MicroRNA Expression Profiles Associated with Mutational Status and Survival in Malignant Melanoma

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Malignant cutaneous melanoma is a highly aggressive form of skin cancer. Despite improvements in early melanoma diagnosis, the 5-year survival rate remains low in advanced disease. Therefore, novel biomarkers are urgently needed to devise new means of detection and treatment. In this study, we aimed to improve our understanding of microRNA (miRNA) deregulation in melanoma development and their impact on patient survival. Global miRNA expression profiles of a set of melanoma lymph node metastases, melanoma cell lines, and melanocyte cultures were determined using Agilent array. Deregulated miRNAs were evaluated in relation with clinical characteristics, patient survival, and mutational status for *BRAF* and *NRAS*. Several miRNAs were differentially expressed between melanocytes and melanomas as well as melanoma cell lines. In melanomas, *miR-193a, miR-338,* and *miR-565* were underexpressed in cases with a *BRAF* mutation. Furthermore, low expression of *miR-191* and high expression of *miR-193b* were associated with poor melanoma-specific survival. In conclusion, our findings show miRNA dysregulation in malignant melanoma and its relation to established molecular backgrounds of *BRAF* and *NRAS* oncogenic mutations. The identification of an miRNA classifier for poor survival may lead to the development of miRNA detection as a complementary prognostic tool in clinical practice.

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INTRODUCTION

Cutaneous malignant melanoma is a highly aggressive disease accounting for the majority of skin tumor-related deaths worldwide. The tumors originate from melanocytes, and are associated with risk factors such as ultraviolet radiation exposure, fair skin type, and predisposing gene mutations. Despite improvements in early melanoma diagnosis, the 5-year survival rate for patients with advanced disease is only 15% (Garbe and Eigentler, 2007; Villanueva and Herlyn, 2008). Currently, the most powerful prognostic factors for cutaneous melanoma at early stage are Breslow's tumor thickness, ulceration of the primary tumor, and sentinel lymph node status.

Activating *BRAF* mutations, which affect the mitogen-activated protein kinase (MAPK) pathway, are detected in 50–70% of melanomas (Davies *et al.*, 2002). *NRAS* mutations are present in 15–30% of melanoma tumors (Chin *et al.*, 1999), and cause constitutive activation of the MAPK pathway independent of BRAF. *NRAS* and *BRAF* mutations rarely co-exist, suggesting that they have biologically equivalent effects in melanoma development (Omholt *et al.*, 2003; Curtin *et al.*, 2005). Inhibition of BRAF or NRAS appears to be a promising route for development of novel therapeutic tools in the treatment of metastatic melanoma.

MicroRNAs (miRNAs) are short non-coding RNAs regulating gene expression in many biological processes, including proliferation, apoptosis, and differentiation (Ambros, 2001; Bartel, 2004; He and Hannon, 2004; Kim, 2005). Expression and functional studies indicate that altered miRNA expression is critical for disease development. Global miRNA expression studies in cutaneous melanoma have provided evidence that miRNAs are deregulated during melanoma progression (Gaur *et al.*, 2007; Mueller *et al.*, 2009). While these studies have been limited to cell lines, virtually nothing is known about the miRNA expression in clinical melanoma materials.

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Abbreviations: M, melanomas; MAPK, mitogen-activated protein kinase; MCc, cell lines derived from cutaneous metastases; MCn, cell lines derived from lymph node metastases; MCp, cell lines derived from primary melanomas; miRNA, microRNA; NM, melanocytes; PAM, prediction analysis of microarrays; SAM, significance analysis of microarrays; wt, wild type

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In this study, we carried out genome-wide miRNA expression profiling in melanoma lymph node metastases and melanoma cell lines in comparison with melanocytes. Subgroups of deregulated miRNAs were found to be associated with *BRAF* mutational status. In addition, we identified a subset of miRNAs associated with significantly shorter melanoma-specific survival.

RESULTS

The miRNA profiles of 16 clinical samples of melanoma lymph node metastases (M-1 to 16), 21 melanoma cell lines from 19 patients and three reference melanocyte cultures (Table 1 and see online Supplementary Table S1) were evaluated using microarray. After normalization and filtration, 167 miRNAs with $\geqslant$80% present calls across the samples remained for further analysis.

miRNA expression patterns in melanomas, melanoma cell lines, and melanocytes

We performed unsupervised hierarchical clustering of the 167 filtered miRNAs among the melanomas, melanoma cell lines, and melanocytes. The analysis grouped the samples into subgroups based on similar miRNA expression patterns without previous knowledge of sample identity. Both melanomas and melanoma cell lines were clearly grouped into separate clusters from melanocytes (Figure 1). We also noted several distinct subgroups among the melanomas and melanoma cell lines, suggesting distinct clinical or biological characteristics of these samples. By hierarchical clustering of the same set of filtered miRNAs among all the samples, we found that the cell lines were grouped separately from the melanomas (see online Supplementary Figure S1). Although the melanoma cell lines displayed substantial differences in miRNA expression, some similarities were found between the melanomas and melanoma cell lines.

