A miRNA-Based Signature Detected in Primary Melanoma Tissue Predicts Development of Brain Metastasis

Doug Hanniford¹,², Judy Zhong²,³, Lisa Koetz¹,², Avital Gaziel-Sovran¹,², Daniel J. Lackaye²,⁴, Shulian Shang²,³, Anna Pavlick²,⁴,⁵, Richard Shapiro²,⁶, Russell Berman²,⁶, Farbod Darvishian¹,², Yongzhao Shao²,³, Iman Osman²,⁴, and Eva Hernando¹,²

Abstract

Purpose: Brain metastasis is the major cause of mortality among patients with melanoma. A molecular prognostic test that can reliably stratify patients at initial melanoma diagnosis by risk of developing brain metastasis may inform the clinical management of these patients.

Experimental Design: We performed a retrospective, cohort-based study analyzing genome-wide and targeted microRNA expression profiling of primary melanoma tumors of three patient cohorts (n = 92, 119, and 45) with extensive clinical follow-up. We used Cox regression analysis to establish a microRNA-based signature that improves the ability of the current clinicopathologic staging system to predict the development of brain metastasis.

Results: Our analyses identified a 4-microRNA (miR-150-5p, miR-15b-5p, miR-16-5p, and miR-374b-3p) prognostic signature that, in combination with stage, distinguished primary melanomas that metastasized to the brain from nonrecurrent and non-brain metastatic primary tumors (training cohort: C-index = 81.4%, validation cohort: C-index = 67.4%, independent cohort: C-index = 76.9%). Corresponding Kaplan–Meier curves of high- versus low-risk patients displayed a clear separation in brain metastasis-free and overall survival (training: P < 0.001; P < 0.001, validation: P = 0.033; P = 0.007, independent: P = 0.021; P = 0.022, respectively). Finally, of the microRNA in the prognostic model, we found that the expression of a key lymphocyte miRNA, miR-150-5p, which is less abundant in primary melanomas metastatic to brain, correlated with presence of CD45⁺ tumor-infiltrating lymphocytes.

Conclusions: A prognostic assay based on the described miRNA expression signature combined with the currently used staging criteria may improve accuracy of primary melanoma patient prognoses and aid clinical management of patients, including selection for adjuvant treatment or clinical trials of adjuvant therapies. Clin Cancer Res; 21(21); 4903–12. ©2015 AACR.

Introduction

Melanoma is a prevalent cancer of increasing incidence globally (1), with a cost of nearly a billion dollars annually to the U.S. health care system (2). Most patients are initially diagnosed with localized melanoma (stages I and II) and are effectively cured through surgical management. Despite generally good prognoses, about 10% and 40% of patients diagnosed with localized lesions, respectively, die from melanoma. In contrast, nearly 40% of patients with stage III (nodal spread) cancers are long-term survivors (3). These outcomes indicate that current histopathology-based staging does not fully capture heterogeneity of outcomes for patients with primary melanoma. Coupling these staging criteria with additional tools (such as molecular markers) has the potential to vastly improve prognostic accuracy for these patients. For those individuals who develop metastatic melanoma, brain metastasis, in particular, is a major clinical burden. Brain metastasis occurs in 5% to 15% of all patients with melanoma and is the cause of up to 50% of melanoma deaths (4). Brain metastasis is rapidly fatal, with median survival of 4 months after diagnosis (5). Approaches to better stratify patients with primary melanoma by their risk of developing brain metastasis may yield substantial benefit to high-risk patients through intensified surveillance, aggressive treatment, and future adjuvant therapies. Moreover, interrogation of molecular alterations within primary tumors of different outcomes may inform our understanding of the biology and
Translational Relevance

Despite recent therapeutic advances, metastatic melanoma is a devastating disease. Stratification of patients with primary melanoma by risk of developing metastatic disease is an ongoing clinical challenge. Relevant to this study, brain metastasis accounts for the majority of melanoma patient deaths, thus identification of patients with early-stage melanoma who are at greatest risk of developing brain metastasis is of paramount importance. In this study, we identified a primary melanoma tissue–based microRNA expression signature that, in combination with an existing histopathology-based prognostic measure [American Joint Committee on Cancer (AJCC) stage], improves prediction of the development of brain metastasis. Moreover, we document that the source of miR-150-5p, a component of the described signature, is predominantly tumor-infiltrating lymphocytes, adding to the emerging evidence linking immune response to patient outcomes in melanoma. A clinical assay derived from this signature could inform the clinical management of patients with melanoma, including selection for more advanced staging, increased surveillance, or clinical trials of adjuvant therapies.

