Gene Expression Signatures as a Guide to Treatment Strategies for In-Transit Metastatic Melanoma

Christina K. Augustine1,6, Sin-Ho Jung3, Insuk Sohn3, Jin Soo Yoo1, Yasunori Yoshimoto7, John A. Olson, Jr.1,4, Henry S. Friedman1,2, Francis Ali-Osman5, and Douglas S. Tyler1,6

Abstract

In-transit metastatic melanoma, which typically presents as multifocal lesions, provides a unique setting to evaluate the utility of gene signatures for defining optimal regional therapeutic strategies and assessing the efficacy of treatment. The goal of this study was to determine whether a single multifocal lesion is representative of residual tumor burden in terms of gene expression signatures predictive of response to therapy. Using microarray-based gene expression profiling, we examined 55 in-transit melanoma lesions across 29 patients with multifocal disease. Principal component analysis, unsupervised hierarchical clustering, one-way ANOVA, binary regression analysis, and gene signatures predictive of oncogenic pathway activation were used to compare patterns of gene expression across all multifocal lesions from a patient. Patterns of gene expression were highly similar ($P < 0.006$; average $r = 0.979$) across pretreatment lesions from a single patient compared with the significantly different patterns observed across patients ($P < 0.05$). The findings presented in this study show that individual melanoma tumor nodules in patients with multifocal disease harbor similar patterns of gene expression and a single lesion can be used to predict response to chemotherapy, evaluate the activation status of oncogenic signaling pathways, and characterize other aspects of the biology of an individual patient’s disease. These results will facilitate the use of gene expression profiling in melanoma regional therapy clinical trials to not only select optimal regional chemotherapeutic agents but to also allow for a more rational identification of candidates for specific targeted therapies and evaluation of their therapeutic efficacy.

Introduction

Malignant melanoma is inherently resistant to chemotherapy and, despite appropriate initial therapy, recurs as in-transit metastases in approximately 6% to 11% of patients (1, 2). These in-transit metastases usually present as multiple nodules throughout the epidermal, dermal, and/or s.c. tissues and generally occur between the primary disease site and the regional lymph nodes. Over-coming innate resistance present in the tumor cell is a critical step toward improving response rates to systemic chemotherapy in melanoma patients with widespread disease as well as to regionally administered chemotherapy in patients with metastatic in-transit disease. There are many novel therapeutic drugs in development that target specific molecular or genetic lesions and which disrupt oncogenic signaling pathways important in melanoma. Although these drugs alone often show only modest antitumor activity, it is likely that when applied in combination with traditional chemotherapies, they will serve to sensitize the tumor cell to chemotherapy. As the number of novel targeted drugs increases, it becomes critical to devise a method for individually evaluating each tumor at both molecular and genomic levels so as to better prescribe a course of treatment that is tailored to each patients disease. Microarray technology provides a powerful means of classifying or stratifying tumors based on their individual gene expression profile. Recent use of gene expression profiling has lead to the identification of patterns that are predictive of oncogenic signaling (3) and clinical outcome (4) as well as gene expression patterns associated with melanoma progression and metastatic dissemination (5).

In-transit melanoma is ideally suited to gene expression profiling because multiple tumor lesions from each patient can be obtained and evaluated to identify gene expression patterns associated with melanoma progression and metastatic dissemination (5).
expression signatures that not only correlate with outcome to regional therapy but that can also be used to predict response to therapy as well as guide treatment decisions. The relationship between these multifocal lesions in terms of patterns of gene expression that are predictive of disease progression and response to therapy, however, is unclear. Several studies have examined the different phases of melanoma progression from radial growth phase to vertical growth phase to metastatic and showed a clear clonal relationship between these growth phases within a patient (6, 7). An analysis of genetic traits across multiple metastatic biopsies from a single patient likewise suggested that each metastasis derived from a common primary tumor supporting a clonal derivation of melanoma (8). A loss of heterozygosity analysis of genomic DNA across a panel of 79 in-transit nodules from 25 melanoma patients (9) showed that multifocal in-transit melanoma lesions were, likewise, of the same clonal origin and that genetic lesions contributing to the localized metastatic spread of in-transit melanoma were uniformly present throughout the nodules in a patient. Although of similar clonal origin with a base of genetic defects that persist throughout the course of disease, melanoma lesions can exhibit increasing number and length of genetic imbalances over time (8, 10), which could, in turn, lead to altered transcriptional profiles. Indeed, radial growth phase and vertical growth phase cells from a single lesion show very different patterns of gene expression (11) reflecting the distinct phenotypes of these two melanoma growth phases. Crucial for the application of gene expression profiling in the clinical setting of in-transit metastasis is the clarification of the concordance of transcriptional patterns across lesions. If transcriptional profiles across these lesions are concordant, then any one can be used to predict oncogenic signaling or response to therapy as well as evaluate treatment efficacy. On the other hand, if these lesions show heterogeneous profiles, the ability to use microarray data in a clinical setting to optimize treatment will be limited at best. The aim of this study was to evaluate the transcriptional relationship of multifocal lesions and to determine the extent to which a single lesion is representative of residual tumor burden in melanoma.