Deregulated miRNAs in melanomas versus melanocytes

To identify the most significant differentially expressed miRNAs between melanomas and normal melanocytes, we performed SAM (Significance Analysis of Microarray) analysis, which resulted in 32 differentially expressed miRNAs with a *q*-value (a measure of the false discovery rate (FDR)) of 0% (see online Supplementary Table S2). Thirteen of the selected miRNAs were overexpressed, and 19 were underexpressed in melanomas (see online Supplementary Table S2). Many of these differentially expressed miRNAs were co-expressed, as illustrated by their close location between each other in the hierarchical clustering (Figure 1).

To further determine the minimal number of miRNAs that can distinguish melanomas from melanocytes, we applied the Prediction Analysis of Microarray (PAM) method on the 167 filtered miRNAs, instead of the SAM-selected miRNAs, to avoid an over-fitting bias induced after selection, as previously described (Simon *et al.*, 2003). The analysis revealed that the expression signature of a minimal number of 10 miRNAs could best distinguish the two groups, resulting in a prediction accuracy of 100% as determined by 10-fold cross validation (see online Supplementary Table S3). All miRNAs in the PAM classifier were also detected by SAM, including underexpression of *miR-192*, *let-7i*, *miR-194*, *miR-211*, *miR-602*, *miR-582*, *miR-454-3p*, and *miR-132*, and overexpression of *miR-126* and *miR-801*.

Table 1. Summary of clinical, histopathological, and genetic characteristics for the melanomas and melanoma cell lines in the miRNA-array screening

Parameter	Melanomas[1]	Cell lines
Number of samples	16	21
Number of patients	16	19
Sex		
Male	14	8
Female	2	11
Age at diagnosis		
Median (range) years	62 (17–82)	51 (22–84)
Histopathology		
SSM	10	9
NM	2	4
Other/n.a.	4	6
Breslow		
$<$1 mm	5	1
1–2 mm	2	4
2.1–4.0 mm	6	3
$>$4 mm	2	6
n.a.	1	5
Survival		
Short (survival range 1–12 months)	8	n.a.
Long (survival range 60–134 months)	8	n.a.
BRAF sequence of exon 15		
Wild-type	8	5
Mutated	8	14
NRAS sequence of exon 2		
Wild-type	12	16
Mutated	4	2
n.a.	—	1

Abbreviations: SSM, superficial spreading melanoma; NM, nodular melanoma; n.a., not available or not applicable.
Clinical and mutation data for melanoma tumors and cell lines were reported in Edlundh-Rose *et al.* (2006), Johansson *et al.* (2009), and Daniotti *et al.* (2004).
[1]Melanomas were obtained from regional lymph node metastasis of patients with cutaneous melanoma.