Materials and Methods

Study population

All patients were enrolled in the Interdisciplinary Melanoma Cooperative Group (IMCG) database of NYU Langone Medical Center (New York). Informed consent was obtained from all patients and approval acquired by the Institutional Review Board of NYU School of Medicine (protocol #10362). IMCG patients are actively, prospectively followed up every 6 months after primary tumor removal and every 3 months after a recurrence. Extensive clinical information is annotated in the IMCG database. Parameters relevant to this study include date of diagnosis, Breslow thickness, ulceration presence, mitotic index, and stage for primary tumors, dates of diagnoses and anatomical locations of metastases, date of last follow-up or death, last clinical status, and cause of death. All patients developing brain metastasis did so during active follow-up. Medical oncologists (A. Pavlick and I. Osman) review all deceased patients’ histories and last clinical statuses to determine if melanoma was the cause of death. Patients were selected using the following criteria. For our initial study cohort (training), we excluded patients who were nonrecurrent/metastatic and whose initial diagnosis occurred within 1 year of the start of the study and thus not expected to reach >3 years of follow-up by the anticipated study conclusion. We excluded all noncutaneous patients with melanoma. From the remaining cases, we randomly selected 92 cases, with a ratio of half nonrecurrent to half recurrent patients. Within recurrent patients, to maximize the chance to detect a prognostic signature of the development of brain metastasis, we selected approximately 50% who had already developed brain metastasis. Subsequent validation cohorts were selected similarly. All patients were treated surgically for removal of primary melanoma lesions.

Clinical specimens

All melanoma specimens were human primary, cutaneous melanoma samples that were collected, formalin-fixed, and paraffin-embedded at the time of surgery (discovery cohort: 1989–2007, validation cohort: 1994–2009, independent cohort: 1997–2010). All tumors were classified according to the 2009 American Joint Committee on Cancer (AJCC) staging system.

RNA extraction

A total of 5 μmol/L sections of FFPE samples (4–12 sections per patient depending on the size of the primary tumor) were attached to PEN-Membrane 2.0-μm slides (Leica) designed for laser capture microdissection. Primary melanoma tissues were macroscopically dissected using disposable scalpels (Feather No. 11) under a dissecting microscope and guided by images of hematoxylin and eosin (H&E) staining of consecutive sections on which a board-certified pathologist (F. Darvishian) marked areas of tumor. Exclusion of surrounding stroma by macrodissection consistently allowed RNA extraction from greater than 80% tumor. RNA extraction was performed using the miRNeasy FFPE Kit (Qiagen) following manufacturer’s recommendations, using the Xylene/Ethanol method for deparaffinization/rehydration.

microRNA microarray expression profiling and data preprocessing

microRNA expression profiling of RNA extracted from FFPE primary melanomas was performed by Exiqon, Inc., blinded to sample characteristics. Briefly, a reference sample was generated
by mixing an equal amount of all samples analyzed, independently for each cohort. RNAs from sample and reference were labeled with Hy3 or Hy5 fluorescent label, respectively, mixed pairwise, and hybridized to the miRCURY LNA arrays (Exiqon). Arrays were scanned using the Agilent G2565BA Microarray Scanner System (Agilent Technologies, Inc.). Image analysis was carried out using the ImaGene software (BioDiscovery, Inc.). The quantified signals were background corrected (Normexp with offset value 10) and normalized using the global Lowess (LOcally WEighted Scatterplot Smoothing) regression algorithm. Data are represented as the log2 transformation of the ratio of median intensities per probe of Hy3-labeled sample to Hy5-labeled reference. MIAME-compliant microarray data were deposited into the Gene Expression Omnibus (GEO) of NCBI under the accession number GSE62372.

**microRNA real-time qPCR and data processing**

Ten nanograms of RNA was reverse-transcribed in 10-µL reactions using the miRCURY LNA Universal RT microRNA PCR, Polyadenylation and cDNA Synthesis Kit (Exiqon). cDNA was diluted 50× and assayed in 10-µL PCR reactions according to the protocol for miRCURY LNA Universal RT microRNA PCR; each miRNA was assayed once by qPCR on the miRCURY Ready-to-Use PCR, Human pick-n-mix panel. Negative controls, excluding template from the reverse transcription reaction, were performed and profiled like the samples. The amplification was performed in a LightCycler 480 Real-Time PCR System (Roche) in 384-well plates. The amplification curves were analyzed using the Roche LC software, both for determination of cycle threshold (Ct by the second derivative method) and for melting curve analysis. The amplification efficiency was calculated using algorithms similar to the LinReg software. All assays were inspected for distinct melting curves and the Tm were checked to be within known specifications for the assay. Measurements that did not pass the service provider’s (Exiqon) threshold (Ct < 37 and at least 5 cycles different from no-template control measurements) were imputed as the lowest value (highest Ct) measured across samples for each miRNA (miR-16-5p: 1 samples, miR-374b-3p: 6 samples). Using NormalFinder (16), we identified 3 miRNAs to be most stably expressed across all samples (hsa-let-7e-5p, miR-30d-5p, and miR-423-3p). The average Ct for these miRNAs was employed as a normalizer for this dataset. A normalized Ct is defined as NormCt = Ct/mean of 3 normalizers − Ct (sample).

**Evaluation of tumor-infiltrating lymphocytes by histologic analysis of primary tumors**

The presence or absence of tumor-infiltrating lymphocytes (TIL) was recorded. The cases with TILs were further subclassified into brisk and non-brisk on the basis of the published criteria (17, 18).