Materials and Methods

Patients and tissue acquisition

In-transit melanoma biopsies were obtained before the initiation of isolated limb infusion (ILI; see Table 1 and Fig. 1A) from 29 patients after general anesthesia was administered using a 4-mm punch. The samples were snap frozen and stored at −133°C until ready for further analysis. All patients were enrolled after obtaining written informed consent and tissue samples were collected according to a protocol approved by the Duke University Medical Center Institutional Review Board.

RNA isolation and microarray processing

Tissue was homogenized and total RNA was isolated (Qiagen RNeasy) from tumor samples. Reverse transcription, labeling, and hybridization to the Affymetrix Human Genome U133plus2 (Hu133+2) GeneChip was done at the Duke University Microarray Core Facility. GeneChips were scanned and the gene expression data were preprocessed as described (12). Gene expression data of tumor samples is available at Gene Expression Omnibus (accession numbers GSE10282 and GSE19293).

Analysis of gene expression data

Expression values across 54,675 probe sets or genes were calculated using the Robust Multichip Average method (13). Robust Multichip Average estimates are based on a robust average of background corrected perfect match probe intensities. Normalization was done using quantile normalization (14). Expression values were transformed by taking Logarithm base 2. We filtered out “AFFX” genes and genes for which there were ≤7 present calls (based on Affymetrix’s present/marginal/absent calls using the ratio of perfect match to mismatch probe intensity). Filtering yielded 32,318 genes that were then used in the subsequent analyses. Pearson’s correlation coefficients were determined using the SAS analysis software.

Intracluster correlation coefficients (ICC) were calculated as described (15). ICC values ranged from 0 (within-patient/cluster variance is much greater than between-patient/cluster variance) to 0.992 (within-cluster variance is much less than between-cluster variance). The top 10 k genes (0.822 ≤ ICC ≤ 0.992) were identified and clustered (Cluster software v3.0; ref. 16) such that samples with similar patterns of expression across these genes were grouped and genes with similar patterns of expression across the 17 patient clusters were grouped. The TreeView software (v1.6) was used to generate a heat map diagram of color-coded relative expression (green, low expression; red, high expression).

One-way ANOVA was used to identify genes associated with BRAf and NRas mutation status. To account for the dependency among multiple lesions of each patient, we used the generalized estimating equation method based on a working independent and identically distributed correlation structure (see appendix for more details; ref. 17). Cluster and the TreeView software were used as described above to perform a hierarchical clustering analysis on BRAf/NRas-associated genes.

Binary regression in combination with a series of gene expression training sets derived from in vitro studies of a family of human primary mammary epithelial cell lines overexpressing several proteins with oncogenic activity [Src, Ras, Myc E2F3, β-catenin, and phosphoinositide 3-kinase (PI3K)] was used to define a prediction of dysregulated signaling in each of these oncogenic pathways (3) across patient biopsy samples. A gene expression signature that correlated with clinical response to melphalan ILI chemotherapy was derived.
using a panel of 52 lesions across 28 patients including 12 patients for whom only one lesion was available. Where more than one lesion was available for a patient, the gene expression data from the multiple lesions were averaged. For each gene, the Spearman rank correlation coefficient between its expression level and response outcome [progressive disease (PD) plus stable disease (SD) versus partial response (PR) plus complete response (CR)] and the asymptotic \( P \) value were calculated.

To define a prediction of sensitivity to chemotherapy with temozolomide across patient biopsy samples, a binary regression analysis was done using gene expression training sets derived from a panel of human melanoma-derived cell lines (see Supplementary Methods; ref. 18).

For predictions of sensitivity to temozolomide and dysregulated pathway signaling, a one-way ANOVA model was used to evaluate between-patient variation.

BRaf and NRas mutation analysis

For each lesion, BRaf and NRas mutation status was determined using standard PCR and DNA sequencing. Where RNA was used, cDNA was synthesized using First-strand cDNA synthesis (Roche). See supplementary Table S1 for BRaf and NRas sequencing primers and Supplementary Methods for details of the PCR reaction.

Quantitative PCR analysis of single gene expression

Gene expression level was measured using SYBR GREEN Real-time PCR. Total RNA was isolated from cell lines as described above. First-strand cDNA synthesis was carried out using the Roche Transcriptor First Strand cDNA Synthesis kit. The PCR was done in a 25 \( \mu \)L volume containing 2 \( \mu \)L of sample cDNA, 12.5 \( \mu \)L ABI SYBR GREEN Master Mix, and 250 nmol/L of the forward and reverse primers. Quantitative PCR and data analysis were done using the Stratagene MX3000P system. See Supplementary Table S1 for primer sequences. Expression values were normalized to \( \beta \)-actin and are expressed as the mean of three measurements.

