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Serum-based miRNAs in the prediction and detection of recurrence in melanoma patients

Nathaniel H. Fleming, BA^{1,7}, Judy Zhong, PhD^{2,3,7}, Inês Pires da Silva, MD^{1,7}, Eleazar Vega-Saenz de Miera, MD^{1,7}, Bobbi Brady, BA, BS^{1,7}, Sung Won Han, PhD^{2,3,7}, Doug Hanniford, PhD^{4,7}, Jinhua Wang, PhD^{5,7}, Richard L. Shapiro, MD^{6,7}, Eva Hernando, PhD^{4,7}, and Iman Osman, MD^{1,7}

¹Ronald O. Perelman Department of Dermatology, New York University School of Medicine, New York, NY 10016, USA

²Department of Population Health, New York University School of Medicine, New York, NY 10016, USA

³Department of Environmental Medicine, New York University School of Medicine, New York, NY 10016, USA

⁴Department of Pathology, New York University School of Medicine, New York, NY 10016, USA

⁵Center for Health Informatics and Bioinformatics, New York University School of Medicine, New York, NY 10016, USA

⁶Department of Surgery, New York University School of Medicine, New York, NY 10016, USA

⁷Interdisciplinary Melanoma Cooperative Group, New York University School of Medicine, New York, NY 10016, USA

Abstract

Background—Identification of primary melanoma patients at the highest risk of recurrence remains a critical challenge, and monitoring for recurrent disease is limited to costly imaging studies. We recently reported our array-based discovery of prognostic serum miRNAs in melanoma. In the current study, we examine the clinical utility of these serum-based miRNAs for prognosis as well as detection of melanoma recurrence.

Methods—Serum levels of 12 miRNAs were tested using qRT-PCR at diagnosis in 283 melanoma patients (training cohort n=201, independent validation n=82; median FU 68.8 months). A refined miRNA signature was chosen and evaluated. We also tested the potential clinical utility of the miRNAs in early detection and monitoring of recurrence using multiple longitudinal samples (pre- and post-recurrence) in a subset of 82 patients (n=225). In addition, we integrated our miRNA signature with publicly available TCGA data to examine the relevance of these miRNAs to melanoma biology.

Corresponding author: Iman Osman, MD, 522 First Avenue, Smilow 405, New York, NY 10016, Phone: 1-212-263-9076, Fax: 1-212-263-9090, Iman.Osman@nyumc.org.

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Informed consent was obtained from all subjects who participated in this investigation.

Results—Four miRNAs (miR-150, miR-30d, miR-15b, and miR-425) in combination with stage separated patients by recurrence-free and overall survival and improved prediction of recurrence over stage alone in both training and validation cohorts (training RFS and OS $p < 0.001$, validation RFS $p < 0.001$, OS $p = 0.005$). Serum miR-15b levels significantly increased over time in recurrent patients ($p < 0.001$), adjusting for endogenous controls as well as age, gender, and initial stage. In non-recurrent patients, miR-15b levels were not significantly changed with time ($p = 0.17$).

Conclusion—Data demonstrate that serum miRNAs can improve melanoma patient stratification over stage and support further testing of miR-15b to guide patient surveillance.

Keywords

Melanoma; MicroRNAs; Biomarkers; Serum Markers; Recurrence

BACKGROUND

Melanoma, the deadliest of skin cancers, is increasing in incidence and remains virtually incurable in the metastatic setting, despite recent advances in targeted therapies¹. While clinical stage is used to stratify patients at time of primary diagnosis², stage alone cannot account for heterogeneity in patients' outcomes, highlighting the need for novel molecular biomarkers that are easily measurable at the time of diagnosis³. In addition, there is a need for biomarkers to monitor primary melanoma patients for disease recurrence, as regular imaging studies are not cost-efficient with estimated costs of over \$20,000 per recurrence in patients diagnosed with stage I and II disease⁴.

Blood-based biomarkers are attractive due to the ease and minimal invasiveness of collecting multiple blood samples compared to repeat tissue sampling. In this regard, we recently reported our array-based screening of 355 microRNAs (miRNAs) to identify miRNAs with potential as predictors of recurrence⁵. While the signature was particularly reliable in stage II patients, the study was limited by an imbalanced patient cohort by stage, making the applicability of the signature to the general patient population uncertain. In addition, as the signature was focused on discovery rather than on clinical applicability, it did not include endogenous controls for normalization of miRNA levels.

In the current study, we examined the levels of 12 pre-selected miRNAs in the serum of melanoma patients collected at the time of primary diagnosis, using expanded training and validation cohorts with extended prospective follow up. In addition, we examined the potential utility of the chosen miRNAs as markers of recurrence and melanoma progression and performed bioinformatics analysis to predict the functional relevance of the identified serum-based miRNAs.