**CD45 immunohistochemistry**

Immunohistochemistry was performed on 5-µm sections of FFPE primary melanoma tissue using mouse anti-human CD45 (leukocyte common antigen, LCA), clone RP2/18 (Cat. 760-2505, Ventana Medical Systems). In brief, sections were deparaffinized in xylene (3 changes), dehydrated through graded alcohols (3 changes 100% ethanol, 3 changes 95% ethanol), and rinsed in distilled water. Antibody incubation and detection were carried out at 40°C on a NexES instrument (Ventana Medical Systems) using Ventana’s reagent buffer and detection kits unless otherwise noted. Endogenous peroxidase activity was blocked with hydrogen peroxide. CD45 antibody was applied neat as directed by the manufacturer and incubated for 30 minutes. Primary antibody was detected with iView biotinylated goat anti-mouse followed by application of streptavidin-horseradish peroxidase conjugate. The complex was visualized with 3,3′-diaminobenzidine and enhanced with copper sulfate. Slides were washed in distilled water, counterstained with hematoxylin, dehydrated, and mounted with permanent media. Appropriate positive and negative controls were included with the study sections. An attending pathologist (F. Davrishian), who was blinded to patients’ clinical data and miR-150 expression measurements, scored CD45 expression as the absolute number of positively stained immune cells demonstrating characteristic lymphocytic morphology in a representative high-power field (HPF; 0.2 mm2) that was selected by scanning each slide at ×40 to find the field with the highest antibody expression. The CD45 expression was assessed in 2 locations: intratumoral and peritumoral (within 0.5 mm from the tumor edge). In addition, the percentage of CD45+ cells, defined as the ratio of CD45+ cells over tumor cells in one high-power field, was calculated in the intratumoral location.

**Statistical analyses**

Using the discovery cohort (n = 92), we analyzed results for 1,360 miRNA, of which data for 578 miRNA were available for cohort analysis 67% samples. All miRNAs were standardized into mean 0 and unit variance variables by subtracting the mean and dividing by the SD within each cohort for scale consistency across cohorts. miRNAs were first ranked by univariate association of expression level for each miRNA with brain metastasis-free survival (BMFS) via Cox proportional hazards regression analysis, with adjustment for tumor stage as a continuous variable. The top 50 ranking miRNAs were used as candidates included in the multivariate Cox proportional hazards model with tumor stage in the regression. The 4 miRNA signature was selected by minimizing Akaiake information criterion (AIC) of the multivariate Cox proportional hazards model through forward stepwise selections (19). In the training cohort, the 4 identified miRNAs were present in all samples with the exception of one patient who had a missing value for miR-15b. The linear coefficients of the miRNAs in the signature were estimated from the remaining 91 training samples with complete data. Subsequent analyses were performed with all samples after data imputation for the 4 microRNA of the signature. Microarray-based measurements (training and validation cohorts) were imputed as the average value of all samples in the cohort (n = 92 and 119, respectively) for a given miRNA. RT-qPCR–based measurements (independent cohort) were imputed as the lowest value detected across samples for each miRNA. The linear combination of model predictors weighted by regression coefficients was defined as the risk score. To test the classifier, regression coefficients of the Cox model were applied to the validation cohort and the independent cohort to obtain their risk scores. The discriminating power of the risk score was evaluated by Harrell C index (20) in the 3 cohorts separately. The risk scores were also evaluated for utility in predicting brain metastasis by identifying the area under the receiver operating characteristic
(ROC) curve in the 3 cohorts separately (21). For this purpose, we defined cases as patients with brain metastasis documented by routine imaging (MRI or CT) of high-risk patients and/or imaging of symptomatic patients with no restriction for time to brain metastasis (as defined by the time from initial primary tumor diagnosis to initial brain metastasis documentation). We defined controls as non–brain metastasis patients (nonrecurrent/nonmetastatic patients and recurrent patients without documented brain metastasis) with ≥3 years of follow-up from initial primary tumor diagnosis. A risk score cut-off using the Youden Index of the ROC curve was chosen to separate patients into high- and low recurrence risk groups (22). The same cut-off was applied to the validation and independent cohorts to stratify patients. Statistical comparison of ROC curves generated from the miRNA + stage classifier to stage alone was performed with Delong test for 2 correlated ROC curves. To compare the brain metastasis-free and overall survival (OS) distributions of the 2 groups in each cohort, data were plotted in Kaplan–Meier survival curves and analyzed statistically by the Wilcoxon test, as it is a more sensitive measure than the log-rank test to compare differences in survival probability between groups that occur in early points in time (23).

Kruskal–Wallis rank-sum test was used to compare the risk scores among nonrecurrent/nonmetastatic, non–brain metastatic, brain metastatic concomitant with or subsequent to other sites, and isolated, first-site brain metastatic patient groups.