Western blot analysis

For protein expression analysis, a piece of tumor tissue was lysed in a modified radioimmunoprecipitation assay buffer (see Supplementary Methods for details). Clarified lysates were analyzed by SDS-PAGE followed by Western blotting and detection using the Visualizer Western Blot Detection kit (Millipore). N-cadherin (CDH2) antibody was obtained from Zymed. O6-Methylguanine-DNA methyltransferase (MGMT) antibody was a kind gift of Dr. D. Bigner (Duke University Medical Center, Durham, NC).

Results

Seventeen patients undergoing ILI for in-transit extremity melanoma were examined for inclusion in the analysis of concordance across multifocal lesions. Twenty-eight consecutive patients (2 through 29) were included in the correlative analysis of response and gene expression and included 12 patients with only one pretreatment biopsy. Of the 29 patients, 15 were male, 14 were female, and ages ranged from 43 to 90 years (average age, 69). Samples were evaluated for the presence of activating BRaf and NRas mutations—two genes frequently mutated in melanoma. Of the 26 patients evaluable for both genes, 11 (42.3%) harbored no mutation in either BRaf or NRas, 5 (19.2%) harbored an activating NRas mutation [exon 2; glutamine to lysine (2) or leucine (1) at amino acid 61], and 10 (38.5%) harbored an activating BRaf mutation (exon 15; valine to glutamic acid at amino acid 600). The mutation status was identical across the multiple lesions analyzed for each patient with the exception of patient 20 for whom 1 lesion (20a) harbored a BRaf mutation whereas the other (20b) did not; both lesions 20a and 20b were NRas wild-type (WT). Additional clinical characteristics of these patients are summarized in Table 1.

Gene expression analysis reveals similar transcriptional profiles across multifocal lesions

Using the 43 multifocal lesions obtained from 17 patients, unsupervised hierarchical clustering was done across 32 \( k \) genes such that samples with similar patterns of expression were grouped. The results reveal the marked relationship between multifocal lesions within a patient. Two primary clusters are evident, each further subdivided according to patient yielding 17 secondary clusters (Fig. 1B). Primary clusters were largely determined by BRaf and NRas mutation status with the exception of BRaf mutant patient 2 who clustered with the six patients harboring WT BRaf and NRas genes. Pearson correlation coefficients were calculated using expression from these 32 \( k \) genes comparing both intrapatient and interpatient lesions. All lesions from a patient were significantly correlated (see Supplementary Table S2A and B). Correlation coefficients \( r \) ranged from 0.957 to 0.993 across lesions within a patient and 0.895 to 0.967 across patients. A scatter plot illustrating the classification of samples based on the first three principal components further illustrates the similarities between tumor samples derived from an individual patient (Fig. 1C; see also Supplementary Fig. S1A) as well as the marked tendency for lesions to cluster based on BRaf and NRas mutation status.

The 32 \( k \) gene list contains several genes that show little variation across patients and are thus not likely to contribute to the phenotypic heterogeneity observed across melanoma patients. To focus the intrapatient and interpatient correlation analysis on genes more relevant in terms of melanoma biology and interpatient heterogeneity, ICCs (15) were measured for each of the 32 \( k \) genes and the top 10 \( k \) genes (ICC values, 0.822-0.992) were identified (see Supplementary Table S3 for a list of the top 50 genes). ICC values close to 1 indicate that
the variance in expression across lesions within a patient is much less than across patients, whereas values close to 0 mean that intrapatient variance is much greater than interpatient variance. In this way, genes with homogeneous patterns of expression within a patient and heterogeneous patterns of expression across patients were identified. Unsupervised hierarchical clustering was done across these 10 k genes (Fig. 2A). As in the initial analysis, intrapatient lesions clustered together and showed similar patterns of gene expression confirming that a single tumor lesion is most similar to other lesions derived from the same patient (intrapatient Pearson’s correlation coefficients, 0.963-0.995; see Supplementary Table S2A). Notably, marked interpatient differences in gene expression patterns were revealed. This can be seen more clearly in the three expanded images (Fig. 2B).