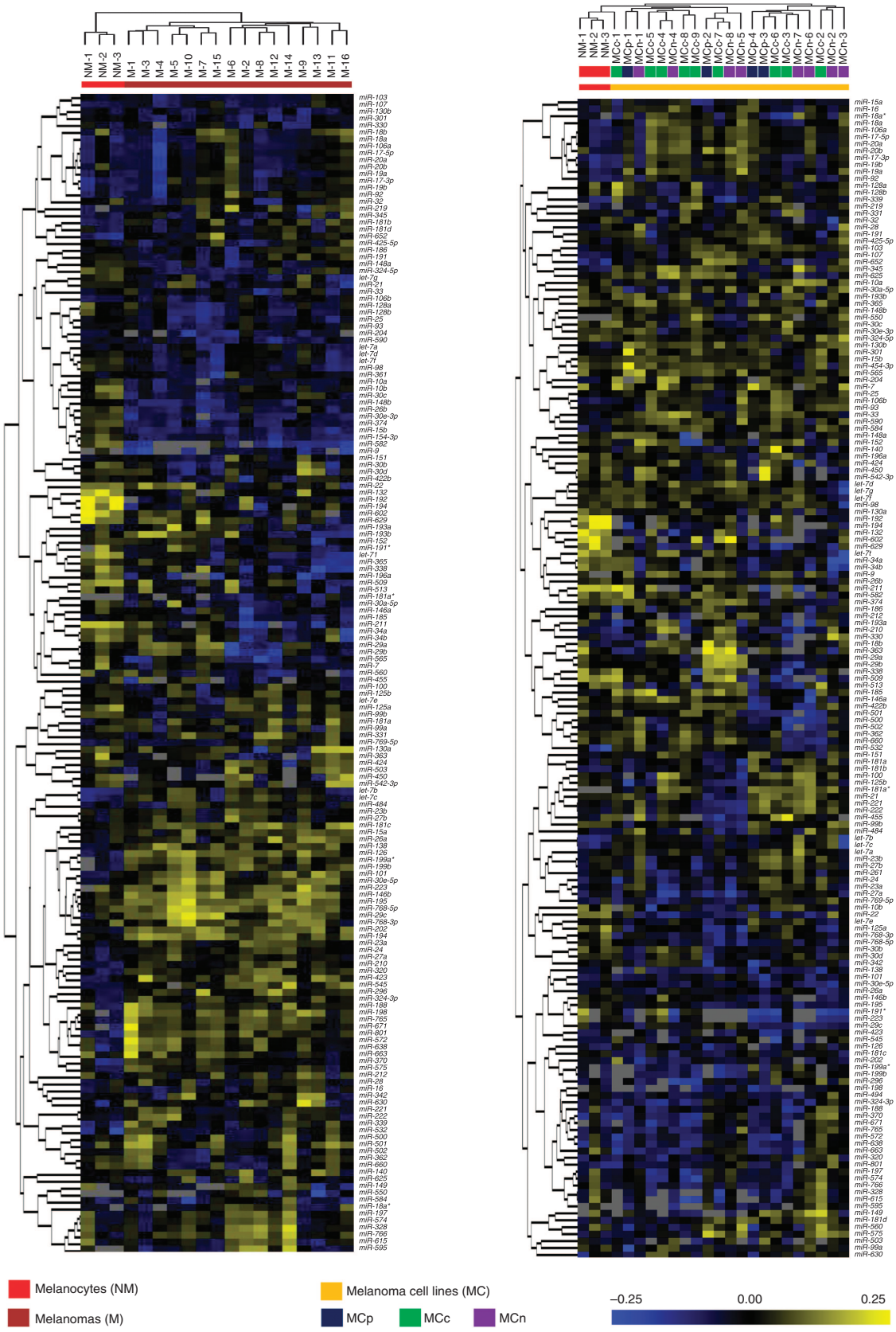

Figure 1. Unsupervised clustering analysis of miRNA expression. Heat maps illustrating unsupervised clustering of miRNA expression profiling in melanocytes (NM) and melanomas (M) (left), as well as melanocytes and melanoma cell lines established from primary tumors (MCp), nodal metastasis (MCn), and cutaneous metastasis (MCc) (right). Clustering was based on all 167 miRNAs remaining after filtration of species with more than 20% missing values across the samples. Median-centered log ratios for each miRNA are represented. The yellow and blue colors indicate relatively high and low expressions, respectively. Missing values are indicated in gray.

Association between miRNA profiles and clinical characteristics of melanoma cases

Using SAM, we analyzed miRNA expression data in relation to clinical characteristics of melanoma cases. We did not observe any differentially expressed miRNAs to be significantly associated with age at diagnosis, gender, or Breslow tumor thickness of the primary tumors (data not shown).

miRNA profiles in relation to *BRAF* and *NRAS* mutations in melanomas

Using SAM, we identified three miRNAs as candidates to discriminate *BRAF* mutant from both *BRAF* and *NRAS* wild-type (wt) melanomas, with an FDR of 0% (see online Supplementary Table S2; Figure 2). Of these, *miR-565* was also identified by SAM analysis as a candidate to distinguish *BRAF* mutant from *BRAF* wt cases (see online Supplementary Table S2; Figure 2). Only *miR-663* was found to be significantly underexpressed in *NRAS* mutant compared with *BRAF/NRAS* wt cases. However, no miRNAs appeared to be deregulated in *BRAF* and *NRAS* mutated compared with wt cases.

To verify the differential expression of *miR-193a* and *miR-338* between *BRAF* and *NRAS* wt and mutant samples observed using microarray data, we evaluated the expression of these two miRNAs in the same series of melanomas (M-1 to 16), as well as in an independent cohort of 16 additional melanomas (M-17 to 32), using qRT-PCR. The strongest association with *BRAF* mutation status was observed for