Two-sample unpaired t test was used to compare log-transformed miRNA expression between brisk and non-brisk TILs. The association between brisk status and B-Met was assessed by $\chi^2$ test. Pearson correlation coefficients were used to characterize the correlation between log-transformed miRNA expression and CD45 expression.

All statistical analyses were performed in R 2.14.0. Specifically, functions from packages ‘survival’ and ‘ROCR’ were used.

## Results

### Brain metastasis prognostic miRNA signature derived from primary melanoma

To explore the potential prognostic value of microRNA in primary melanoma, we performed global and targeted microRNA expression profiling of total RNA extracted from FFPE primary cutaneous melanoma patient tissues. Table 1 presents a detailed summary of clinicopathologic features of patient groups included in this study. The 3 patient cohorts were composed of 92 (training), 119 (validation), and 45 (independent) individuals. Localized melanomas (stages I and II) comprise 77.2%, 62.2%, and 95.6% of patients, respectively. For each cohort, approximately 50% nonrecurrent and 50% recurrent patients were selected, and among the latter, approximately 50% had already developed a documented brain metastasis. Median follow-up time for control patients (nonrecurrent or recurrent without brain metastasis) was approximately 90 months (range, 10–231), 50 months (range, 12–177), and 70 months (range, 16–100), respectively, with >90% of surviving patients exceeding 3 years of follow-up after initial diagnosis.

Using global miRNA expression profiling data from our training cohort, we established a prognostic model of the development of brain metastasis. To do so, we identified the top-ranking miRNAs by univariate association of expression with BMFS via Cox proportional hazards regression analysis with adjustment for tumor stage. We used this candidate set in multivariate Cox proportional hazards regression analysis, including tumor stage coded as a continuous variable, to establish a model predictive of BMFS. This analysis revealed a 4-miRNA signature (miR-15b-5p, miR-150-5p, miR-16-5p, and miR-374b-3p) with adjustment for tumor stage (Table 2) that robustly predicts brain metastasis development [Harrell’s C-index of 81.4%; 95% confidence interval (CI), 69.6%–93.2%]. In contrast, a model using clinical stage alone achieved Harrell’s C-index of 69.0%. The same set of miRNAs was identified

| Table 1. Summary of clinical characteristics of patients used in this study |
|---------------------|-----------------------|-----------------------|-----------------------|-----------------------|
| Characteristic      | Training              | Validation            | Independent           |
|                     | All cases (n)         | Nonrecurrent/Brain    | Nonrecurrent/Brain    | Nonrecurrent/Brain    |
|                     | (n=271)               | metastasis            | metastasis            | metastasis            |
|                     |                       | (n=113)               | (n=122)               | (n=76)                |
| All cases (n)       | 271                   | 113                   | 122                   | 76                    |
| Ulceration status   | 224                   | 103                   | 108                   | 67                    |
| No                  | 27                    | 10                    | 8                     | 10                    |
| Yes                 | 24                    | 12                    | 22                    | 67                    |
| Stage at diagnosis  | 132                   | 66                    | 68                    | 38                    |
| I                   | 14                    | 3                     | 2                     | 4                     |
| II                  | 29                    | 10                    | 13                    | 4                     |
| III                 | 1                     | 8                     | 11                    | 1                     |
| IV                  | 9                     | 11                    | 11                    | 1                     |
| Thickness, mm       | 2.2                   | 2.9                   | 3.1                   | 2.2                   |
| Median              | 2.2                   | 2.9                   | 3.1                   | 2.2                   |
| Range               | 0.9–11                | 1.1–12                | 0.85–30               | 0.85–24               |
| Nodular subtype     | 22                    | 16                    | 20                    | 23                    |
| Nodular             | 22                    | 16                    | 20                    | 23                    |
| SSM                 | 19                    | 4                     | 3                     | 21                    |
| Other               | 3                     | 2                     | 3                     | 1                     |
| Follow-up, mo       | 90.5                  | 88.0                  | 31.5                  | 53.0                  |
| Median              | 90.5                  | 88.0                  | 31.5                  | 53.0                  |
| Range               | 31–124                | 10–231                | 15–198                | 12–133                |
| Alive               | 84.1%                 | 40.9%                 | 3.8%                  | 83.0%                 |
| Dead                | 15.9%                 | 59.1%                 | 96.2%                 | 17.0%                 |

Abbreviation: SSM, superficial spreading melanoma.

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Table 2. Members, HRs, and P values of the 4-miRNA signature predicting development of melanoma brain metastasis derived from Cox proportional hazards modeling.

<table>
<thead>
<tr>
<th>miRNA</th>
<th>HR (95% CI)*</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>hsa-miR-150-5p</td>
<td>0.31 (0.14–0.68)</td>
<td>0.003</td>
</tr>
<tr>
<td>hsa-miR-15b-5p</td>
<td>4.50 (1.48–13.68)</td>
<td>0.008</td>
</tr>
<tr>
<td>hsa-miR-16-5p</td>
<td>0.11 (0.05–0.45)</td>
<td>0.002</td>
</tr>
<tr>
<td>hsa-miR-374b-3p</td>
<td>0.57 (0.39–0.84)</td>
<td>0.004</td>
</tr>
</tbody>
</table>

NOTE: miRNA expressions obtained from the discovery cohort.
*HRs and 95% CIs of the standardized miRNA expressions obtained from the discovery cohort.

coding stage as a categorical value or using a logistic regression–based approach (Supplementary Tables S1 and S2).