Table 1. Clinical characteristics of data set

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>No. of lesions</th>
<th>Mutation status</th>
<th>Disease stage before ILI*</th>
<th>Age</th>
<th>Sex</th>
<th>3-mo response†</th>
<th>Disease burden‡</th>
<th>Prior systemic therapies§</th>
<th>Size of primary tumor</th>
<th>Extremity location†</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3</td>
<td>Wt</td>
<td>Wt</td>
<td>90</td>
<td>M</td>
<td>LTF/U</td>
<td>High</td>
<td>TMZ</td>
<td>Unknown</td>
<td>LLE</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>Mutant</td>
<td>Wt</td>
<td>85</td>
<td>F</td>
<td>CR</td>
<td>Low</td>
<td>None</td>
<td>5 mm</td>
<td>LLE</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>Mutant</td>
<td>Wt</td>
<td>50</td>
<td>F</td>
<td>PD</td>
<td>High</td>
<td>IFN</td>
<td>0.49 mm</td>
<td>LLE</td>
</tr>
<tr>
<td>4</td>
<td>4</td>
<td>Wt</td>
<td>Wt</td>
<td>79</td>
<td>M</td>
<td>PR</td>
<td>High</td>
<td>None</td>
<td>Unknown</td>
<td>LLE</td>
</tr>
<tr>
<td>5</td>
<td>3</td>
<td>Mutant</td>
<td>CAA&gt;AAA</td>
<td>73</td>
<td>M</td>
<td>PD</td>
<td>Low</td>
<td>None</td>
<td>3.5 mm</td>
<td>LLE</td>
</tr>
<tr>
<td>6</td>
<td>2</td>
<td>Mutant</td>
<td>Wt</td>
<td>60</td>
<td>F</td>
<td>PD</td>
<td>Low</td>
<td>None</td>
<td>1.85 mm</td>
<td>LLE</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>Mutant</td>
<td>CAA&gt;CTA</td>
<td>67</td>
<td>F</td>
<td>CR</td>
<td>Low</td>
<td>None</td>
<td>Unknown</td>
<td>LLE</td>
</tr>
<tr>
<td>11</td>
<td>3</td>
<td>Mutant</td>
<td>CAA&gt;AAA</td>
<td>55</td>
<td>F</td>
<td>SD</td>
<td>High</td>
<td>None</td>
<td>9.0 mm</td>
<td>LLE</td>
</tr>
<tr>
<td>12</td>
<td>2</td>
<td>Mutant</td>
<td>Wt</td>
<td>49</td>
<td>M</td>
<td>PD</td>
<td>High</td>
<td>IFN; DTIC; TMZ</td>
<td>Unknown</td>
<td>LLE</td>
</tr>
<tr>
<td>13</td>
<td>3</td>
<td>Mutant</td>
<td>Wt</td>
<td>76</td>
<td>M</td>
<td>CR</td>
<td>High</td>
<td>None</td>
<td>1.9 mm</td>
<td>LUE</td>
</tr>
<tr>
<td>14</td>
<td>2</td>
<td>Wt</td>
<td>Wt</td>
<td>78</td>
<td>M</td>
<td>PR</td>
<td>Low</td>
<td>None</td>
<td>Unknown</td>
<td>LLE</td>
</tr>
<tr>
<td>15</td>
<td>3</td>
<td>Wt</td>
<td>Wt</td>
<td>85</td>
<td>F</td>
<td>PD</td>
<td>Low</td>
<td>None</td>
<td>3.5 mm</td>
<td>LLE</td>
</tr>
<tr>
<td>16</td>
<td>3</td>
<td>Mutant</td>
<td>Wt</td>
<td>63</td>
<td>F</td>
<td>CR</td>
<td>High</td>
<td>None</td>
<td>1.8 mm</td>
<td>LLE</td>
</tr>
<tr>
<td>17</td>
<td>2</td>
<td>Mutant</td>
<td>Wt</td>
<td>43</td>
<td>M</td>
<td>PD</td>
<td>High</td>
<td>IFN</td>
<td>Unknown</td>
<td>LLE</td>
</tr>
<tr>
<td>18</td>
<td>2</td>
<td>Wt</td>
<td>Wt</td>
<td>83</td>
<td>M</td>
<td>PD</td>
<td>Low</td>
<td>None</td>
<td>0.8 mm</td>
<td>LUE</td>
</tr>
<tr>
<td>19</td>
<td>2</td>
<td>Wt</td>
<td>Wt</td>
<td>66</td>
<td>F</td>
<td>PD</td>
<td>High</td>
<td>IFN; CAR; TAX</td>
<td>Unknown</td>
<td>LLE</td>
</tr>
<tr>
<td>20</td>
<td>2</td>
<td>Mutant/wt</td>
<td>Wt</td>
<td>70</td>
<td>F</td>
<td>CR</td>
<td>Low</td>
<td>IFN</td>
<td>2.4 mm</td>
<td>RUE</td>
</tr>
</tbody>
</table>