PATIENTS AND METHODS

Patient Accrual and Sample Collection

A cohort of 283 primary (AJCC stage I-III²) melanoma patients prospectively enrolled in the New York University Interdisciplinary Melanoma Cooperative Group (IMCG) from September 2002 to September 2011 was identified. All recurred patients in the database with

primary serum samples available were included. To ensure a reasonable balance of each clinical stage in the non-recurred group, patients were identified according to clinical stage (I, II, and III), with target accrual for stage II and stage III patients proportionally higher than in the general melanoma population. Within each stage, patients with available primary serum samples were identified based on length of follow-up. A subset of 82 patients was reserved for an independent validation cohort. Patient demographics and primary tumor pathological characteristics were obtained for each patient at the time of enrollment. Recurrence and survival information are prospectively obtained via active follow-up every six months for all patients. The study was approved by the Institutional Research Board at NYU in accord with a Federalwide Assurance approved by the Department of Health and Human Services, and all patients provided informed written consent at the time of enrollment.

Blood was collected in BD Vacutainer serum tubes from each patient at the time of diagnosis and immediately stored at 4°C. Additionally, in a subset of 82 patients from the training and validation cohorts, multiple longitudinal blood samples drawn during regular clinical follow-up visits were collected. Within two hours of collection, samples were centrifuged for 10 minutes at 750 x g. The supernatant serum was divided into 1 mL aliquots and stored in cryovials at -80°C. Levels of 18-pre selected miRNAs (12 as biomarker candidates, 5 as normalizer candidates, and 1 to assess sample hemolysis) were measured using qPCR (see Supporting Information section for detailed methods). The 12 candidate biomarker miRNAs were all identified in our previously published data as predictive of recurrence in multivariable discovery models and/or as statistically associated with disease recurrence longitudinally⁵. The 5 potential normalizers were identified as stably expressed or unaffected by hemolysis in our previous array data and validated in preliminary testing in control and melanoma patients (Supporting Information Table S1). Following the miRNA qPCR, two miRNAs, miR-30c-5p and miR-181a-5p, were selected as the most stable miRNA using NormFinder⁶. Test miRNA Cp values were normalized to the average Cp of the two most stable miRNAs.

Statistical Methods

Using the training cohort (n=201), the 12 candidate miRNAs were ranked by univariate association of serum levels with recurrence via logistic regression analysis with adjustment for tumor stage (I, II, or III) as a categorical variable. All miRNAs were standardized into mean 0 and unit variance variables for scale consistency across cohorts. The 4 miRNA-signature was selected by minimizing Akaike's information criterion (AIC) of the logistic regression model through backward stepwise selection. The linear combination of model predictors weighted by regression coefficients was defined as the risk score. The selected miRNA signature was evaluated by identifying the area under the Receiver Operating Characteristic (ROC) curve (AUC). An optimal risk score cutoff, maximizing specificity subject to a minimum value for sensitivity at 80% was chosen to classify patients into high and low risk groups⁷⁻⁹. Kaplan-Meier survival curves and log-rank tests were used to compare the recurrence free survival and overall survival distributions of the two groups. Regression coefficients of the logistic regression model were then applied to an independent

validation cohort (n=82) to classify patients. The same cutoff was used to classify patients into high- and low-risk groups. All statistical analyses were performed in R 2.14.0.

To test the ability of the signature miRNAs to monitor for melanoma recurrence, we measured miRNA levels, including the signature miRNAs and the normalizers (miR-30c and miR-181a), in 82 patients (34 recurred, 48 non-recurred) with at least two serum samples available, drawn at different time points (n=225 samples total). All available samples from the 82 patients were analyzed; of note, 26 recurred patients had serum samples tested both pre- and post-recurrence. For each normalized miRNA in the identified signature, we used a linear mixed effect model to analyze the normalized serum levels of the miRNA as a function of time since recurrence (negative values representing sampling time pre-recurrence), adjusting for age, gender, and initial tumor stage at diagnosis in the recurred patients. In addition, we fit a separate linear mixed effect model in the non-recurred patients to analyze miRNA levels as a function of time since diagnosis.