From the training cohort, we defined risk scores for individual patients as the linear combination of the 4 miRNAs and tumor stage weighted by their regression coefficients in the Cox model. The same set of coefficients was applied to microarray or targeted RT-qPCR data, respectively, of 2 additional patient cohorts (validation [n = 119] and independent [n = 45]) to obtain risk scores. In combination with stage, this brain metastasis-distinguishing miRNA classifier displayed Harrell's C-index of 67.4% (95% CI, 56.3%–78.5%) and 76.9% (95% CI, 58.4%–95.4%) in the validation and independent cohorts, respectively (Table 3). Using the same patient cohorts, a model using clinical stage alone achieved Harrell's C-index of 65.8% and 72.1%, respectively.

ROC curves support prognostic potential of miRNA signature

To further evaluate the prognostic performance of the miRNA signature obtained by Cox analysis, we calculated the AUC of the ROC curves for development of brain metastasis. For this purpose, we excluded patients for whom we did not have at least 3 years of follow-up, unless they had a documented brain metastasis. We defined cases as patients with documented brain metastasis regardless of time to brain metastasis (n = 26, 31, and 13), and controls as patients who did not have documented brain metastasis and for whom we had ≥3 years of follow-up since initial melanoma diagnosis (n = 59, 61, 31). In combination with stage, this brain metastasis-distinguishing miRNA classifier displayed an AUC of 81.5% (95% CI, 70.3%–92.6%), 64.1% (95% CI, 52.5%–75.8%), and 78.7% (95% CI, 63.1%–94.2%) in the training, validation, and independent cohorts, respectively (Table 3). In contrast, using the same patient cohorts, a model using clinical stage alone yielded AUCs of 70.4% (95% CI, 59.6%–81.2%), 79.3% (95% CI, 63.1%–94.2%), and 78.7% (95% CI, 63.1%–94.2%) for all patients in each cohort. High- and low-risk groups plotted in Kaplan–Meier survival curves displayed significant differences in the 3 patient cohorts for BMFS (P < 0.001, P = 0.033, and P = 0.021, respectively) and overall survival (P < 0.001, P = 0.007, and P = 0.022, respectively; Fig. 1).

Finally, to further examine the prognostic value of the miRNA signature beyond stage alone, we estimated the signature's performance within individual at-diagnosis stages for each cohort. We observed that the model performed best for patients with stage II tumors (Supplementary Fig. S2 and Supplementary Table S4). These results may suggest that the miRNA signature would be more beneficial to patients with melanoma whose tumors have not advanced to clinically evident nodal disease, although it may also reflect that these cohorts contained greater numbers of these tumors.

Collectively, our results demonstrate that expression of a small set of miRNA, measured from primary melanoma tissues at initial melanoma diagnosis, may improve the prognostic capacity of AJCC stage for the development of brain metastasis.

miRNA signature reflects brain-specific tropism for some melanomas

Our group recently identified a subgroup of 36% of melanoma brain metastasis patients whose tumors spread to the brain as the isolated, first site of visceral metastasis (24). This subset of patients suggests that some melanomas may be clinicopathologically and/or molecularly distinct and may develop molecular alterations that dictate tissue tropism of metastatic cells. To examine whether the described miRNA signature is reflective of this possibility, we further stratified the studied patients with melanoma from all cohorts into 4 groups: nonrecurrent/nonmetastatic (n = 115), non–brain metastatic (n = 67), brain metastatic concomitant with or subsequent to other sites of metastasis (n = 51), and brain metastatic as the isolated, first site of visceral metastasis (n = 16). We found that the median signature risk scores reflect the difference among these 4 groups (P < 0.001, Kruskal–Wallis rank-sum test; Fig. 2). This subanalysis suggests that the described signature may be reflective of brain-specific tropism for some melanomas.

miR-150-5p levels correlate with the presence of TIL

We further explored the prognostic value of miR-150-5p because it is not expressed in melanoma cell lines and short-term cultures (25, 26) but well detected in melanoma tissues.

Table 3. Four-miRNA signature in combination with stage improves accuracy of prognosis of brain metastasis development.