Patients included in analysis of gene signature of LPAM response

<table>
<thead>
<tr>
<th>Patient No.</th>
<th>No. of lesions</th>
<th>Mutation status</th>
<th>Disease stage before ILI*</th>
<th>Age</th>
<th>Sex</th>
<th>3-mo response†</th>
<th>Disease burden‡</th>
<th>Prior systemic therapies§</th>
<th>Size of primary tumor</th>
<th>Extremity location†</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>1</td>
<td>Wt</td>
<td>Mutant CAA&gt;CGA</td>
<td>78</td>
<td>M</td>
<td>PD</td>
<td>Low</td>
<td>None</td>
<td>2.25 mm</td>
<td>LLE</td>
</tr>
<tr>
<td>8</td>
<td>1</td>
<td>Wt</td>
<td>Wt</td>
<td>69</td>
<td>M</td>
<td>PR</td>
<td>High</td>
<td>IFN</td>
<td>7.0 mm</td>
<td>LLE</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>Wt</td>
<td>Wt</td>
<td>66</td>
<td>M</td>
<td>PD</td>
<td>High</td>
<td>None</td>
<td>11.0 mm</td>
<td>LLE</td>
</tr>
<tr>
<td>21</td>
<td>1</td>
<td>Wt</td>
<td>Wt</td>
<td>87</td>
<td>F</td>
<td>PR</td>
<td>High</td>
<td>None</td>
<td>Unknown</td>
<td>LLE</td>
</tr>
<tr>
<td>22</td>
<td>1</td>
<td>Nd</td>
<td>Wt</td>
<td>69</td>
<td>M</td>
<td>PD</td>
<td>High</td>
<td>IFN; TMZ; THAL; IL-2</td>
<td>2.0 mm</td>
<td>LLE</td>
</tr>
<tr>
<td>23</td>
<td>1</td>
<td>Nd</td>
<td>Nd</td>
<td>84</td>
<td>F</td>
<td>SD</td>
<td>High</td>
<td>None</td>
<td>3.6 mm</td>
<td>RUE</td>
</tr>
<tr>
<td>24</td>
<td>1</td>
<td>Wt</td>
<td>Nd</td>
<td>68</td>
<td>F</td>
<td>CR</td>
<td>High</td>
<td>None</td>
<td>Unknown</td>
<td>RUE</td>
</tr>
<tr>
<td>25</td>
<td>1</td>
<td>Wt</td>
<td>Wt</td>
<td>75</td>
<td>M</td>
<td>CR</td>
<td>High</td>
<td>None</td>
<td>0.9 mm</td>
<td>RUE</td>
</tr>
<tr>
<td>26</td>
<td>1</td>
<td>Wt</td>
<td>Mutant CAA&gt;AAA</td>
<td>65</td>
<td>F</td>
<td>CR</td>
<td>High</td>
<td>IFN</td>
<td>4.0 mm</td>
<td>LLE</td>
</tr>
<tr>
<td>27</td>
<td>1</td>
<td>Mutant</td>
<td>Wt</td>
<td>46</td>
<td>F</td>
<td>CR</td>
<td>Low</td>
<td>IFN</td>
<td>Unknown</td>
<td>RUE</td>
</tr>
<tr>
<td>28</td>
<td>1</td>
<td>Mutant</td>
<td>Wt</td>
<td>53</td>
<td>M</td>
<td>PD</td>
<td>Low</td>
<td>IL-2</td>
<td>3.2 mm</td>
<td>LLE</td>
</tr>
<tr>
<td>29</td>
<td>1</td>
<td>Wt</td>
<td>Wt</td>
<td>66</td>
<td>M</td>
<td>PD</td>
<td>High</td>
<td>None</td>
<td>6.0 mm</td>
<td>LLE</td>
</tr>
</tbody>
</table>

Abbreviation: Nd, not determined.

*Disease stage before ILI: IIIB, in-transit disease only. IIIC, in-transit disease; + positive regional lymph node with lymph node excision. IV, in-transit disease; + positive regional lymph node with lymph node excision; + distant disease.
†3 month response. LTF/U - lost to follow-up.
‡Disease burden: low, <10 lesions (all lesions less than 3 cm diameter); high, >10 lesions or at least one lesion >3 cm diameter.
§Prior Systemic Therapies: THAL, thalidomide; DTIC, dacarbazine; IL-2, interleukin-2; CAR, carboplatin; TAX, taxol; TMZ, temozolomide; IFN, interferon.
∥Extremity location: LLE, left lower extremity; RLE, right lower extremity; LUE, left upper extremity; RUE, right upper extremity.
Seven genes important in melanoma biology and which showed prominent differences in expression across patients were selected for further expression analysis across 12 lesions (four patients) using standard qPCR. Relative expression of these seven genes as measured by qPCR confirmed the pattern of gene expression revealed by the microarray (Supplementary Fig. S1B and C). Gene expression was also confirmed at the level of protein expression for one patient for whom each of the two lesions was divided into two pieces, one each for RNA and protein analysis. Protein expression of N-cadherin (CDH2) and MGMT, important in melanoma biology and chemoresistance, respectively, paralleled that of gene expression across both lesions (Supplementary Fig. S2A). These results show not only the marked variability in gene expression that can exist across melanoma patients but also that within a patient, unique transcriptional profiles are retained across metastatic lesions within the extremity.