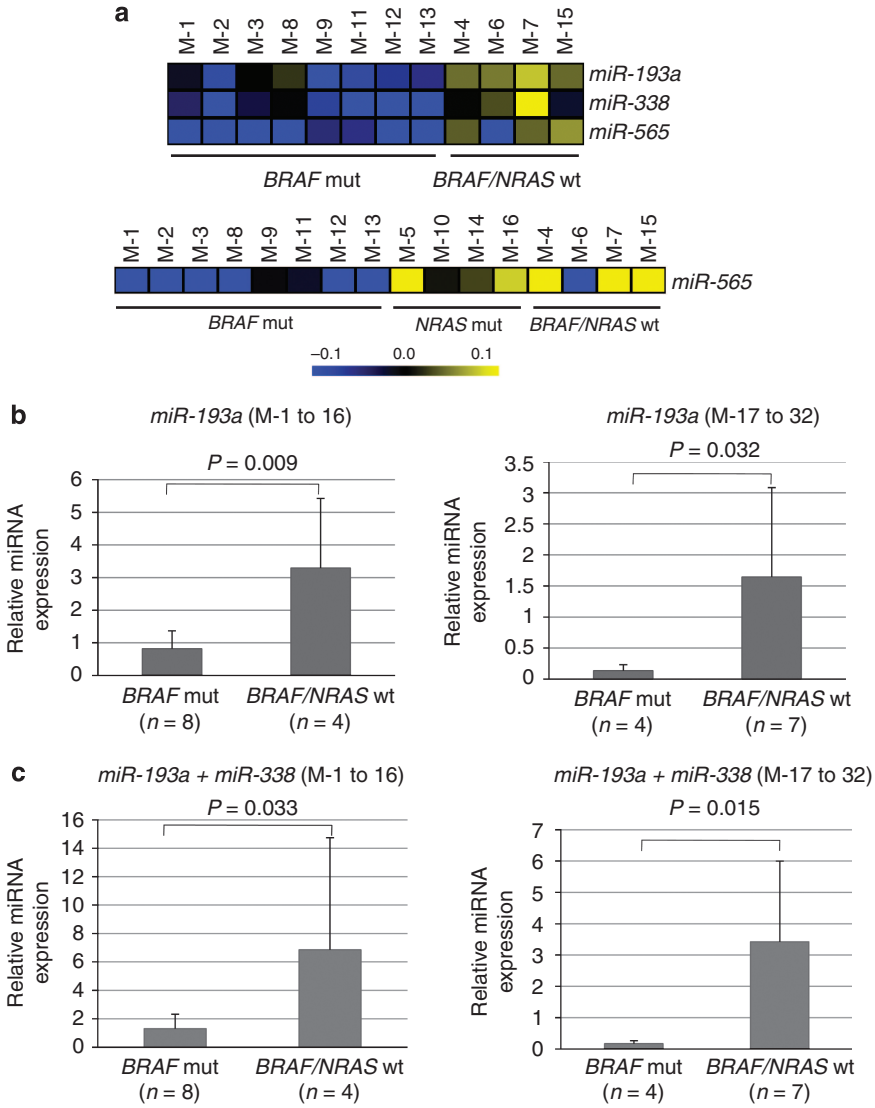

Figure 2. miRNA expression profiles related to *BRAF* and *NRAS* mutational status. (**a**) Heat maps of array-based expression for the classifier of three specific miRNAs obtained after SAM analysis. Comparisons for *BRAF* versus *NRAS/BRAF* wt samples (top) and *BRAF*-mutated melanomas versus *NRAS* mutated and *NRAS/BRAF* wt melanomas (bottom) are illustrated. The yellow and blue colors indicate relatively high and low expressions, respectively. (**b**) Average *miR-193a* expression in *BRAF*-mutated and *BRAF/NRAS* wt cases from two independent sets of cases measured by qRT-PCR. (**c**) Average expression level of *miR-193a* and *miR-338* assessed by qRT-PCR in relation to *BRAF* mutational status in two independent cohorts of patients. miRNA expression levels between the groups were compared using *t*-tests.

miR-193a, which showed significantly reduced levels in both melanoma series (M-1 to -16, $P=0.009$; M-17 to 32, $P=0.032$; *t*-test) (Figure 2). *miR-338* alone was not sufficient to distinguish *BRAF* mutant from *BRAF/NRAS* wt cases (M-1 to 16, $P=0.376$; M-17 to 32, $P=0.169$; *t*-test), but could in combination with *miR-193a* accurately discriminate between the two classes of melanomas ($P<0.05$ for both series; Figure 2).

miRNA signature of survival in melanomas

We then determined whether the miRNA expression signature could be used to predict melanoma-specific survival. Among the 16 melanomas analyzed in the microarray experiments, eight patients had a short survival measured from diagnosis of regional lymph node metastases (range 1–12 months) and the remaining had a good prognosis and long survival (range 60–134 months) after diagnosis. For survival analysis, we considered patient M-4 as ''censored'' because of disease-unrelated death 7 months after diagnosis.

Performing SAM analysis, we identified six candidate miRNAs that could predict disease outcome with an FDR of 0% and a SAM survival score >1.5 (a modified score statistic for a univariate Cox proportional hazards model). Melanomas from patients with short survival showed underexpression of *miR-191,* whereas *miR-193b, miR-365*, *miR-338, let-7i*, and *miR-193a* were overexpressed (see online Supplementary Table S4). This association was further assessed by Kaplan–Meier survival curves and log-rank analysis. Low expression of *miR-191* was associated with short survival ($P<0.001$, log-rank test) as well as high expression of *miR-193b* ($P=0.026$), *let-7i* ($P=0.004$), and *miR-365* ($P=0.043$). On the other hand, *miR-193a* ($P=0.109$) and *miR-338* ($P=0.06$) did not reach statistical significance (see online Supplementary Figure S2).

To verify microarray data, we measured the expression of four miRNAs using qRT-PCR. Three of these showed a significant correlation between microarray and qRT-PCR-based expression values as assessed by Pearson correlation analysis, that is, *miR-193b* ($Cor=0.8625$; $P<0.0001$), *let-7i* ($Cor=0.4822$, $P=0.0252$), and *miR-365* ($Cor=0.4353$, $P=0.041$), whereas *miR-191* ($Cor=0.2896$, $P=0.1612$) did not show significant correlation. However, Kaplan–Meier survival curves and log-rank analysis showed a significant association with survival for *miR-191,* in concordance with array-based results, whereas *let-7i* and *miR-365* are not statistically significant.

