (KIF23, CDC20, PRC1, CCNB2, CDK1, KIF11, CCNB1), DNA damage-induced 14-3-3 Sigma Signaling (CCNE2, CCNB2, CDK1, CCNB1) and G2M DNA damage checkpoint regulation (TOP2A, CCNB2, CDK1, CHEK1, CCNB1), Cyclins and cell cycle regulation (Cyclins A2, B1, B2, E2 and CDK1), Eumelanin Biosynthesis (TYR, DCT) and Melanocyte Development and Pigmentation Signaling (TYRP1, TYR, DCT, SOX10), the role of CHK Proteins in cell Cycle Checkpoint Control (CDK1, CHEK1, RCF3), the antiproliferative role of TOB in T Cell signaling (CCNA2, CCNE2) and the p53 signaling (BIRC5, CHEK1, SERPINE2). Table 3 lists the top pathways and the corresponding level of significance.

DISCUSSION

Our study sought to identify genes that are differentially expressed in the SLN microenvironment of patients who underwent lymphatic mapping for melanoma staging in the presence or absence of micrometastatic disease. Twenty five genes that achieved differential expression of highest statistical significance were found to be common among the most significant pathways. The major canonical pathways were implicated in immunosuppression, melanoma carcinogenesis and metastasis. Further study of this signature and related pathways may illuminate the understanding of mechanisms of metastasis and immune evasion and may correlate with diagnosis and clinical outcome.

The top canonical pathways represented mitotic roles of the Polo Like Kinases (KIF23, CDC20, PRC1, CCNB2, CDK1, KIF11, CCNB1) involving key regulators of the cell cycle and regulators of several different aspects of mitosis providing important insights into the process of oncogenesis [15]. The next top pathways were also closely linked with several overlapping differentially expressed genes including DNA damage-induced 14-3-3 Sigma Signaling (CCNE2, CCNB2, CDK1, CCNB1) that play a critical role in DNA damage-induced checkpoints by controlling the biological activity of several key cell cycle checkpoint proteins [16]. Next were G2M DNA damage checkpoint regulation (TOP2A, CCNB2, CDK1, CHEK1, CCNB1), Cyclins and cell cycle regulation (Cyclins A2, B1, B2, E2 and CDK1) and the p53 signaling (BIRC5, CHEK1, SERPINE2) involving key components of cell cycle regulation and tumor suppression. In fact, melanoma harbors one of the highest DNA genomic instability ratios and increased expression of CHEK1 among other checkpoint/repair pathways genes is associated with carcinogenesis and metastasis^[17]. Similarly, linked and overlapping pathways identified included the role of CHK Proteins in cell Cycle Checkpoint Control (CDK1, CHEK1, RCF3) and the antiproliferative role of TOB in T Cell signaling (CCNA2, CCNE2). Finally, as expected significant pathways also included Eumelanin Biosynthesis (TYR, DCT) and Melanocyte Development and