To identify messenger RNAs (mRNAs) most closely associated with the signature miRNAs, we obtained the 20,531 mRNA (level 3 normalized RNAseq results on gene levels) and 1,046 miRNAs (level 3 normalized results) on 325 Skin Cutaneous Melanoma (SKCM) cases from the Cancer Genome Atlas (TCGA) Data Portal (<http://tcga-data.nci.nih.gov/tcga/findarchives.htm>). We first filtered mRNAs with no between-sample variations (n=405 mRNAs). All miRNAs and mRNAs were first log₂ transformed, then standardized into mean 0 and unit variance variables for scale consistency in the multivariate regression. An *L1* penalized regression approach (Lasso) was used to identify the mRNAs associated with each miRNA with the miRNA as dependent variable. A linear coefficient and p-value were estimated for each mRNA predictor in the Lasso regression, reflecting the mRNA's association strength and significance to the miRNA. A threshold of association correlation = 0.1 and p=0.05 were used to define the set of significantly associated mRNAs of each miRNA. The Lasso regression was implemented by *glmnet()* function in R. As additional analysis to identify potential direct mRNA targets, we also filtered the results to only those mRNAs (a) negatively associated with each serum miRNA and (b) predicted to be targeted by the serum miRNA using TargetScan software¹⁰.

For each miRNA, we analyzed the selected potential target mRNAs using gene set enrichment analysis software package (GSEA; <http://www.broadinstitute.org/gsea/index.jsp>) to find the enriched molecular pathways. Pathways were selected with significant p-values (<0.01) and FDR (<0.01). Key genes in each selected pathway were identified.

RESULTS

Table 1 illustrates the demographic and clinicopathological characteristics for the training (n=201) and independent validation (n=82) cohorts, demonstrating that the two cohorts are well-matched in all categories, including recurrence and survival status. The median time of follow-up for survivors was 90.9 months (range, 17.5–128.9 months) in the training cohort, and 39.0 months (range, 0.8–95.0 months) in the validation cohort.

A serum-based miRNA signature predicts recurrence

Using serum miRNA data from a training cohort of 201 melanoma patients, we developed a prognostic model of melanoma recurrence, which we subsequently validated in an independent patient cohort. The 4-miRNA signature (miR-150, miR-15b, miR-425, miR-30d) normalized by two endogenous controls (miR-30c and miR-181a) can effectively distinguish recurrent cases from non-recurrent cases. In combination with stage, this miRNA classifier displayed an area under the receiver operating characteristic (ROC) curve AUC=0.760 (95% CI= (0.687, 0.833)) in the training cohort. Applying this classifier to the independent validation (n=82) cohort yielded AUC of 0.790 (95% CI = (0.684, 0.897)). In contrast, using the same patient cohorts, a model using clinical stage alone achieved AUCs of 0.704 and 0.759, respectively. AUC values of the miRNA-based predictive model within each clinical stage can be found in Supporting Information, Table S2.

We defined risk scores for individual patients as the linear combination of model predictors weighted by regression coefficients. Based on the cut-off value selected to classify patients into high- and low-risk groups, the sensitivity was 80.9% and 84.6% and the specificity was 60.1% and 66.1%, respectively, in the two cohorts (Figure 1). For patients predicted to be negative for recurrence by the miRNA signature in the two cohorts (negative predictive value), 87.4% and 90.2% patients have not recurred, which supports the clinical sensitivity of the signature.

We repeated the training and validation processes in the subsets of recurrent patients and non-recurrent patients with at least 3 years of follow-up of recurrence free survival (n=200 and 59 in the training and validation cohorts, respectively). The resulting miRNA signature identified was the same as those selected when all patients were used. Using this follow-up restriction, the AUCs obtained with the same miRNA + stage classifier applied to the training and validation cohorts were 76 and 80%, respectively. Collectively, these results demonstrate that serum levels of a small set of miRNAs can improve prediction of recurrence when used in combination with clinical stage.

Four-miRNA classifier accurately stratifies patients according to recurrence-free survival and overall survival

To assess the ability of the miRNA signature to stratify patients by survival, high- and low-risk groups were plotted in Kaplan-Meier survival curves and statistical analysis performed by log-rank tests. High- and low-risk groups displayed clear differences in recurrence free survival ($p<0.001$) and overall survival ($p<0.001$) (Figure 2). When applying the same cutoff risk scores to the independent validation cohort (n=82), a significant segregation of patients based on recurrence free survival ($p<0.001$) and overall survival ($p=0.005$) was observed (Figure 2). These analyses underscore the robustness of our miRNA-based classifier in predicting melanoma patient outcome.

miR-15b levels are significantly increased over time from pre-recurrence to post-recurrence

To test the ability of the signature miRNAs to monitor for melanoma recurrence, we measured miRNA serum levels in 82 patients with at least two serum samples available

(n=225 samples total), drawn at different time points pre- and post-recurrence. Starting pre-recurrence and moving to post-recurrence, serum levels of miR-15b were significantly increased over time in recurrent patients ($p < 0.001$, Figure 3; see also Supporting Information, Figure S1), adjusting for levels of endogenous controls as well as patients' age, gender, and initial tumor stage at diagnosis in the linear mixed effect model. In non-recurrent patients, miR-15b levels were not significantly changed with time since diagnosis ($p = 0.17$, Supporting Information Figure S2), suggesting that miR-15b has potential to act as a specific early marker for melanoma recurrence.