<table>
<thead>
<tr>
<th>Subgroup</th>
<th>Training</th>
<th>Validation</th>
<th>Independent</th>
</tr>
</thead>
<tbody>
<tr>
<td>All samples (n)</td>
<td>92</td>
<td>19</td>
<td>45</td>
</tr>
<tr>
<td>C-index (95% CI)</td>
<td>81.4% (69.6%–93.2%)</td>
<td>67.4% (56.3%–78.5%)</td>
<td>76.9% (58.4%–95.4%)</td>
</tr>
<tr>
<td>Stage</td>
<td>69.0% (58.7%–79.3%)</td>
<td>65.8% (55.4%–76.1%)</td>
<td>72.1% (59.9%–84.4%)</td>
</tr>
<tr>
<td>≥3 years follow-up (n)</td>
<td>85</td>
<td>92</td>
<td>44</td>
</tr>
<tr>
<td>AUC (95% CI)</td>
<td>81.5% (70.3%–92.6%)</td>
<td>64.1% (52.5%–75.8%)</td>
<td>78.7% (63.1%–94.2%)</td>
</tr>
<tr>
<td>Stage</td>
<td>70.4% (59.6%–81.2%)</td>
<td>59.2% (47.8%–70.6%)</td>
<td>67.6% (52.0%–83.2%)</td>
</tr>
</tbody>
</table>
measured in this study and others (27, 28), which likely contain mixed cell-type populations. Because miR-150-5p is highly expressed in mature B and T cells (29, 30), we hypothesized that differences in its expression between primary melanomas of different outcomes may be reflective of TILs, rather than of a tumor cell intrinsic property. Indeed, we found that miR-150-5p levels were significantly lower in samples classified as "non-brisk" (n = 27) than in "brisk" (n = 55) TIL response (P = 0.006, Fig. 3A) in a set of 82 primary melanoma samples (23 metastatic to brain, 20 metastatic to other sites, and 39 nonmetastatic), with lower miR-150-5p detection associated to higher brain metastasis occurrence (P = 0.025, Fig. 3B). To independently confirm the correlation between miR-150-5p levels and hematopoietic cell infiltrates, we stained 48 primary melanomas, which we had previously analyzed for miR-150-5p expression and TILs, for the leukocyte marker, CD45. We found that miR-150-5p positively correlates with the absolute and relative number of intratumoral CD45+ cells (P = 0.007 and P = 0.016, respectively, Fig. 3C and D and Supplementary Fig. S3). Collectively, these results indicate that miR-150-5p expression detected in primary melanoma tissues is likely to derive from TILs. Moreover, these data suggest that defective immune response to a primary melanoma or active immune evasion by melanoma cells may enable increased brain metastasis potential for some patients’ tumors.

Discussion

In this study, we evaluated the prognostic capacity of miRNA expression from primary cutaneous melanoma tissues, particularly for its ability to predict the development of brain metastasis. We analyzed miRNA expression for approximately 250 primary melanoma patient tumors, which to our knowledge, represents one of the largest molecular profiling studies of primary melanoma to date, a tumor type for which access to large numbers of tissues with extensive clinical follow-up is rare. Using these datasets, we developed and validated a 4-miRNA (miR-15b-5p, miR-150-5p, miR-16-5p, miR-374b-3p) prognostic model that, combined with tumor stage, classes patients with primary melanoma by risk of developing brain metastasis.

Defining an accurate prognosis for patients with primary melanoma is a key clinical goal. Currently, management of these patients is guided exclusively by gross and histopathologic criteria, which clinicians use to select patients for more extensive staging (including sentinel lymph node evaluation), intensified surveillance, and/or adjuvant therapy (e.g., IFNα). However, low- and moderate-risk primary melanoma patients with histopathologically similar tumors can have vastly different outcomes, supporting the notion that current staging can be improved to better capture this heterogeneity. In supplement of histopathologic features, molecular changes within tumors of similar staging
Primary Melanoma miRNA Signature Predicts Brain Metastasis

Indicative of organ-specific tropism as opposed to general metastatic capacity. In support of this notion, we found that the miRNA signature risk scores reflect the differences between primary tumors that metastasized to brain as the isolated, first visceral site of metastasis compared with those that metastasized to brain concomitant with or subsequent to other sites of metastasis. This correlation is consistent with the concept that brain tropism may be encoded in some primary melanomas.

Interestingly, signature member miR-150-5p was well detected in primary melanoma tissues; however, its expression is not detectable in isolated melanoma cultures (25). We reasoned that this paradox might be explained by an extrinsic non-melanoma cell source for miR-150-5p detected in melanoma tissues. MiR-150-5p is highly expressed in and a key regulator of mature B and T cells (29, 30, 40), suggesting TILs may be a key source of detected miR-150-5p. Supporting this hypothesis, we found that miR-150-5p levels significantly correlated with CD45+ TILs in primary melanoma patient samples. The immune system has long been known to restrain some melanomas, and recent studies have found that TIL response associates with various parameters of melanoma progression and outcome (41–44). Moreover, the remarkable efficacy achieved for some patients using immune checkpoint inhibitors (ipilimumab, nivolumab, pembrolizumab) clearly shows the intricate relationship between melanoma cells and host immune response (45, 46). Our findings support this concept of immune cells as key repressors of melanoma progression generally, and perhaps of brain metastasis, specifically. As a biomarker, miR-150-5p expression may represent a quantitative way to measure TIL infiltration and prognostic stratification of high- and low-risk patients. Moreover, if TIL infiltrate represents a good predictor of response to immunotherapies, then miR-150-5p expression may also represent a valuable predictive biomarker.