To further validate this prognostic miRNA signature, we performed qRT-PCR analyses for *miR-191* and *miR-193b* in an independent cohort of 16 melanomas (M-17 to 32; see online Supplementary Table S1). Univariate Cox proportional hazard models were fitted to evaluate the association of miRNA expression with survival. We confirmed significant associations for *miR-191* ($P=0.0004$) and *miR-193b* ($P=0.0099$). Patients with low expression of *miR-191* and high expression of *miR-193b* were found to have significantly shorter survival (*miR-191*, $P=0.005$; and *miR-193b*, $P=0.004$; log-rank test) (Figure 3).

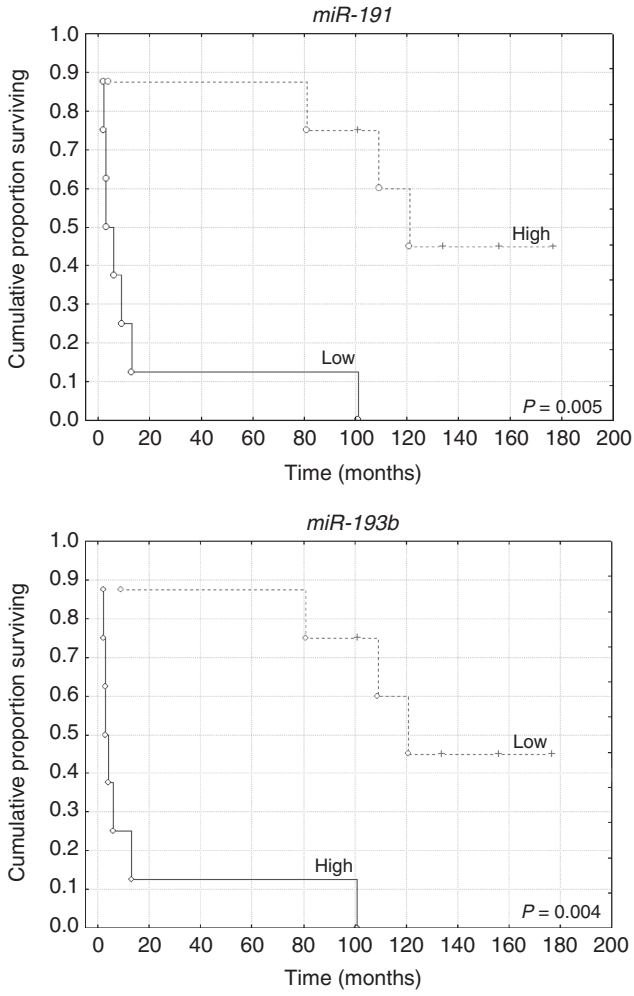

Figure 3. Associations between miRNA expression levels and survival. Kaplan–Meier plots illustrating significant associations between high *miR-193b* and low *miR-191* expression and poor melanoma-specific survival in the second set of melanoma cases (M-17 to 32). High or low expression levels of the two miRNAs were determined by qRT-PCR analyses. Differences in survival were calculated using log-rank test.

Comparison of miRNA profiles in melanomas and melanoma cell lines

In the microarray experiments, we found that the melanocytes, melanoma cell lines, and melanomas were clustered into separate branches (see online Supplementary Figure S1). We next identified miRNAs that are differentially expressed between the melanocytes and all melanomas (including both clinical samples and cell lines) using SAM. Nine miRNAs could distinguish the melanomas and the cell lines from the non-neoplastic counterpart (see online Supplementary Table S5). Of these, seven (*miR-192*, *miR-194*, *miR-132*, *miR-602*, *miR-211*, *let-7i*, and *miR-509*) were also significantly underexpressed in melanomas compared with melanocytes.

We also compared the miRNA expression of melanomas (that consisted of lymph node metastases) with the miRNA expression of melanoma cell lines established from nodal metastases (MCn). Thirty-nine miRNAs were found to be

Table 2. Array results of miRNAs commonly underexpressed in melanomas (M) and cell lines (MCn) compared to melanocytes

	Normalized expression value (mean ± SD)		
miRNA	**NM**	**M + MCn**	***P*-value**
miR-211	0.0197 ± 0.0143	−0.0106 ± 0.1165	<0.0001
miR-509	0.1499 ± 0.0229	−0.0042 ± 0.0998	<0.0001
miR-132	0.1786 ± 0.0488	−0.0100 ± 0.0776	0.0094
miR-194	0.1996 ± 0.0798	−0.0178 ± 0.0763	0.0326
miR-192	0.2681 ± 0.0989	−0.0021 ± 0.0598	0.0379
miR-602	0.2063 ± 0.1001	−0.0207 ± 0.0913	0.0446

Abbreviations: NM, melanocytes; M, melanomas; MCn, melanoma cell lines from nodal metastases.
P-values were determined by unpaired *t*-test and values of $P<0.05$ were considered significant.

differentially expressed between the two groups on the basis of SAM analysis (see online Supplementary Table S5). By comparing the list of miRNAs for melanomas versus melanocytes (see online Supplementary Table S2) and MCn versus melanocytes (see online Supplementary Table S5), we found six miRNAs that were commonly downregulated in both melanomas and melanoma cell lines of nodal metastases (Table 2). Interestingly, *miR-211* and *miR-509* were the most significantly downregulated miRNAs in nodal metastases ($P<0.0001$).