Pigmentation Signaling (TYRP1, TYR, DCT, SOX10) implicated in melanogenesis. *KIF11* and *KIF23* code for kinesin 5 (EG5) and kinesin 6 (MKLP1) respectively. These are members of a superfamily required for duplicate centrosome separation, bipolar mitotic spindle formation and chromosome movement during mitosis. Overexpression occurs in many highly proliferative cancers and so as resistance to taxanes spindle targeting drugs. Pursue of EG5 inhibition as anticancer treatment was extensive but results for melanoma treatment among other malignancies have been disappointing to date^[18]. *MCM4* belongs to the mini-chromosome maintenance proteins family. It is a key member of the pre-replication complex who has helicase activity for DNA unwinding and subsequent replication and its function is regulated by S phase checkpoint inhibitors^[19]. Molecular studies suggest increased levels in multiple precancerous lesions and malignancies^[19]. For melanoma, MCM4 increased expression correlates with increased tumor thickness, shorter distant-metastasis free and overall survival and poor clinical outcome^[20]. *CDC20* is a mitotic coactivator of the anaphase-promoting complex/cyclosome (APC/C) and its main role is promoting the initiation of anaphasis and chromosome separation via ubiquitination of anaphase inhibitors. CDC20 overexpression has been documented in many cancers and is reported to invertly correlate with p53 function^[21]. *CDC6* is a member of the pre-replicative complex and is required for DNA loading with mini chromosome maintenance (MCM) protein as an essential step in the initiation of DNA synthesis. It is tightly regulated and inactivated by cdk1 after S phase onset in order to prevent re-replication. Overexpression is common in carcinogenesis as it promotes DNA hyper replication represses the p15^{INK4b}, p16^{INK4a} and ARF tumor suppressive genes (INK4/ARF locus) as well as E-cadherin to promote epithelial to mesenchymal transition (EMT) a key event in the process of metastasis^[22]. This consists with the observation of CDC6 higher expression in melanoma metastatic lesions compared to the primary tumor^[23]. *PRC1* is a “non-motor” protein participating in the formation of the mitotic spindle. It interacts with several “motor” proteins like MKLP1 regulating their expression and function. It is also required for spindle localization of the Aurora A kinase, another vital protein for centrosome maturation and spindle assembly. Overexpression in cancer highly correlates with chromosomal instability^[24]. *BIRC5* (survivin) is an inhibitor of apoptosis, promotes cell cycle progression and has been correlated to tumor recurrence and chemotherapy resistance in many tumors. Survivin plays a role in melanomagenesis and its expression in the SLN has been reported to be inversely correlated with disease progression and survival in stage III melanoma patients^[25]. *Cyclin A2* (CCNA2) is a key regulator of both S/M phases during the cell cycle. CCNA2 upregulation is a common event in cancer and has been shown to correlate with tumor thickness, cell proliferation rate, progression and survival particularly in superficial spreading melanomas^[26]. *Cyclin B2* (CCNB2) is also involved in the G2/M phase transition and its tumor overexpression or elevated circulating mRNA levels are associated with dismal outcome in many tumors^[27]. *CCNE2* along with CCNE1 are necessary for the formation of the pre-replication complexes on DNA in order for the cells to leave quiescence and enter the cell cycle. They also promote the G1/S phase transition through retinoblastoma phosphorylation and centrosome duplication. Data from breast cancer suggest that CCNE2 is not related to proliferation but instead with other oncogenic attributes such as invasion and drug resistance and is independently associated with early metastases and poor survival^[28]. *CDK1* regulates through sequential binding to CCNA and CCNB1 the cell cycle transition

from late S phase and is required for entry into Mitosis. CDK1 overexpression is prominent in primary invasive and in metastatic melanoma lesions^[23]. *CHEK1* is part of the DNA damage checkpoint regulating machinery and is mainly sensitive for single strand DNA breaks. Upon detection, it arrests cells cycle in late S or G2/M transition, allowing damage restoration. It also confers resistance to DNA damaging therapies (e.g. alkylating agents, radiation). Since other checkpoints like p53 are defective in melanoma, efforts are made in inhibiting CHEK1 either alone or in combination with DNA damaging agents to induce apoptosis in many tumor types^[29]. *DCT* or tyrosinase related protein 2 (TRP-2) belongs to the melanin production associated genes and expression is consistent across all stages of melanoma. It confers resistance to apoptosis either from oxidative stress or from drugs like cisplatin or radiation therapy^[30]. *RFC3* is one of five replication factor subunits that form a multi-protein complex known as Activator 1, which acts as a clamp loader of the proliferating cell nuclear antigen (PCNA) onto primed DNA. PCNA is required for processive DNA chain elongation during Synthesis^[31]. RFC3 is overexpressed in many cancers mainly of endothelial origin, correlates with dismal disease survival and associates with the mitogenic transcription factor MYC, a known oncogene in melanoma vertical growth phase and metastasis^[31]. *RRM2* is the key regulatory component of ribonucleotide reductase (RNR), an enzyme responsible for deoxyribonucleotide triphosphate (dNTP) production during Synthesis. RRM2 downregulation is both necessary and sufficient for the establishment and maintenance of senescence-associated cell growth arrest. In BRAF or NRAS mutated nevi, RRM2 may overcome oncogenic BRAF or NRAS-induced senescence. Further, in melanoma patients with BRAF or NRAS mutant melanoma, high RRM2 expression correlates with poor overall survival^[32]. *Serpin E2* (Protease Nexin-1 (PN-1), belongs to the Serpin protein superfamily and acts as a protease inhibitor mainly for thrombin, plasmin and plasminogen activators, all factors associated with tissue remodeling. Although theoretically its ability to reduce proteolysis and extracellular matrix degradation would protect against cancer cell invasion and metastasis, serpinE2 overexpression leads to upregulation of matrix-metalloproteinase MMP9 which cleaves serpinE2 to allow protease mediated remodeling^[33]. SerpinE2 is highly upregulated upon MAPK pathway stimulation, its overexpression is common in many cancers with such pathway oncogenic alterations^[33]. *SOX10* is an essential factor for specification, survival and differentiation of neural crest derived lineages including melanocytes. It controls both melanogenesis and melanocyte survival and also plays a role in cell cycle progression via MITF (microphthalmia-associated transcription factor) regulation and SOX10 ablation leads to G1 cell cycle arrest as well as promoting^[34] melanoma cell invasion and metastasis^[34,35]. *TOP2A* is a group of highly conserved enzymes that catalyze the ATP-dependent transport of one intact DNA double helix through another during replication and chromosome segregation. It belongs to the DNA defects repair genes and overexpression or aberrant function regulation is associated with chromosome instability. TOP2A has been extensively studied in melanoma, expression correlates with tumor thickness, risk of recurrence, metastasis, resistance to alkylating chemotherapeutic agents and is considered an independent prognostic factor of poor clinical outcome.^[17] *TTK* (or Monopolar spindle 1, MPS1) kinase has an essential role for the spindle assembly check point preventing cell cycle progression from the metaphase to the anaphase when chromosomes are improperly attached to the mitotic spindle. This is crucial for survival of aneuploid cells especially in the presence of other check point deficient