Signature miRNAs are associated with immune signaling, melanogenesis, and the cell cycle

To understand the biological relevance of the signature miRNAs, we performed data mining of the TCGA Skin Cutaneous Melanoma dataset to define the set of significantly associated mRNAs with each miRNA (Figure 4). In order to identify all downstream effects, positively and negatively associated mRNAs were initially included¹¹. We further identified the key functional roles of the miRNA-associated mRNAs (Table 2). Genes most strongly associated with miR-150 include immune system related genes, cytokine signaling genes, and numerous G protein-coupled receptor signaling genes. miR-30d was associated with MITF, tyrosinase and other genes involved in melanogenesis and developmental biology. miR-15b was associated with aurora B kinase, centromere protein A, and several other factors involved in the cell cycle and DNA replication. Finally, miR-425 had a small number of associated mRNAs (n=2) that included CKS2 and FAM54A, genes which have been implicated in breast cancer¹².

We further narrowed the analysis to identify potential direct targets of the key miRNAs. As miRNAs typically repress the expression of specific target mRNAs, we focused on those mRNAs that were (a) negatively associated with each serum miRNA and (b) predicted to be targeted by the serum miRNA using TargetScan software¹⁰. This analysis yielded a number of interesting putative target mRNAs for miR-30d in particular, including ITGA5, SERPINE1, and ADAM19.

DISCUSSION

This study tested serum levels of a subset of pre-selected miRNAs in the blood of melanoma patients at the time of primary diagnosis and longitudinally over time. Our data demonstrate that a signature composed of four miRNAs combined with stage had superior prognostic capabilities over stage alone and accurately stratified patients by recurrence-free and overall survival. Second, our results demonstrated that serum levels of miR-15b can potentially be used for early detection and monitoring of disease recurrence in melanoma patients. Finally, our bioinformatics analysis of TCGA data revealed that the signature miRNAs are predicted to have involvement in key processes relevant to melanoma biology, including immunogenic signaling, melanogenesis, and DNA replication.

Our finding that serum-based miRNAs provide improved prognostic value in primary melanoma patients has important implications for the management of these individuals. The use of levels of circulating miRNAs in the clinical setting at the time of diagnosis could

determine surveillance strategies by informing clinicians of the risk of disease recurrence. With the high sensitivity of our predictive model, patients with a low risk score can reasonably be spared aggressive adjuvant treatment regimens and costly imaging studies, even those with more advanced clinical stage. Meanwhile, for stage I patients, regular surveillance is currently highly cost-inefficient on a population level due to their relatively low recurrence rate⁴. Nevertheless, stage I cases account for a substantial proportion of total recurrences due to their high volume of between 56–71% of melanoma diagnoses^{13, 14}, and identification of those specific patients at a higher risk could impart extraordinary cost savings without compromising patient care.

Our results also demonstrate that serum levels of miR-15b have potential utility for the early detection of melanoma recurrence when measured regularly following primary treatment. In our cohort, serum levels of miR-15b increased steadily in the six months prior to recurrence and then decreased shortly after recurrence, possibly reflecting early progression of disease followed by response to treatment. No significant change was observed in non-recurrent patients over time. While these results represent a small number of patients and require prospective validation in an independent cohort, this use of blood-based miR-15b has the potential to significantly improve the efficiency of follow-up care for primary melanoma patients. Evidence suggests that regular follow-up imaging studies alone are not efficient at detecting recurrent disease, due to high false-negative and false-positive rates as well as prohibitive cost when employed regularly^{4, 15}. As such, a regularly administered blood-based miRNA assay can serve as an inexpensive and non-invasive method to identify patients at need for targeted imaging.

The increase of miR-15b levels just prior to recurrence is consistent with multiple lines of evidence suggesting a role of miR-15b in melanoma progression and metastasis. Genes associated with miR-15b revealed in our TCGA analysis include cell cycle and mitotic regulation genes, most notably aurora B kinase and centromere protein A. In addition, miR-15b presence in melanoma tissues has been associated with poorer recurrence-free and overall survival in a retrospective analysis¹⁶. Interestingly, despite our finding that miR-15b levels increased at the time of recurrence, our data show that lower levels of miR-15b are a negative prognostic factor at the time of diagnosis. These results suggest that miR-15b levels are involved in different functional processes at various stages of melanoma disease. Specifically, the increase in miR-15b at the time of recurrence is consistent with its putative functions in progression and metastasis, while the low levels at the time of diagnosis could reflect a different process that has yet to be identified.