DISCUSSION

Here, we report frequent dysregulation of miRNA expression in lymph node metastases of malignant melanomas with implications for patient survival and associations to mutational status. Our findings show differential miRNA expression pattern in melanomas compared with melanocytes, and thus extend previous observations of miRNA expression in melanoma cell lines (Mueller *et al.*, 2009).

We found substantial differences in miRNA expression patterns between the cell lines and the clinical samples, although both entities were clearly distinct from the melanocytes used as non-neoplastic counterparts. Possible explanations include modifications of miRNA transcription as a direct effect of culturing conditions such as exposure to growth factors, as well as those resulting from acquired genetic changes following adaptation to *in vitro* culturing. Alternatively, the differences might be due to the heterogeneity of cell types within the tumor samples. Nevertheless, a common miRNA expression signature was observed between the cell lines and clinical samples, indicating that relevant gene regulatory networks are conserved in cell lines after successive passages in culture.

Although we found significant correlation between microarray and qRT-PCR for most of the miRNAs, some discrepancies were observed. One possible explanation might be due to differences in detection sensitivity and probe designs for microarray (e.g., the probe length for *miR-191* is only 16-nucleotides (nt), whereas the mature miRNA is 23-nt long). Besides, the different normalization processes might account for discrepancies observed between the two platforms: microarray uses a global normalization for comparison across the samples, whereas qRT-PCR data are normalized using one reference gene that is assumed to be constant in all samples analyzed.

Altered miRNA expression in melanoma

SAM and PAM comparisons of melanomas and melanocytes identified 10 significant differentially expressed miRNAs. Seven of these were also differentially expressed in the melanoma cell lines compared with melanocytes. Two of the most significant miRNAs, *miR-192* and *miR-194*, which are located close to each other, were found to have low expression in melanoma. This is consistent with recent studies (Gaur *et al.*, 2007; Mueller *et al.*, 2009), which showed that *miR-192* and/or *miR-194* were also downregulated in melanoma cell lines, further supporting their relevance in melanoma. The co-expression of the clustered *miR-192* and *miR-194* suggests a possible co-regulation by common transcriptional factor(s) and common function(s). In line with this hypothesis, Hino *et al.* (2008) showed that the *miR-192* to *194* cluster is encoded by a single primary miRNA transcript and induced by hepatocyte nuclear factor-1α, HNF-1α. HNF-1α is an important regulator of various genes specific to the intestine, and it is also expressed in other tissue types, including human skin melanocytes (Schallreuter *et al.*, 2003). Furthermore, *miR-192* was found to be regulated by p53, and has an important role in cell cycle control and proliferation (Braun *et al.*, 2008; Georges *et al.*, 2008; Song *et al.*, 2008). The role of the *miR-192* to *194* cluster in melanoma cells and its regulation by HNF-1α/p53 are certainly worthy of further investigation.

Another miRNA, *miR-211*, was significantly less abundant in the melanomas and melanoma cell lines compared with the normal counterparts. This miRNA was also found to be downregulated in a highly invasive melanoma cell line compared with its isogenic cell line with low invasive potential (Mueller *et al.*, 2009). Interestingly, *miR-211* is transcribed from the *TRPM1* gene. Loss of TRPM1 expression strongly predicts melanoma progression and poor clinical outcome in human cutaneous melanoma (Duncan *et al.*, 2001; Hammock *et al.*, 2006); thus, it is an important prognostic marker. Although the role of TRPM1 in melanoma progression is unknown, a recent study suggested that TRPM1 function is critical for melanocyte pigmentation (Oancea *et al.*, 2009). Given that *miR-211* is likely co-transcribed with its host gene *TRPM1*, further investigation of the clinical and biological significance of *miR-211* in melanoma development is warranted.

A recent study by Segura *et al.* (2009) showed overexpression of *miR-182* in a subset of melanoma cell lines and some metastatic melanoma samples. In our microarray data, the expression of *miR-182* was undetectable in all melanocytes and half of the melanoma tumors. The remaining melanoma tumors show low *miR-182* expression. Therefore, we could not evaluate its significance by statistical analysis.

The authors also showed that ectopic expression of *miR-182* promotes lung metastasis in a mouse model, suggesting a possible role for *miR-182* in distant metastasis (e.g., in lung), which may not have a significant role in regional lymph node metastasis.

miRNA signature of poor survival in melanoma

We showed that high expression of *miR-193b* and low expression of *miR-191* in melanomas were significantly associated with poor survival. *miR-193b* is also highly expressed in head and neck squamous cell carcinoma (Avissar *et al.*, 2009), and has been shown to be associated with high risk of metastatic disease in uveal melanoma (Worley *et al.*, 2008). Besides its expression association, virtually nothing is known about the function of *miR-193b*. Computational methods (miRanda, TargetScan 5.1, and PicTar_vertebrate) predict hundreds of *miR-193b* target mRNAs (Supplementary Table S6). Among the predicted targets, PTEN, an important tumor suppressor that negatively regulates the Akt-signaling pathway, has been implicated in melanoma tumorigenesis and progression. Importantly, altered PTEN expression is common in primary melanoma and associated with metastatic disease (Stahl *et al.*, 2003; Mikhail *et al.*, 2005; Dankort *et al.*, 2009). However, the functional roles of *miR-193b* and its target(s) remain to be further investigated.