mechanisms like TP53 and correlates with high tumor grade and resistance to antimitotic agents.^[36] *Tyrosinase* is the principal enzyme in the process of melaninogenesis. Expression is present in all stages of melanoma development and some reports suggest that in metastatic disease percentage of positivity slightly drops^[37]. It's one of the standard 4 markers for melanoma diagnosis with IHC and detection is highly specific for melanoma disease. *TYRPI* appears to have a less prominent role in melaninogenesis but acts more as a tyrosinase activity regulator and protector of melanocyte against oxidative stress and apoptosis in regional involved LN has been correlated with shorter relapse free survival and dismal outcome in stage III melanoma patients.^[38]

An interesting observation in our analysis has been the implication of a number of differentially expressed genes in the regulation of host immunity towards melanoma, including *CCL20*, *SGK1*, *CCNB1* and *TYMS*. *CCL20* is a strong chemotactic cytokine for CCR6⁺ expressing memory lymphocytes and plasmotoid dendritic cells (PDC). This is potentially an early event in melanoma development and melanoma tumors that produce CCL20 attract PDC that may initiate a specific antitumor response^[39]. *SGK1* belongs to the AGC kinase family and is a downstream effector of the PI3K pathway. Its function is regulated via post-translational modifications by the mTOR complex. Besides its role in homeostasis through membrane regulation of Na⁺/K⁺ turnover, SGK1 promotes oncogenesis by phosphorylating the tumor suppressing gene p27 activates MDM2 (murine double-minute homolog 2)-dependent p53 ubiquitination affecting cell cycle checkpoint arrest and apoptosis^[40] and shifts immune response towards TH2 differentiation, inhibiting interferon- γ mediated tumor rejection^[41]. *CCNB1* regulates mitotic spindle assembling during G2/M phases and under normal conditions its expression remains nuclear at very low levels whereas in cancer cells is overexpressed residing in the cytoplasm, a phenomenon attributed to p53 dysfunction. Additionally, *CCNB1* has been long identified as a tumor antigen and can elicit specific T and B cell antitumor responses^[42]. *TYMS* is a crucial enzyme for DNA synthesis and catalyzes the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP). Overexpression is a common finding in melanoma, primary or metastatic and may have an adverse effect in immune therapies^[43].