The other miRNAs found in our signature predictive of recurrence are also predicted to play roles in key melanoma processes. Our group recently reported that miR-30d plays a role in melanoma invasion and metastasis¹⁷, a hypothesis which is further supported by our TCGA analysis revealing that miR-30d is associated with mRNA levels of microphthalmia-associated transcription factor (MITF), involved in melanogenesis and a potential melanoma oncogene^{18, 19}. Furthermore, we identified a number of mRNAs predicted to be directly targeted by miR-30d, including ITGA5, SERPINE1 and ADAM19, which have been implicated in melanoma and/or other cancer progression^{20–22}. For miR-150, our analysis showed that putative downstream mRNAs associated include numerous chemokine

receptors and ligands that are heavily involved in immune signaling pathways and the development and activation of immune cells. Interestingly, it was recently shown *in vivo* that miR-150 is released in the external milieu upon T cell activation, resulting in a decrease of its intracellular levels and an increase of its levels in the serum, as a consequence of lymphocyte activation²³. Accordingly, it is possible that miR-150 found in melanoma serum is reflective of tumor-infiltrating lymphocytes (TILs), accounting for the positive association between miR-150 upregulation and lower risk of recurrence, possibly via melanoma-related chemokines such as CXCR3 and CCL21^{24–26}. Finally, although miR-425 has been mentioned in the context of non-melanoma cancers^{27, 28}, its precise role in melanoma remains unclear and TCGA analysis revealed only a small number of connections to putative downstream genes (CKS2, FAM54A) that may play a role in breast cancer¹². Our data suggest that further investigation of miR-425 is warranted as a novel potential biomarker in melanoma.

One question that remains unresolved is the source of circulating miRNAs. For example, in our current signature, lower levels of miR-30d were associated with a higher risk of melanoma recurrence, despite earlier work from our group suggesting a role for miR-30d in tumor progression¹⁷. This discrepancy suggests that circulating miRNAs may not always be shed directly from tumor tissue. Indeed, other studies have also reported differences between miRNA levels in tumor tissue and circulating levels of miRNAs as measured in serum^{29, 30}, and in our own group's work in human melanoma tissues, we have observed only partial concordance between tissue-based and serum-based miRNAs^{31, 32}. As such, the functional roles of miR-30d and the other signature miRNAs need to be further explored with regard to their levels in circulating fluids.

Similarly, our results have identified different miRNAs from other groups that have reported results from serum-based miRNAs in melanoma^{33–36}. However, unlike the other reports in the literature, our study is unique in identifying miRNAs that are relevant to recurrence risk when measured at the time of primary diagnosis. Other studies have measured miRNAs in metastatic melanoma compared to disease-free patients^{33–35} or compared primary melanoma patients with healthy controls³⁶; while these methodologies have important biological relevance, they do not speak to the potential utility of miRNAs to predict recurrence at the time of diagnosis.