It is evident (Figs. 1 and 2) that activating mutations in BRaf and NRas dramatically affect transcriptional profiles. Regression and ANOVA methods were used (see Materials and Methods) to identify a panel of genes with expression patterns that correlate with BRaf mutation status. Two thousand one hundred sixty-eight genes were significantly correlated (q value, <0.001) with mutation status. Unsupervised clustering was done on the top 200 genes across all 43 lesions such that genes with similar patterns of expression are grouped and samples with similar expression patterns across these genes are grouped (see Supplementary Table S4 for a list of the top 200 genes; Fig. 3A). Notably, all lesions from a patient clustered together forming 16 groups (note that the two lesions from patient 20 did not cluster together consistent with the discordant BRaf mutation status for this patient). Furthermore, all patients clustered based on BRaf mutation status forming three groups—BRaf and NRas WT, BRaf mutant and NRas WT, and BRaf WT and NRas mutant. Although
BRaf mutant/NRas WT lesion 20a clustered with similar lesions, BRaf/NRas WT lesion 20b did not.

Expression of micropthalmia-associated transcription factor (MITF) and calcium selective transient receptor potential channel TRPM1, proteins important in melanoma biology, showed marked differences across patients but homogeneous patterns across lesions from a patient (Fig. 3B and C). A significant, nonlinear correlation between TRPM1 expression and MITF expression ($P = 0.0000$; Fig. 3D) was observed as well as a significant correlation between TRPM1 expression and BRaf/NRas mutation status ($P < 0.001$; one-way ANOVA analysis; see Supplementary Fig. S2B). There is also a trend toward lower MITF expression with BRaf mutation ($P < 0.001$).

**Transcriptional patterns predictive of dysregulated intracellular signaling**

Recent work has shown that unique patterns of gene expression can be identified that correlate with dysregulated signaling in several oncogenic pathways such as Src tyrosine kinase, Ras G protein, and Myc and E2F transcription factors (19). By applying a binary regression analysis, these gene signatures can be used to predict the extent to which a tumor harbors expression patterns that are consistent with dysregulated signaling in one or more of these pathways. We used these “gene signatures of dysregulated signaling” to define a predicted probability for each of these pathways in the melanoma samples. Because previous work has shown...
a link between pathway activation and sensitivity to drugs that target a component of the pathway (19, 20), one goal of an analysis such as this is to determine whether a patient harbors high or low activity of any given pathway, thereby identifying them as candidates for targeted therapy. However, the clinical utility of these signatures in the setting of in-transit extremity melanoma will depend on the level of concordance

Figure 3. A, regression and ANOVA was performed for 32 k genes across all 43 lesions. The generalized estimating equation method based on a working independent and identically distributed correlation structure was used to account for the dependency among multiple lesions of each patient. The top 200 genes most highly correlated with BRaf mutation status are illustrated in the heat map in which each row represents a gene and each column a sample. Normalized expression is color coded in which red is high and green is low relative expression. BRaf and NRas mutation status is indicated below; sample 20B (far right) is BRaf WT /NRas WT. See Supplementary Table S4 for list of genes with corresponding q and P values. Expression of MITF (B) and TRPM1 (C) is shown across all samples. Patients are noted on the X-axis and are grouped according to BRaf and NRas mutation status. D, expression of MITF (measured using the microarray genechip and normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) is plotted as a function of TRPM1 (measured using the microarray genechip and normalized to glyceraldehyde-3-phosphate dehydrogenase). Regression analysis was done to account for the nonlinearity in the data (solid line; P = 0.000). Each symbol color refers to a patient.
observed across lesions from a patient. To address this, predicted probabilities of dysregulated signaling in Ras, Src, E2F, Myc, PI3K, and β-catenin pathways were compared across all multifocal lesions. When comparing intrapatient lesions, concordance was considered strong if the range of predictions across lesions did not exceed 0.3. As shown in Fig. 4A, predicted Ras pathway activation ranged from 0.35 to 0.80 and there was a strong level of concordance across intrapatient lesions in 94% of patients. The predicted probability of dysregulated signaling in the Src and PI3K pathways (Fig. 4A, right, and B, left) showed marked differences across patients (0.04-0.97 and 0-0.89, respectively) with strong levels of concordance observed in 88% and 82% of patients (Src and PI3K, respectively). E2F and Myc pathway activation predictions ranged between 0.13 and 0.75 (E2F) and 0.23 and 0.87 (Myc; Fig. 4B, right, and C, left) and concordance was strong in 82% and 100% of patients (Myc and E2F, respectively). β-Catenin pathway activation showed marked differences across patients ranging from no predicted pathway activation up to 0.996 (Fig. 4C, right) and concordance was strong in 70% of patients. When mean predictions were compared across all 17 patients, significantly different patterns were observed (one-way ANOVA: P < 0.001; see Supplementary Table S5).