Early efforts in molecularly defining metastatic disease present in SLN that cannot be detected by conventional pathology used the same markers as with IHC staining trying to identify microscopic disease that could not be revealed by classic pathology examination. A median yield of 13–52% of additionally identified “molecular SLN metastasis” often correlated with clinical outcome (reduced relapse free and overall survival) but never made it to the clinic to be incorporated in diagnostic/prognostic criteria^[44,45]. Even after CLND, postoperative lymphatic drainage of affected areas with persistent expression of melanoma differentiation genes seems to be prognostic of early relapse^[46]. Establishing a “gene expression signature” that can predict recurrence risk and/or treatment response is rational. Many researchers looked into primary tumor gene expression profiles. Among them, Winnepenninckx et al, retrospectively evaluated 83 primary melanoma tumors and established a 254 gene signature associated with distant-metastasis free survival in 58 patients.^[20] A following report that included 60 patients updated follow up of at least 4 years concluded that the most important pathways in predicting distant-metastasis free

survival are those associated with DNA repair and specifically S-phase checkpoint and post-replicative DNA repair mechanisms, an observation consistent with our results.

Conway et al, identified osteopontin expression as the most significant predictor of relapse free survival (RFS) in a cohort of 254 primary melanomas (0.75mm thick) before and after adjustment for other risk factors. Validation in a second cohort of 218 patients also correlated osteopontin expression with reduced RFS and higher probability of SLN involvement. Additional genes closely correlated to osteopontin and with the most significant difference between relapsers and non-relapsers included BIRC5, TOP2A, CCNA2, TK1 and RAD51, also consistent with our results^[47]. Efforts to investigate gene expression inside the SLN are more recent. Koh et al, tried to define a distinct expression profile of genes between primary melanoma and metastatic SLN, evaluating 10 primary and 9 SLN lesions although only 4 of them were paired (same patient). Most of the patients had documented systematic disease at the time of diagnosis. Of the 174 genes upregulated in the SLN those with the biggest fold difference correlated with host's immune response like tumor antigens (MAGE family antigens) and FC like receptors^[48]. Itakura et al, molecularly characterized 23 SLN (10⁺/13⁻) and 11 tumor free non-SLN into two different patterns according to gene expression of inflammation related genes. Interestingly, SLNs had a similar immunosuppressive pattern (higher IL-10, IL12 receptor b2, CCR5, TNF R2 expression) irrespectively of the presence of metastatic disease or not compared to non-SLN. Between SLN positive or negative for metastatic disease, positive nodes expressed higher levels of IL-13, leptin, lyphotoxin b-receptor (LTbR) and macrophage inflammatory protein 1 β (MIP1 β or CCL4) and lower levels of IL-11R α . These results were further validated in 21 additional SLN (9⁺/12⁻) and show that SLN microenvironment is affected by melanoma even in the absence of actual disease^[49].

To date, the risk of relapse assessment for patients with localized melanoma is based solely on surgical and pathology criteria like Breslow thickness, ulceration, lymphatic involvement and mitotic rate. The SLN status remains the most important prognostic factor. Pathology examination of SLN uses IHC antibodies for tumor cell staining (S100, MART-1, HMB45, tyrosinase) or classic H&E stains. This approach is inherently limited because microscopic tumor foci can be missed in the midsection parts of the examined SLN. On the contrary, pursuing genes expressed in the tumor microenvironment may overcome this obstacle^[50]. In uveal melanoma, a gene expression profile (GEP) assay stratifying this subset of melanomas according to risk of metastasis as predicted by a 15-gene signature is increasingly used to guide patients' treatment^[51]. Gerami et al, tried to expand GEP in an effort to predict risk recurrence in stage I–II patients with cutaneous melanoma. He developed a 27 gene signature and evaluated archived FFPE primary melanomas from 220 patients with at least 5 years of follow up (less if a metastatic event was evident). In 119 stage I patients, negative and positive prognostic values (i.e. cases called class 1 and class 2 meaning no risk or risk of metastasis) were 95% and 56% respectively while for 109 stage II patients (45 stage IIA, 42 stage IIB, 14 stage IIC) negative and positive prognostic values were 67%/90%, 43%/96% and 33%/100% respectively indicating that increasing Breslow thickness led to a higher “false” prediction of relapse^[52]. The same group compared the prognostic value of SLN status with that of the expanded GEP. SLN status had a greater positive prognostic value (PPV) 55vs.50% for distant metastasis but less negative (NPV)

67vs.82%. Disease free survival (DFS) and distant metastasis free survival (DMFS) were similar for SLN⁺, GEP class 2 patients, and for patients with SLN⁻ but classified as GEP class 2. DFS and DMFS varied from 79% and 82% for GEP class 1 to 55% and 64% for SLN⁻ patients indicating a higher impact of GEP in predicting “less aggressive” disease. In accordance, for 9 patients with SLN⁺ but GEP 1 score, DFS and DMFS were intermediate (53% for both)^[53].

