Acta Derm Venereol 2016; 96: 29–34

INVESTIGATIVE REPORT

Identification of a Circulating MicroRNA Profile as a Biomarker of Metastatic Cutaneous Melanoma

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No specific biomarkers for prognostication or evaluation of tumour load in melanoma have been reported to our knowledge. MicroRNAs (miRNAs) are strongly implicated in oncogenesis and tumour progression, and their circulating forms have been studied as potential biomarkers in oncology. The aim of this prospective study was to identify a melanoma-specific profile of plasma miRNAs. A screening phase, using RNA microarray, was conducted on plasma from 14 patients with metastatic melanoma and 5 healthy subjects. Selected miRNAs were analysed by reverse transcription quantitative PCR (RTqPCR) in 2 independent training and validation cohorts including, respectively, 29 and 31 patients and 16 and 43 control subjects. A profile of 2 miRNAs (miR-1246 and miR-185) significantly associated with metastatic melanoma with a sensitivity of 90.5% and a specificity of 89.1% was identified. This plasma miRNA profile may become an accurate non-invasive biomarker for melanoma. ***Key words: biological markers; melanoma; neoplasm metastasis; microRNAs; REMARK.***

Accepted May 27, 2015; Epub ahead of print Jun 3, 2015

Acta Derm Venereol 2016; 96: 29–34.

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Melanoma, which arises from melanocytes, is one of the most aggressive forms of skin cancer. There is currently no curative therapy for advanced stages of the disease. Clinical, histopathological and imaging data are among the methods used for prognostication and monitoring of melanoma. Histopathological studies measuring tumour depth (Breslow thickness) provide the most important prognostic indicator, but do not allow precise predictions of clinical outcome in patients with primary melanoma (1). No specific or sensitive biomarkers for tumour load in melanoma have been clinically used to our knowledge (2).

MicroRNAs (miRNAs) are small non-coding RNAs that act as post-transcriptional regulators of gene expression and control many critical cellular processes. Numerous studies have reported aberrant expression of miRNAs in a range of different pathologies, with striking alterations in tumour tissues (3). Profiling of miRNAs has contributed to the molecular classification of tumours according to cancer type and prognosis (4, 5). Some miRNAs may be specific to the melanocytic lineage (6) and miRNA signatures have potential as clinically relevant biomarkers in metastatic melanoma (7).

miRNAs can be detected in urine, serum and plasma, allowing non-invasive identification of individuals with cancer (8–10). miRNAs display several properties that make them good biomarkers (stability in body fluids, protection from endogenous RNase activity, resistance to prolonged incubation at room temperature, and resistance to multiple freeze-thaw cycles) (8). Circulating miRNAs show constant homogeneous expression in healthy individuals (11). Growing evidence shows that measurements of miRNAs in serum or plasma can provide valuable non-invasive biomarkers for detection of various human cancers (12).

The present study used microarray analysis and reverse transcription quantitative PCR (RTqPCR) assays to define the miRNA expression profile in plasma from patients with metastatic melanoma and compared it with that of healthy donors. The aim of this study was to identify a plasma miRNA profile specifically associated with metastatic melanoma. As no sensitive biomarker has yet been developed for melanoma, this miRNA profile could serve as a useful biomarker for diagnosis, prognosis and/or follow-up of patients with metastatic melanoma.

MATERIAL AND METHODS (for a complete version see Appendix S1[1])

Study design and subjects

A 3-step case-control study was conducted (Fig. S1[1]). Training and validation phases were analysed using 96.96 Dynamic Array integrated fluidic circuits (Fluidigm France, Les Ulis, France), as described below.

Plasma from 64 control subjects and 73 patients with histologically-proven metastatic melanoma was collected after obtaining

[1]https://doi.org/10.2340/00015555-2156

written informed consent. The clinical and demographic characteristics of the patients and control subjects in each group are summarized in Table I. All patients were free from treatment for at least 4 weeks before blood sample collection. Tumours were staged according to the American Joint Committee on Cancer (AJCC) 2009 classification (1). Control samples were obtained from volunteer blood donors. Due to the design of the study, lactate dehydrogenase (LDH) was not analysed in the screening group.