CONCLUSION

Our data demonstrated the prognostic utility of a set of serum-based miRNAs in stratifying patients according to their risk of melanoma recurrence. miR-15b showed potential for early monitoring for recurrence with good specificity. Bioinformatics analysis revealed that the miRNAs predictive of recurrence may regulate several key aspects of melanoma biology, including immune signaling pathways, melanogenesis, and the cell cycle.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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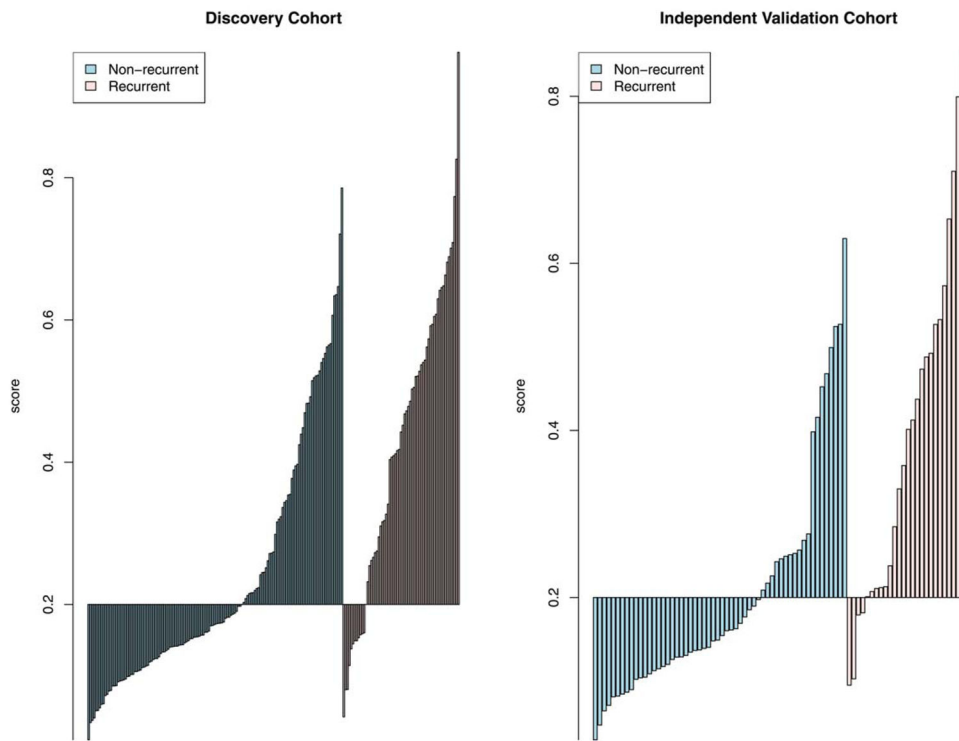


Figure 1. Sensitivity and specificity for prediction of recurrence using a risk score derived from miRNA-based predictive model

An optimal risk score cutoff (horizontal line) maximizing specificity subject to a minimum value for sensitivity at 80% was chosen to classify patients into high and low risk groups.

(A) Training cohort, (B) Independent validation cohort.