Previous studies mostly retrospectively investigated archive formalin embedded primary lesions. This approach had the caveat of RNA degradation during fixation and storage. Diverse populations examined (thin/thick melanomas, clinical staged tumors, stage IV tumors at diagnosis) and lack of quantitative validation make these results difficult to interpret. Common candidate genes are different within studies generally indicating these limitations. Our results include most of the pathways implicated in previous studies linking melanoma progression to higher expression of genes correlating to DNA replication, repair and checkpoint inhibitor pathways. To our knowledge, this is the first molecular prospective study of a homogenous patient population based on Breslow thickness evaluating SLN microenvironment molecular status and clinical data with estimated risk of relapse and response to adjuvant treatment will be reported upon maturation.

CONCLUSION

In our investigation of the SLN microenvironment, a 25 gene signature is significantly differentially expressed between the SLN⁺ and SLN⁻ groups. These genes are related to melanoma oncogenesis and to immunosuppression. The expression profile provides a signature of nodal involvement and warrants further investigation towards understanding the mechanisms and melanoma metastasis and in relation to melanoma metastasis diagnosis. Association with clinical outcome will be investigated as follow up of our patient cohort matures.

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SLN and Non-SLN Procurement for Molecular Profiling

Figure 1.

A summary of patient management and tissue harvesting and processing under the study's protocol.

Figure 2. Heatmap of eighty nine probe sets for genes that were differentially expressed at significant levels (1.5–27 fold; q value <0.05) between sentinel lymph nodes (SLN) involved by melanoma (+) and SLN–.

Figure 3. Heatmap of the probe sets for 25 genes that were significantly differentially expressed between sentinel lymph nodes (SLN) involved by melanoma (+) compared to SLN- and belonged to the top (most significant) canonical pathways.

Table 1

Patient demographics and disease characteristics.

Variable	No. of Patients (%)
Age, years; Median (range)	54 (16-73)
Cutaneous primary	48 (100)
Gender	
Female	20 (42)
Male	28 (58)
Performance status (ECOG)	
0	42 (88)
1	6 (12)
Ulceration of primary	
Yes	29 (60)
No	19 (40)

ECOG: Eastern Cooperative Oncology Group

Table 2

Genes that belonged to top pathways and were significantly differentially expressed in SLN+ vs.

Gene ID	Gene Description
BIRC5	Baculoviral inhibitor of apoptosis repeat-containing 5 (Survivin)
CCL20	Chemokine (C-C motif) ligand 20
CCNA2	Cyclin A2
CCNB1	Cyclin B1
CCNB2	G2/mitotic-specific cyclin B2
CCNE2	Cyclin E2
CDC20	Cell division cycle 20
CDC6	Cell division cycle 6
CDK1	Cyclin-dependent kinase 1
CHEK1	Checkpoint kinase 1
DCT	Dopachrome tautomerase
KIF11	Kinesin family member 11
KIF23	Kinesin family member 23
MCM4	Minichromosome maintenance complex component 4
PRC1	Protein regulator of cytokinesis 1
RFC3	Replication factor C (activator 1) 3
RRM2	Ribonucleotide reductase M2
SERPINE2	Serpin peptidase inhibitor, clade E member 2
SGK1	Serum/glucocorticoid regulated kinase 1
SOX10	SRY (sex determining region Y)-box 10
TOP2A	Topoisomerase (DNA) II alpha 170kDa
TTK	TTK protein kinase
TYM S	Thymidylate synthetase
TYR	TYR tyrosinase
TYRPI	Tyrosinase-related protein 1

Table 3

Top Canonical Pathways

Pathway	Adjusted P value
Mitotic Roles of Polo-Like Kinase	0.0000
DNA DAMAGE-IDUCED 14-3-3 Sigma Signaling	0.0001
G2/M DNA Damage Checkpoint Regulation	0.0001
Cyclins and cell Cycle regulation	0.0012
Eumelanin Biosynthesis	0.0023
Melanocyte Development Signaling	0.0096
Role of CHK in Cell Cycle Checkpoint Control	0.0199
Antiproliferative Role of TOB in T Cell Signaling	0.0401
p53 Signaling	0.062

Abbr; CHK; checkpoint kinases; TOB; transducer of ERBB2