Predicting response to melphalan chemotherapy

We used a panel of 52 melanoma biopsies (28 patients) for which 3-month response to regional infusion with melphalan (LPAM-ILI) was known to identify a panel of genes whose expression correlated with response to LPAM-ILI in melanoma patients. Of the 52 lesions, 40 included lesions across 16 patients with expression data from multiple tumor nodules (response data were not available for patient 1). Unsupervised hierarchical clustering was done across the 100 genes identified as being most highly correlated with LPAM-ILI response (P ≤ 0.005; see Supplementary Table S6). The heat map diagram (Fig. 5A) illustrates the pattern of clustering across patient samples and genes.
Patients responding to LPAM-ILI (CR/PR) clustered together, whereas nonresponding patients (stable and PD; SD/PD) clustered together. Genes that negatively correlated with resistance to LPAM (−0.527 ≤ r ≤ −0.740; decreased expression in responders compared with nonresponders) showed a distinct cluster compared with genes positively correlated with resistance (0.519 ≤ r ≤ 0.731). All lesions from a patient clustered together and were highly correlated (0.925 ≤ r ≤ 0.993; see Supplementary Table S7). Studies are ongoing to validate this “LPAM gene signature” across a larger, independent patient set and to evaluate the predictive utility of this response signature.

**Transcriptional pattern predictive of sensitivity to temozolomide**

Using a gene expression signature of temozolomide resistance derived from a panel of human melanoma cell lines (see Supplementary Methods, Supplementary Table S8, and Supplementary Fig. S3) we evaluated the homogeneity of predicted temozolomide resistance as well as MGMT expression across the multiple lesions from these in-transit melanoma patients. MGMT was homogeneously expressed across lesions from a patient (Supplementary Fig. S4A) and predicted probability of resistance to temozolomide was strongly concordant in 94% of patients (Fig. 5B). A significant correlation was...
observed between predicted temozolomide resistance and MGMT expression (Supplementary Fig. S4B). Although correlating predicted with measured temozolomide resistance is not possible due to the absence, in our data set, of patients treated with temozolomide in the setting of a clinical trial (in which response outcomes are measured in a standardized method), studies are ongoing to validate this “temozolomide gene signature” in a clinical trial of regional temozolomide infusion treatment of extremity melanoma.

Discussion

In-transit melanoma presents a unique setting in which to evaluate the ability of gene expression profiling to predict therapeutic opportunities. Unlike other solid tumors, in-transit melanoma typically presents as multiple nodules. A critical question about the utility of gene expression profiling as a tool to make therapeutic and prognostic predictions in melanoma is the relationship between each nodule on a patient in terms of transcriptional profiles. The goal of this study was to specifically address this question “can a single biopsy sample derived from one multifocal lesion be used to characterize a patient’s tumor in terms of response to therapy?” Our results show that for a patient with metastatic melanoma confined to the extremity, each multifocal lesion displays transcriptional patterns that are significantly correlated with other nodules presenting in that extremity.

Microarray technology and gene expression signatures are increasingly being used as a tool to predict tumor progression, and underlying biology and patterns of gene expression predictive of oncogenic signaling can be used to characterize a patient’s melanoma. It is therefore important to understand the relationship between melanoma nodules at the transcriptional level. In a previous study, global gene expression patterns (across >10,000 genes) in primary and matched metastatic lesions showed no statistically significant differences (5). Our study extends this observation further across a larger in-transit metastatic melanoma patient sample and, importantly, across a panel of gene expression signatures with clinical utility in terms of predicting therapeutic response. The results presented show that one lesion can yield information about the transcriptional profile of a patient’s tumor, which can then be used to help guide an appropriate course of treatment.

The data presented illustrates the effect mutations in BRAf and NRas have on patterns of transcription—at multiple levels of evaluation patients clustered based on their mutation status. The similarity in expression between BRAf mutant and NRas mutant lesions across the 200 “BRAf discriminatory” genes (Fig. 3A) is striking and suggests common oncogenic signaling patterns in samples with these mutations. Although WT/WT lesion 20b failed to cluster with matching WT/WT lesions, the gene expression pattern across this lesion suggests that it harbored a high level of oncogenic signaling akin to the matching BRAf-mut/NRasWT lesion 20a. Our results corroborate previous work in which gene expression signatures that correlate with BRAf mutation status were documented (21–23). The genes identified in the present study show little correlation with the BRAf discriminating genes previously identified; however, these gene signatures were developed using expression data obtained from cultured melanoma-derived cell lines rather than tissue obtained directly from patient. This is important as different culture conditions could significantly alter signaling in the Raf-Ras-mitogen-activated protein kinase pathway (24)—this point is underscored by the fact that although multiple groups have looked for, and many have identified, gene expression patterns that correlate with BRAf mutation status, the results have been conflicting (21, 23, 25). The clinical significance of a BRAf mutation signature in terms of stratifying patients based on likely progression or therapeutic response is unclear. A recent phase II clinical trial with the BRAf kinase inhibitor, sorafenib, did not show any relationship between BRAf mutation status and response, although it is worth noting that the best response observed in this trial was SD (26). Mutant BRAf, however, is clearly oncogenic (27, 28) and associated with up to 70% of human melanomas (of the 26 patients evaluated in this study 38.5% of patients harbored the BRAf mutation) and can lead to enhanced proliferation and survival (24). In the context of the present study, the top 200 genes identified as being most highly correlated with BRAf and NRas mutation status further show that multiple tumor nodules in a patient are highly correlated at a level in which prognostic and response predictions are likely to be made.