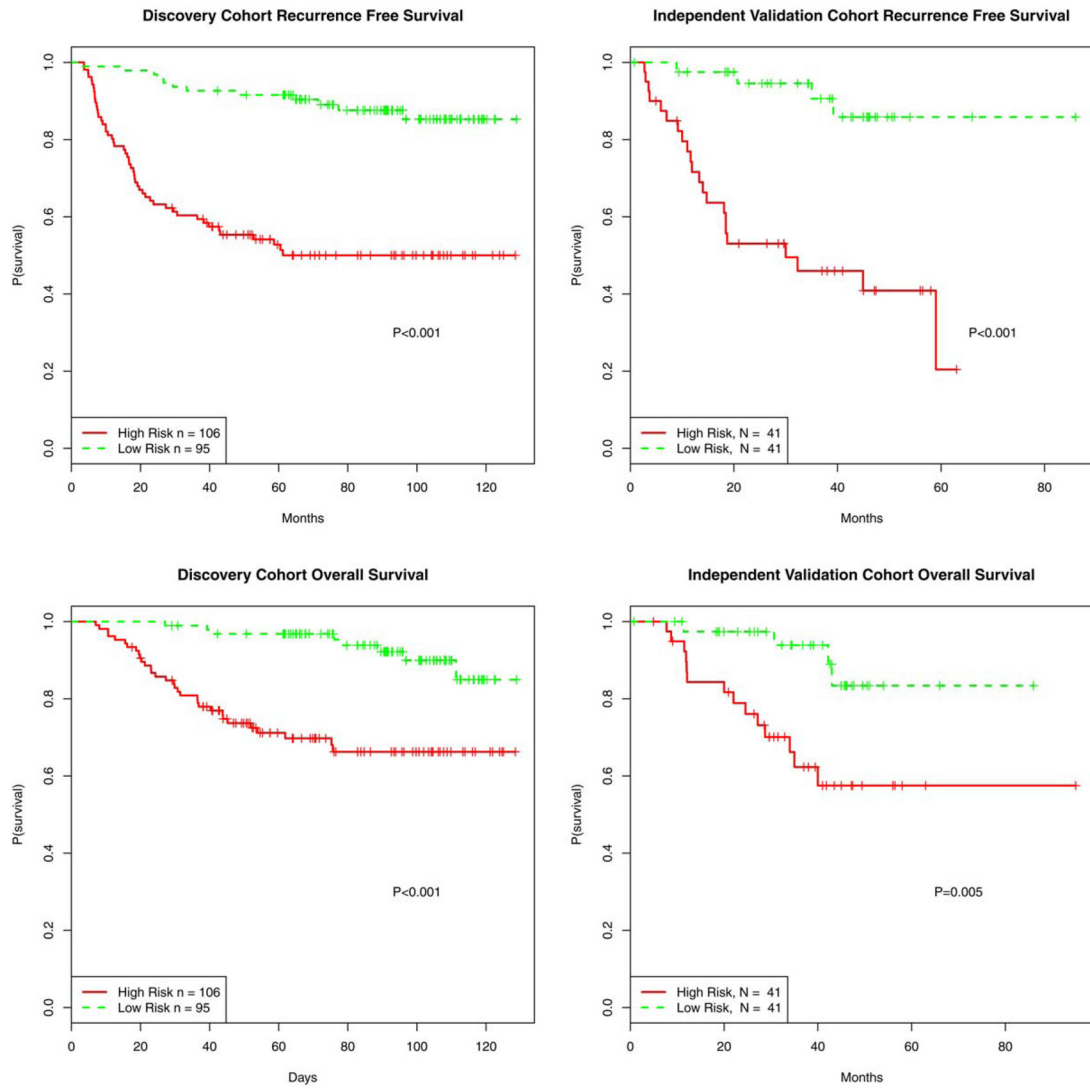


Figure 2. Recurrence-free and overall survival of patients stratified by the miRNA-based predictive model

Using the risk score previously defined by the miRNA-based predictive model, high and low risk groups were plotted in Kaplan-Meier survival curves and statistical analysis performed by log-rank tests.

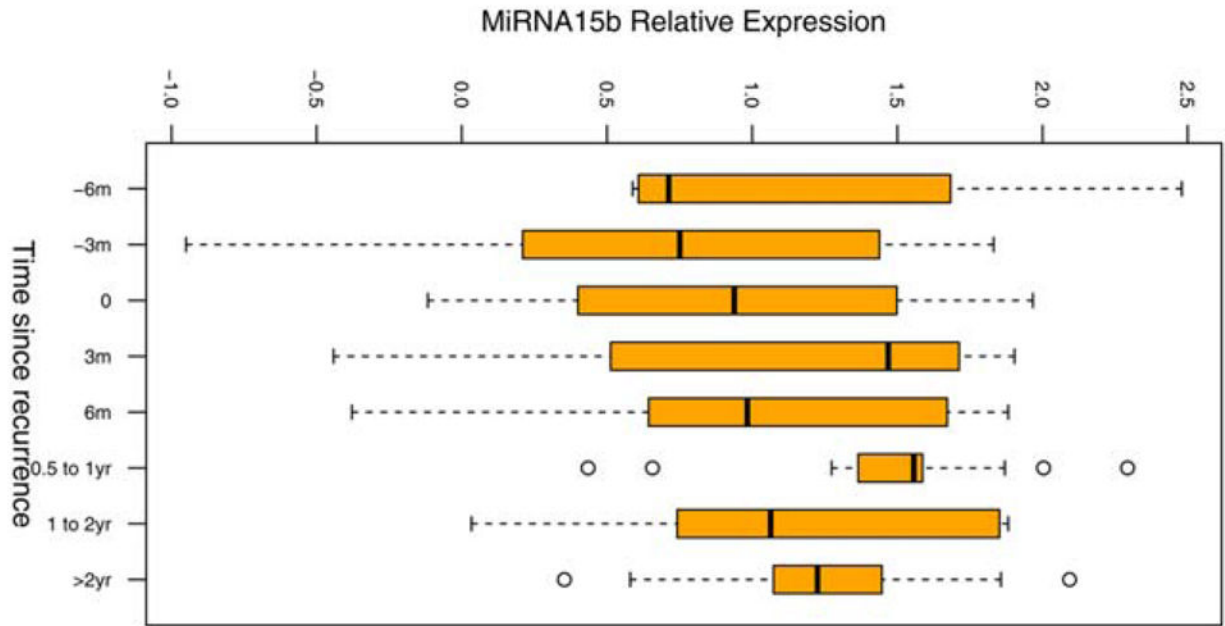


Figure 3. Normalized levels of miR-15b measured in multiple serum samples from recurrent patients over time

Serum miR-15b levels were measured in a subset of patients. Recurrent patients (n=34) are shown. Each patient had at least two serum samples, drawn at different time points, tested. t=0 at date of first recurrence. Serum level of miR-15b increases leading up to recurrence.