We found reduced levels of *miR-191* to be associated with short survival in melanoma, which is in contrast to a previous report showing that high expression of *miR-191* was associated with poor survival in acute myeloid leukemia patients (Garzon *et al.*, 2008). Although high *miR-191* expression is found in several cancer types, including the colon, lung, pancreas, prostate, and stomach (Volinia *et al.*, 2006; Yanaihara *et al.*, 2006), it remains possible that the activity of specific miRNA varies in different tissues or cell types. For example, inhibition of *miR-191* caused increased cell proliferation in HeLa cervical cancer cells, but decreased proliferation in A549 lung cancer cells (Cheng *et al.*, 2005). Furthermore, Mueller *et al.* (2009) found high expression of *miR-191* in a melanoma cell line stably transfected with antisense targeting of *SNAIL* compared with its parental cell line. Snail, a member of the Snail family of transcription factors, has been shown to be involved in epithelial–mesenchymal transition (a hallmark of invasiveness) (Batlle *et al.*, 2000; Cano *et al.*, 2000). The Snail knockdown mutant lost its invasive propensity because of loss of repression of E-cadherin expression by Snail (Kuphal *et al.*, 2005). The high expression of *miR-191* in the non-invasive melanoma cell line (i.e., Snail knockdown) corresponds well with a good prognosis and long survival in melanoma patients. Computational analysis revealed a number of predicted targets for *miR-191* (Supplementary Table S6), which have been shown to be involved in melanoma progression, e.g., FZD5 (Bachmann *et al.*, 2005) and BDNF (Innominato *et al.*, 2001).

miRNA expression associated with *BRAF*/*NRAS* mutation

We found two significant associations when comparing *NRAS/BRAF* mutant tumors with those that were wild type for both genes: (i) low expression of *miR-193a*, *miR-338*, and *miR-565* was associated with cases carrying a *BRAF* missense mutation at the commonly involved residue V600; and (ii) reduced expression of *miR-663* was associated with *NRAS*-mutated cases. In contrast, there were no significant differentially expressed miRNAs between *BRAF* versus *NRAS* mutated melanomas. Furthermore, we did not find any miRNAs that were commonly deregulated in *BRAF*/*NRAS*-mutated melanomas compared with *BRAF*/*NRAS* wild-type cases. This observation suggests that BRAF and NRAS might regulate distinct signaling pathways, in addition to the common MAPK-signaling pathway. The finding is consistent with global mRNA expression array studies (Daniotti *et al.*, 2004; Pavey *et al.*, 2004; Giordano *et al.*, 2005). Together, the data would be in agreement with a model where BRAF and NRAS contribute to melanoma development through partly overlapping and partly distinct pathways (Dumaz *et al.*, 2006; Eskandarpour *et al.*, 2009; Huang and Marais, 2009; Jaiswal *et al.*, 2009; Old *et al.*, 2009).

miR-193a was observed as the strongest discriminator between *BRAF*-mutated and wild-type melanomas. Interestingly, this miRNA was also reported as downregulated in $BRAF^{V600E}$-mutated thyroid cancer cell lines compared with normal thyroid tissue (Cahill *et al.*, 2007). *miR-193a* is the only common deregulated miRNA associated with *BRAF* mutation in both papillary thyroid cancer (Cahill *et al.*, 2007) and our melanoma series, suggesting that *miR-193a* may have an important and common role in BRAF-associated events in MAPK and/or other signaling pathways. It is noted that some of the predicted targets by computational analysis are associated with MAPK pathway (Supplementary Table S7). Elucidation of the targets of *miR-193a* may lead to a further understanding of the pathological role of *BRAF* mutation, and development of novel therapeutic targets.

Prospect for clinical utility of miRNAs as biomarkers

Our study shows frequent dysregulation of miRNAs in melanomas with identification of a classifier for poor survival. On confirmation of the prognostic value in independent melanoma series, miRNA detection could be developed into complementary diagnostic tools in clinical pathology. In this context, detection of miRNA expression would have several advantages compared with e.g., detection of coding gene transcripts. Mature miRNA species are comparatively stable and preserved in paraffin-embedded samples commonly used in clinical routine. In addition, analyses of miRNA species in individual cells may be applied on paraffin-embedded melanoma tissues that are frequently heterogeneous with related problems of tumor cell representation.

MATERIALS AND METHODS

Melanoma cell lines and melanocytes

Generation, characterization, and culturing conditions for the 21 melanoma cell lines included in the study have been previously reported in detail (Daniotti *et al.*, 2004). The cell lines were derived from primary melanomas (MCp-1 to 4), lymph node metastases

(MCn-1 to 8), or cutaneous metastases (MCc-1 to 9) of 19 patients with cutaneous malignant melanoma. Three lines (MCp-2, MCn-8, and MCc-7) originated from the same patient. Melanoma cell lines had been propagated for 5–51 passages (median = 18). Information of originating tumor phenotypes and genetic status for *BRAF* and *NRAS*, as previously determined (Daniotti *et al*., 2004), is given in Supplementary Table S1. Human melanocyte cultures (NM-1 to 3) (PromoCell, Heidelberg, Germany) from three different individuals were included as non-neoplastic provenience. Ethics approval was obtained for the use of all samples.