A molecular classifier capable of reliably ascribing risk at the time of initial melanoma diagnosis of a future disease outcome could be a valuable tool to improve melanoma patient management. This concept is particularly important for prediction of brain metastasis, which causes the majority of deaths from melanoma. Adjuvant therapies effective at preventing development of brain metastasis are currently lacking, thus the clinical utility of the described signature is unclear at present. However, ongoing (NCT01667419, NCT01682213) and future clinical trials will determine the usefulness of BRAF and MEK inhibitors (vemurafenib, dabrafenib, and trametinib) and immunotherapies (anti-CTLA4, -PD1/PDL1) as adjuvant therapies for patients with primary melanoma. Moreover, some of these therapeutics have already benefited patients with existing brain metastases (47–49), thus treatment options efficacious in the adjuvant setting for high-risk primary melanoma patients may soon be available. We believe that development of a prognostic assay that informs selection of patients who would benefit most from effective adjuvant therapies and identify high-risk patients for clinical trials would ideally be ready for use when such therapies become available. However, translation of this finding into a clinically useful prognostic assay requires a number of future steps. Independent replication of our results with additional patient sets from other institutions would strengthen the reliability of our findings. Furthermore, development of a technical platform amenable to reproducible and precise quantification (such as RT-qPCR) is required to

Figure 2. Median risk scores support that the 4-miRNA classifier is partially reflective of brain tropism. Patients from all cohorts were divided into 4 groups (nontumors (n = 115), non-brain recurrent (n = 67), concomitant brain metastatic (n = 51), and isolated, first-site brain metastatic (n = 16)) and risk scores plotted in box and whiskers format (A). Whiskers are min-max, excluding outliers (defined as above 1.5 interquartile range (IQR) away from the 25% and 75% quantiles). Statistical analysis was performed by Kruskal–Wallis rank-sum test.

Hold immense promise to better understand, diagnose, prognosticate, and develop treatments for melanoma.

The utility of miRNA as biomarkers of cancer outcomes has increasingly been explored. Studies of a variety of cancers have identified associations between miRNA and clinical outcomes (31–34) with limited use in prognostic modeling (35, 36). miRNA as prognostic biomarkers have not been comprehensively explored in primary melanoma tissues. Using a candidate approach, Satzger and colleagues found that high abundance of miR-15b-5p, consistent with the signature herein, correlates with poor recurrence-free and OS and is an independent prognostic parameter (14, 15). Moreover, our group previously identified a miRNA signature, of which miR-15b-5p and miR-150-5p are members, measured in sera of patients with primary melanoma at initial diagnosis that is prognostic for disease recurrence (38, 39). In contrast to these reports, the current study focused on miRNA in primary melanoma tissues as prognostic biomarkers for the development of site-specific (brain) metastasis. We speculate that certain molecular alterations in some primary melanomas may be
advance this assay toward clinical use. In support of this translation, we find a good correlation between risk scores for identical samples derived from microarray and RT-qPCR data ($r = 0.412$, $P < 0.001$). Finally, a large, multicenter prospective study to assess the robustness of this prognostic signature in the general melanoma population would solidify feasibility of conversion of our findings to a clinical prognostic test.

In this study, we elected to develop a model incorporating miRNA expression in combination with tumor staging. Staging incorporates known prognostic factors for primary melanoma (tumor thickness, ulceration, and mitotic index) into a unified prognostic tool. Moreover, its inclusion allowed us to assess the significance of miRNA signatures in predicting brain metastasis survivals for patients with the same stage. However, the

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**Figure 3.** MiR-150-5p expression correlates with CD45$^+$ TILs in primary melanoma tissues. MiR-150-5p levels were measured by RT-qPCR in a subset of primary melanoma samples ($n = 82$; $23$ brain metastasis, $20$ extracranial metastases, and $39$ nonrecurrences). Differences in miR-150-5p expression were evaluated by 2-tailed unpaired $t$ test for samples with "non-brisk" ($n = 27$) versus "brisk" ($n = 55$) TIL responses ($P = 0.006$; A) and brain metastasis ($n = 23$) versus non-brain metastasis ($n = 59$; $P = 0.025$; B). A subset of 48 primary melanomas [brain metastasis ($n = 12$), non-brain metastasis ($n = 36$)] previously analyzed for miR-150-5p expression and TILs were stained for the leukocyte marker, CD45. MiR-150-5p levels were plotted against the absolute (C) and relative (D) numbers of intratumoral CD45$^+$ cells ($P = 0.007$ and $P = 0.016$, respectively).
significance of stage in the multivariate model may reflect a lead
time bias associated with detecting stage I or II patients before they
reach a more advanced stage. In addition, while our model
qualitatively improved upon the predictive capacity of clinical
stage alone in ROC/AUC analyses of BMFS, these differences were
only statistically significant in the training cohort. Our data suggest
that the described classifier partially reflects tumor aggressiveness,
thus it is a possibility that inclusion of more patients with highly
aggressive tumors (but who have not yet developed brain meta-
tasis) in our control groups than would be expected in the general
melanoma population lessened the statistical power of the clas-
sifier in the described analyses. This possibility implies that the
accuracy of the signature may improve when applied on expanded
cohort that better resemble the general primary melanoma patient
collection. Alternatively, inclusion of additional nonre-
test stage I and II tumors in our analyses may have yielded
different and/or additional microRNA with brain metastasis prog-
nostic potential. Finally, while our model is a basis for the making
of a useful clinical assay, the PPV is low currently. We opted to
identify a risk score cutoff to maximize the NPV, as "false negatives"
are a much less desirable outcome of a clinical prognostic assay.
The low PPV may also partially reflect the need for longer follow-up
for control patients, who may still develop brain metastasis, which
occurred as late as over 10 years after initial melanoma diagnosis in
the patient populations studied here. We expect to continue to
monitor these patients and examine the classifier’s accuracy over
time based on their risk scores defined here.