MITF is a transcription factor important in melanocyte differentiation and regulates the expression of genes involved with melanin production and cell survival (29). The role that MITF plays in the progression from melanocyte to melanoma is complex in that it is an amplified oncogene important for survival in some tumors but also induces the expression of tumor suppressor proteins (30–33). More recently MITF mutations have been identified in primary metastatic melanomas; however, the effects on cell proliferation and gene regulation during melanomagenesis is unclear (34). TRPM1 (melastatin) has been shown to negatively correlate with the aggressiveness of melanoma and to show reduced expression in metastatic melanomas (35). Recently, it was shown that MITF can bind to the promoter region and increase expression of TRPM1 (36), consistent with our observations. Previous reports (37) have shown that oncogenic BRAf-induced increases in extracellular signal-regulated kinase activity can lead to decreased MITF expression. Our results corroborate these reports and suggest a relationship between Raf-Ras-mitogen-activated protein kinase pathway signaling, MITF expression, and TRPM1 expression.

The failure of melanoma patients to respond to chemotherapy is thought to be largely due to resistance mechanisms present in the tumor cell. To underscore the
importance of resistance mechanisms in the failure of chemotherapy, drugs that target specific resistance pathways have been shown to improve the response rate of melanoma to traditional chemotherapy when used in combination (38–40). Molecular or genetic markers that can be used prospectively to predict resistance to chemotherapy reagents also have the potential to improve response rates by allowing treatment to be targeted to the patient most likely to respond. Clinical trials across an independent patient set are ongoing to evaluate the utility of the LPAM response genes described in this study in terms of predicting resistance to LPAM. Temozolomide, a second generation alkylating agent with a mechanism of action similar to dacarbazine, is an effective antitumor agent in an animal model of regional therapy for the treatment of extremity melanoma and we have shown that 40% of tumor xenografts are preferentially sensitive to either LPAM or temozolomide (41). Indeed, our results presented here suggest that five of nine patients (55%) not responsive to LPAM might be more likely to respond to temozolomide (patients 3, 6, 12, 17, and 18 predicted sensitive to temozolomide; see Fig. 5B). It has been further shown that in vitro response to temozolomide in melanoma cell lines is significantly correlated with activity of the DNA repair enzyme MGMT (18). A clinical trial to study temozolomide in the setting of regional melanoma therapy is under way and will be used to evaluate both MGMT expression and the temozolomide gene signature presented in this study across an independent patient set with known outcomes to identify the most robust marker of response to temozolomide.

Optimizing tumor response to chemotherapy will be facilitated by knowledge of the underlying dysregulated biology that drives the tumor phenotype. Therapy can be tailored to target not only molecules known to be altered or mutated but signaling pathways that are dysregulated due to a convergence of multiple genetic and environmental events. The development of gene expression signatures that can predict which signaling pathways are dysregulated provides another means to evaluate and characterize tumor biology (3). Although the results presented here did not reveal distinctive patterns of pathway activation across this melanoma patient set future studies will aim to confirm the predictions of pathway activation more systematically and across a larger melanoma patient population and verify that, in melanoma, a prediction of altered pathway signaling correlates with response targeted to that pathway.

Serial biopsies obtained over the course of treatment can, in theory, be obtained and evaluated for changes in response to therapy providing a measure of the effectiveness of a therapy. Although there are a few examples of the use of sequential biopsies to evaluate responses to therapy in melanoma (42, 43), an implicit assumption was made in these studies that all lesions were, at least before any treatment, transcriptionally equivalent. The results presented here open the door for further studies of this nature by showing that across most patients with metastatic in-transit melanoma, multifocal lesions are homogeneous in terms of transcriptional profile.

In conclusion, our results show that in the setting of in-transit extremity melanoma, concordant patterns of gene expression are displayed across multifocal tumor nodules. This information will have important clinical application because any lesion obtained from a patient with in-transit extremity melanoma can be used to evaluate the transcriptional profile of that patient’s tumor and, ultimately, help guide treatment strategies that are tailored to each individual patient.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

Durham VA Medical Center Institute for Medical Research Grant (C.K. Augustine), VA Merit Review Grant (D.S. Tyler), and a grant from the Institute for Genomic Sciences and Policy, Duke University (D.S. Tyler, F. Ali-Osman, and H.S. Friedman).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received 08/20/2009; revised 01/15/2010; accepted 02/09/2010; published OnlineFirst 04/06/2010.

References


Molecular Cancer Therapeutics

Gene Expression Signatures as a Guide to Treatment Strategies for In-Transit Metastatic Melanoma


Mol Cancer Ther  Published OnlineFirst April 6, 2010.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-09-0764

Supplementary Material
Access the most recent supplemental material at:
http://mct.aacrjournals.org/content/suppl/2010/04/05/1535-7163.MCT-09-0764.DC1

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.