Melanomas

A total of 32 samples from fresh frozen regional lymph node metastases of patients with cutaneous melanoma were studied (M-1 to 32; Supplementary Table S1). The study was approved by the ethics committee of Karolinska University Hospital and conducted in adherence to the Declaration of Helsinki protocols. All tumors were obtained with informed consent of the patients and were all present and collected at the time of initial diagnosis. All patients had regional lymph node metastases (AJCC stage III disease) and represented cases with poor or good prognosis in terms of melanoma-specific survival, and all tumors have been characterized for genetic status of *BRAF* exon 15 and *NRAS* exon 2 as previously reported for the majority of cases (Edlundh-Rose *et al*., 2006; Johansson *et al*., 2009).

RNA extraction

Total RNA was extracted using mirVana miRNA Isolation Kit (Applied Biosystems/Ambion, Austin, TX), and RNA concentrations were determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE).

miRNA microarray experimentation and analyses

miRNA expression profiling was performed using Human Agilent's miRNA Microarray system (Agilent, Santa Clara, CA) with probe sets for 470 human miRNAs (miRBase release 9.1). In brief, 120 ng of total RNA were fluorescence-labeled with Cyanine 3-pCp, and hybridized onto the arrays for 18–20 h at 55 °C. Slides were scanned in an Agilent microarray scanner G2565BA and the images obtained were processed with Feature Extraction Software 9.5.3.1 (Agilent). Intensity values were processed using Cluster 3.0 software whereby data were normalized, log transformed, and median centered. Only normalized miRNAs with less than 20% missing values across the samples were included in the further analysis. The microarray data have been deposited at NCBI Gene Expression Omnibus (GEO accession number, GSE19387).

Hierarchical clustering (average linkage with uncentered correlation) of the samples and miRNAs was performed using Cluster 3.0 software and visualized with Treeview v1.60. Significance Analysis of Microarrays (SAM) (http://www-stat.stanford.edu/Btibs/SAM/) was applied to determine the association of each miRNA with sample groups, melanoma-specific survival, as well as clinical and genetic parameters including age at diagnosis, gender, Breslow tumor thickness, and *BRAF*/*NRAS* mutational status. A total of 1000 permutations were performed to estimate false discovery rates for the resulting score statistics. Prediction Analysis of Microarrays (PAM) (http://www-stat.stanford.edu/Btibs/PAM/) was used to construct a classifier to predict sample groups based on miRNA expression.

Quantitative Reverse Transcription-PCR (qRT-PCR)

Expression of selected mature miRNAs was assessed by qRT-PCR. cDNA was prepared from total RNA using Taqman MicroRNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). Predesigned TaqMan MicroRNA Assays for *miR-191* (ID 002299), *miR-193b* (ID 002367), *let-7i* (ID 002221), *miR-338-3p* (ID 002252), *miR-365* (ID 001020), and *miR-193a-3p* (ID 002250) were purchased from Applied Biosystems and qRT-PCR was performed using an Applied Biosystems 7500 Fast Real-Time PCR System. All reactions were run in triplicate and normalized to the expression of *RNU6B* (ID 001093). Relative expressions were determined with the ΔC_T method and reported as $2^{-\Delta CT}$.

Statistical analysis of individual miRNAs

Microarray and qRT-PCR results were compared using Pearson's correlation: the microarray and qRT-PCR measurements were computed, and *P*-values were estimated by permuting the samples. Unpaired *t*-test was used to compare miRNA expression in different groups of samples. SAM survival analysis was used to determine association of miRNAs with survival. *P*-values were obtained for the Cox score statistics using the χ^2 distribution. At retrospective follow-up, survival information was collected for the 32 cases of melanoma tumors (see online Supplementary Table S1). Survival was defined as the time from the first diagnosis of regional lymph node metastasis until the date of death or last follow-up; melanoma-specific survival was the outcome of interest, and therefore cases that died of a cause other than disease were treated as censored. Fourteen cases died of the disease within 1–13 months (poor prognosis), two cases died of unknown or melanoma-unrelated cause of death, and 16 cases survived for 60 months or more (good prognosis). Tumor samples were classified into different groups with high or low expression of each miRNA, according to the median level of expression. Kaplan–Meier plots were used to graphically display the association of miRNAs with the outcome. Survival curves for each group were compared using the log-rank test. Univariate Cox proportional hazards models were used to evaluate the association of miRNA expression with survival. All *P*-values obtained in this study were 2-tailed. *P*-values <0.05 were considered significant. Statistical calculations were performed using Statistica 8.0 (StatSoft, Tulsa).

CONFLICT OF INTEREST

The authors state no conflict of interest.

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SUPPLEMENTARY MATERIAL

Supplementary material is linked to the online version of the paper at http://www.nature.com/jid

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