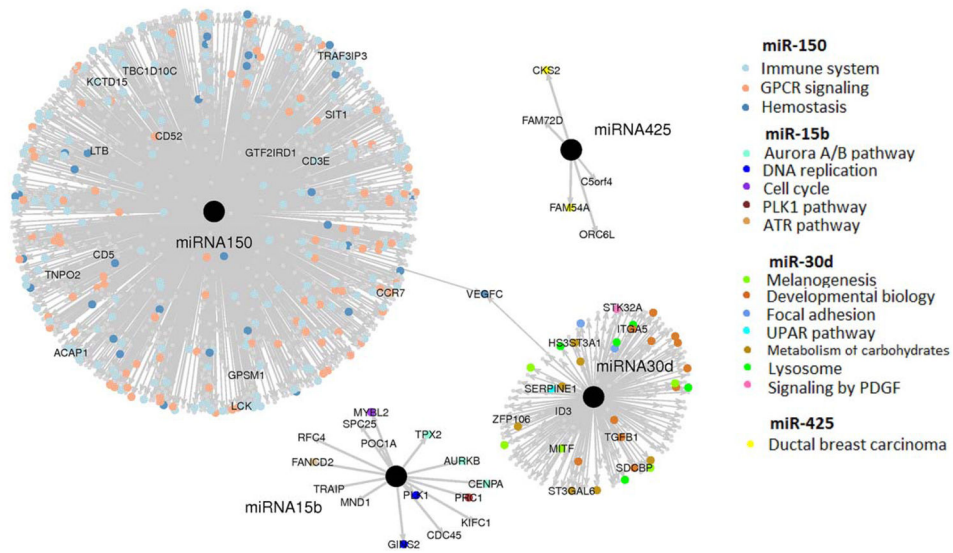


Figure 4. Key mRNAs and functional roles associated with signature miRNAs as identified in TCGA skin cutaneous melanoma dataset

An L1 penalized regression approach (Lasso) was used to identify the mRNAs associated with each miRNA. A linear coefficient and p-value were estimated for each mRNA predictor in the Lasso regression, reflecting the mRNA's association strength and significance to the miRNA. mRNA targets with the strongest association with signature miRNAs are identified by colored dots representing their predicted functional significance according to GSEA analysis.

Table 1

Baseline demographic and primary tumor characteristics of Training and Independent Validation Cohorts

Characteristic n (%)	Training Cohort (n=201)	Independent Validation Cohort (n=82)	Total (n=283)
Gender			
Male	112 (56)	52 (63)	164 (58)
Female	89 (44)	30 (37)	119 (42)
Age, median (range)	61 (21–96)	59.5 (25–88)	
Thickness, median (range)	1.5 (0.16–30)	1.3 (0.27–28)	
Ulceration			
Present	65 (32)	24 (29)	89 (31)
Absent	136 (68)	58 (71)	194 (69)
Mitoses			
Present	141 (70)	60 (73)	201 (71)
Absent	58 (29)	22 (27)	80 (28)
Unclassified	2 (1)	0 (0)	2 (1)
Initial Stage & Recurrence Status			
I	89 (44)	41 (50)	130 (46)
<i>Recurrent</i>	14 (16)	6 (15)	20 (15)
<i>Non-Recurrent</i>	75 (84)	35 (85)	110 (85)
II	52 (26)	20 (24)	72 (25)
<i>Recurrent</i>	15 (29)	7 (35)	22 (31)
<i>Non-Recurrent</i>	37 (71)	13 (65)	50 (69)
III	60 (30)	21 (26)	81 (29)
<i>Recurrent</i>	33 (55)	13 (62)	46 (57)
<i>Non-Recurrent</i>	27 (45)	8 (38)	35 (43)
Follow-up time in months, median (range)	90.9 (17.5–128.9)	39.0 (0.8–95.0)	68.8 (0.8, 128.9)
Status as last follow-up			
Alive	157 (78)	62 (76)	219 (77)
Died, of Melanoma	39 (19)	18 (22)	57 (20)
Died, Other Causes	5 (2)	2 (2)	7 (2)

Table 2

Summary of major functional pathways associated with signature miRNAs

microRNA	Gene Set Description	# of Genes	Key Genes	P-value	FDR Q-Value
miR-150	Immune System	107	VAV1, RASGRP1/2	<0.001	<0.001
	Cytokine Signaling in Immune System	27	PTAFR	<0.001	<0.001
	Signaling by GPCR	53	CCXR3, CCL21	<0.001	<0.001
miR-30d	Melanogenesis	5	MITF, GNAI2, TYR	<0.001	0.005
	Developmental Biology	11	TGFB1, ITGA5	<0.001	<0.001
miR-15b	Cell Cycle	6	AURKB, CENPA	<0.001	<0.001
	Cell Cycle (mitotic)	6	AURKB, CENPA	<0.001	<0.001
	DNA Replication	5	AURKB, CENPA	<0.001	<0.001
miR-425	Up-regulated in ductal carcinoma tumor cells	2	CKS2, FAM54A	<0.001	0.005