In summary, we developed a prognostic miRNA classifier that, in
combination with stage, stratifies patients with early-stage primary
melanoma by their risk of developing brain metastasis in 3 patient
cohorts. The described miRNA signature represents a first step to
developing a useful molecular, brain metastasis prognostic assay for
melanoma. We believe such a test has the potential to improve
clinical care and outcomes of patients with primary melanoma.

Disclosure of Potential Conflicts of Interest

D. Hanniford and E. Hernando are listed as co-inventors on a provisional
patent application on the method of use of the microRNA signature as a
prognostic tool for primary melanoma described in this manuscript, which,
if accepted, will be owned by the New York University Langone Medical Center.
No potential conflicts of interest were disclosed by the other authors.

Authors' Contributions

Conception and design: D. Hanniford, J. Zhong, A. Gaziel-Sovran, S. Shang,
Y. Shao, I. Osman, E. Hernando

Development of methodology: D. Hanniford, J. Zhong

Acquisition of data (provided animals, acquired and managed patients,
provided facilities, etc.): D. Hanniford, A. Pavlick, R. Shapiro, R. Berman,
I. Osman

Analysis and interpretation of data (e.g., statistical analysis, biostatistics,
computational analysis): D. Hanniford, J. Zhong, L. Koetz, A. Gaziel-Sovran,
S. Shang, F. Darvishian, Y. Shao, E. Hernando

Writing, review, and/or revision of the manuscript: D. Hanniford, J. Zhong,
L. Koetz, A. Pavlick, R. Berman, Y. Shao, I. Osman, F. Hernando

Administrative, technical, or material support (i.e., reporting or organizing
data, constructing databases): D. Hanniford, L. Koetz, D.J. Lackaye, R. Shapiro

Study supervision: E. Hernando

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References


2. Guy GP Jr, Ekwueme DU, Tangka FK, Richardson LC. Melanoma treatment
2012;43:537–45.

3. Balch CM, Gershenwald JE, Soong SJ, Thompson JF, Atkins MB, Byrd DR,
et al. Final version of 2009 AJCC melanoma staging and classification.

4. Flanagan JC, Hlavacek LB, Chiang VL, Kluger HM. Advances in therapy for

Determinants of outcome in melanoma patients with cerebral metastases.

6. Iorio MV, Croce CM. MicroRNA dysregulation in cancer: diagnostics,
monitoring and therapeutics. A comprehensive review. EMBO Mol Med

7. Bovell L, Shannagam C, Katkouri VR, Zhang B, Vogtmann E, Grizzle WE,
et al. microRNAs are stable in colorectal cancer archival tissue blocks. Front

Enhanced stability of microRNA expression facilitates classification of
FFPE tumour samples exhibiting near total mRNA degradation. Br J Cancer

9. Peiro-Chova L, Pena-Chilet M, Lopez-Guerrero JA, Garcia-Gimenez JL,

analysis of microRNA expression of RNA extracted from fresh frozen

11. Klopfeisch R, Weiss ATA, Gruber AD. Excavation of a buried treasure -
DNA, mRNA, microRNA and protein analysis in formalin fixed, paraffin


and cutaneous melanoma: from discovery to prognosis and therapy.
Carcinogenesis 2012;33:1823–32.

multi-miRNA targeting of ApoE drives LRP1/LRP8-dependent

15. Gaziel-Sovran A, Segura MF, Di Meco R, Collins MK, Hanniford D, Vega-
Saenz de Miera E, et al. miR-30b/30d regulation of GalNAc transferases
enhances invasion and immunosuppression during metastasis. Cancer
Cell 2011;20:104–18.

16. Andersen CL, Jensen JL, Orntoft TF. Normalization of real-time quantita-
tive reverse transcription-PCR data: a model-based variance estimation

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## Clinical Cancer Research

**A miRNA-Based Signature Detected in Primary Melanoma Tissue Predicts Development of Brain Metastasis**

Doug Hanniford, Judy Zhong, Lisa Koetz, et al.


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