All tumour samples were obtained from the tumour bank of the department of pathology of our hospital (CHU, Toulouse, France) after obtaining written informed consent. All tumour samples were from patients whose plasma was analysed. The specimens consisted of 10 formalin-fixed, paraffin-embedded (FFPE) biopsies of metastatic melanomas. For all tumour samples, a board-certified pathologist had confirmed the diagnosis and delineated tumour zones. Manual macrodissection of the tumour and surrounding healthy tissue was performed on 4 10-µm thick tissue sections from each specimen.

MicroRNA extraction and analysis

For microarray analysis and reverse transcription RTqPCR analysis, RNA extraction was performed using the miRNeasy extraction kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. For details, see Appendix S1[1].

Tissue RNA was extracted using the miRNeasy FFPE kit (Qiagen) according to manufacturer's instructions(see Appendix S1[1]).

Total RNA was ligated to a RNA-linker-Cy3-dye using Ambion® T4 RNA ligase (Life Technologies, Saint Aubin, France). Overnight hybridization of labelled RNA was performed with locked nucleic acid (LNA)-modified microarray slides (Exiqon, V7). Slides were then scanned and microarray images were analysed. A logarithmic transformation of the signal values was first applied (see Appendix S1[1]).

miRNA expression analysis using the 96.96 dynamic array (see Appendix S1[1])

Tissue miRNA expression analysis by RTqPCR using the CFX96 PCR System (see Appendix S1[1])

Statistical analysis (see Appendix S1[1])

RESULTS

Melanoma miRNA screening phase

This multiphase single-centre case-control study was conducted in 3 consecutive steps: screening, training and validation (Fig. S1[1]). In the screening phase, a sensitive array was used for miRNA expression profiling, based on 576 human LNA-modified capture probes (16), to identify miRNAs predominantly present in plasma from patients with metastatic melanoma. The expression of plasma miRNAs in 14 patients and 5 healthy donors was compared (Table I). Out of the 184 miRNAs detected on the array, 12 were down-regulated and 27 were up-regulated in patients with melanoma compared with healthy donors (Table SI[1]). In addition, several miRNAs were detected only in patient samples (Table SII[1]). Data for the number of positive spots for each miRNA over the 14 patient samples and the number of patients with positive results for each of these miRNAs are summarized in Table SII[1]. Some of the miRNAs were well represented, such as miR-27b (with 17 positive signals in 10 patients/20 spots). In contrast, only one positive spot per patient was detected for some miRNAs, such as miR-1248.

Only the overexpressed miRNAs in patient samples were selected for further investigation. miRNAs that met the following criteria were considered selective enough to warrant further evaluation: (*i*) those with a patient/control ratio ≥ 2 (Table SI[1]), (*ii*) those with a patient/control ratio ≥ 1.7 in at least 7/14 patients (Table SI[1]); (*iii*) those exclusively present in patients, detected at least 8/28 times (Table SII[1]); and (*iv*) miR1292, miR-2110, miR-517c, miR-668, miR-1537 and miR-518d, exclusively present in patients and detected in $< 8/28$ times (Table SII[1]), but which had a fluorescence intensity > 90. A total of 25 miRNAs were considered selective and were assessed further using RTqPCR.

In addition, a panel of miRNAs for which expression had previously been reported to be altered in melanoma cells or tissues was analysed. Finally, miRNAs previously reported as reference genes, or those found to be highly stable in both patient and control samples in the screening phase, were also analysed. A total of 51 miRNAs (Table SIII[1]) was evaluated in both the training and validation cohorts using a REMARK-compliant methodology (17).

Table I. *Demographic and clinical characteristics of patients and controls*

	Screening		Training		Validation	
Variable	Controls $n=5$	Patients $n=14$	Controls $n=16$	Patients $n=28$	Controls $n=43$	Patients $n=31$
Age[a], years, median; (IQR)	43 (29; 58)	67 (45; 79)	43 (30; 52)	61 (54; 79)	43 (34; 53)	60 (44; 69)
Sex ratio (M/F)	0.66	0.75	1.29	0.81	1.26	1.21
Stage IIIc – M1a[a], *n*		3		9		3
Stage IVa, n		11		20		28
Breslow thickness[b], mm, median; (IQR)		3 (1.5; 6)		2.5 (1.5; 3.4)		2.2 (1.2; 4.2)
Ulceration[b] (present), *n*		4		6		8
Mitotic rate $\geq 1/mm^2$[b], *n*		3		3		10
BRAF V600 mutated, *n*		1		5		21
NRAS mutated, *n*		–		4		2
LDH factor vs. upper limit of normal (=234 IU/l), median; (IQR)	nd[c]	nd[c]	0.71 (0.60; 0.74)	0.78 (0.63; 0.97)	0.70 (0.62;0.75)	1.07 (0.73;1.80)

[a]At the time of blood sample collection; [b]At the time of diagnosis; [c]Due to the design of the study LDH could not be analysed in the screening group (nd).
IQR: interquartile range; LDH: lactate dehydrogenase; IU: International Units.

Differential expression of melanoma miRNAs in the training cohort

RTqPCR analysis of the 51 above-mentioned miRNAs was performed using Fluidigm® microfluidic technology on plasma from a training cohort of 28 patients and 16 healthy donors (see Table I). miRNAs were first analysed according to the criteria defined in the Methods section. Twenty-six miRNAs were retained for further analysis. Due to technical issues with amplification, some of the miRNAs selected initially were excluded, for example miR-27b and miR-302d.

The most stable miRNAs, miR-103, miR-423-3p and miR-191, to be used for normalization were selected by geometric averaging of the Cq values of all miRNAs analysed from both cohorts (18). Bivariate Wilcoxon analysis identified 5 miRNAs (miR-185, miR-20a, let-7b, miR-338-3p and miR-1246) that differed significantly between patients with melanoma and healthy donors, with a threshold of 5% (Table II). The receiver operating characteristic (ROC) curves were generated and the area under curve (AUC) analysed for these 5 miRNAs (Fig. S2[1]). All 5 displayed an AUC >0.7, with miR-1246 showing an AUC of 0.95 ($p<0.001$), suggesting that this miRNA alone would be a good marker.

Prediction performance analysis was used to evaluate the diagnostic value of these miRNAs. For each single miRNA, an optimal threshold was determined (using linear discriminant analysis). The categorisation of the patients was then performed according to this threshold. For the combination of miR-185 and miR-1246, a random forest model was constructed and the patients were classified according to this model. As shown in Table III, the use of each of miRNA alone allowed the correct classification of a maximum of 79% of patients. Surprisingly, the results of ROC curve analysis were not confirmed. None of the 5 miRNAs on their own (including miR-1246) was efficient enough to predict "cancer/non-cancer" status. Thus, we evaluated whether a combination of circulating miRNAs could predict "cancer/non-cancer" status. A combination of miR-1246 and miR-185 gave good prediction performances.

The Kolmogorov–Smirnov test confirmed the validity of the results. The statistical distribution of miRs was tested and the statistical distance calculated. Interestingly, among 10 tested miRNAs analysed, the maximum difference between the cumulative distributions was 0.9310, which was obtained between miR-1246 and miR-185, showing that the distance between these 2 miRNAs was wide. The wide statistical distance between the miRNAs that we identified strengthens our results, suggesting that the use of both miRNAs increases the sensitivity of the profile, by summing the values of 2 independent biomarkers.

Table II. *Plasma microRNAs that differed between patients with metastatic melanoma (Cancer) and healthy donors (Control) in the training cohort*

miRNA	Selection criteria	Cancer	Control	Ratio[a]	*p*
hsa-miR-185	Bibliography	0.6045	0.9780	0.62	1.98E-03
hsa-miR-20a	Bibliography	2.2537	3.0143	0.75	1.51E-02
hsa-let-7b	Bibliography	0.1094	0.0739	1.48	1.21E-02
hsa-miR-338-3p	Patients>Healthy[b]	0.0423	0.0152	2.78	9.68E-03
hsa-miR-1246	Patients>Healthy[b]	0.2333	0.0512	4.56	2.14E-08

Bivariate Wilcoxon analysis, with a threshold of 5%.
[a]Ratio of mean expression of each miRNA in patients with metastatic melanoma and healthy donors. [b]In the screening phase.

Table III. *Risk score analysis of patients with metastatic melanoma (Cancer) and healthy donors (Control) For details see Appendix S1*[1]

	Cancer	Control	% well-classified
Training cohort			
hsa.miR.185			
Cancer	22	6	79
Normal	6	10	63
hsa.miR.20a			
Cancer	20	8	71
Normal	6	10	63
hsa.let.7b			
Cancer	12	16	43
Normal	3	13	81
hsa.miR.338.3p			
Cancer	7	21	25
Normal	0	16	100
hsa.miR.1246			
Cancer	18	10	64
Normal	0	16	100
hsa.miR.1246/hsa-miR-185			
Cancer	28	0	100
Normal	0	16	100
Validation cohort			
hsa.miR.1246/hsa-miR-185			
Cancer	27	2	93
Normal	7	36	83

Sensitive detection of metastatic melanoma using a miRNA profile

We assessed whether using a combination of miRNAs would provide a more sensitive and specific approach for distinguishing the 2 populations. Multivariate analysis using the random forest algorithm and repeated cross-validation (10-fold, repeated 30 times) was initially performed on the training cohort to test the performance of a miRNA model in differentiating the patients and controls. This method allowed accurate differentiation of patients from healthy donors with high sensitivity (91%) and good specificity (80%). After cross-validation, all the training data were used to construct a random forest assignment model that led to a profile of 2 miRNAs: miR-1246 and miR-185. Furthermore, risk score analysis showed that none of the control or cancer samples was misclassified when using the 2 miRNA profile (Table III).

Validation phase

To verify the accuracy and specificity of these results, the miRNA profile described above was analysed using

an independent validation cohort of 31 patients and 43 healthy donors. Two of the melanoma samples were excluded due to missing data. After risk score analysis, only 2 cancer samples in the validation cohort were misclassified (Table III). The (miR-1246 + miR-185) prediction model was very reliable and performed significantly better than LDH measurements (Fig. 1). Repeated cross-validation on both cohorts revealed good performance, with a sensitivity of 90.5% and a specificity of 89.1%.

Prognostic value of the plasma miRNA profile

To evaluate the prognostic value of the plasma miRNAs that we identified, their impact on survival was investigated. After analysis of the levels of each miRNA in the training and validation cohorts combined, the Kaplan–Meier survival curves were plotted and compared using the log-rank test. In bivariate analysis, miR-20a, miR-152, miR-2110 and miR-185 displayed a protective effect, whereas LDH and staging according to AJCC classification were associated with lower survival rates (Fig. S3[1]). To adjust for possible confounding factors, a multivariate analysis was constructed according to the Cox model, including all variables for which bivariate analysis revealed a significant impact on survival: WHO performance status (> 1), LDH (>limit of normal) and age (> 65 years). After correction, the protective effect of miR expression on survival disappeared.

Origin of miRNAs

Plasma miRNAs identified as being deregulated in patients with melanoma may originate either from the tumour or from the tumour microenvironment. To better characterize their origin, we used RTqPCR to analyse the expression of the most discriminating plasma miRNAs (miR-20a, miR-185, miR-338-3p and miR-1246) in the peritumoural and tumoural zones of tumour samples from 10 patients. These miRNAs were detected in both the peritumoural and tumoural areas of the tumour tissues (Fig. S4[1]). miR-1246 appeared to be expressed at similar levels in both zones of the tumour; miR-185 and miR-20a expression displayed non-significant tendencies to higher expression in the tumoural zones than in the peritumoural zones; and expression of miR-338p was significantly higher in the tumoural zones ($p = 0.05$).

DISCUSSION

This study identifies co-detection of miR-185 and miR-1246 in plasma as significantly associated with metastatic melanoma. This miRNA profile enables the accurate differentiation of patients and healthy donors, with a sensitivity of 90.5% and a specificity of 89.1%. As no specific biomarker of melanoma has been validated, the (miR-185+miR1246) profile may be used as a marker for tumour load or residual disease in monitoring patients.

To our knowledge, LDH is the only validated circulating biomarker used in patients with metastatic melanoma (1, 19). LDH has a very low specificity and provides information of limited clinical relevance (19). Circulating miRNAs display properties that make them good candidates for biomarkers: remarkable stability in plasma, resistance during prolonged storage at room temperature (8), constant expression in the blood of control subjects, and the absence of any impact of age on expression (20). Circulating miRNAs can be used to discern pathology and origin, notably in cancer (8–10), and thus could be useful tools for monitoring disease. We identified 4 plasma miRNAs that are differentially expressed between patients with metastatic melanoma and healthy donors. miR-185 and miR-20a have been implicated in melanoma pathology (6, 21–24). To our knowledge, miR-1246 and miR-338-3p have not previously been reported as differentially expressed in the plasma of patients with melanoma vs. control donors.

Used individually, these 4 miRNAs were rather poor predictors of melanoma status. However, we identified a profile of plasma miRNAs (miR-185+miR-1246) that had higher accuracy than individual miRNAs. This plasma miRNA profile may be of use as a specific biomarker in patients with melanoma, enabling the monitoring and/or screening of patients.

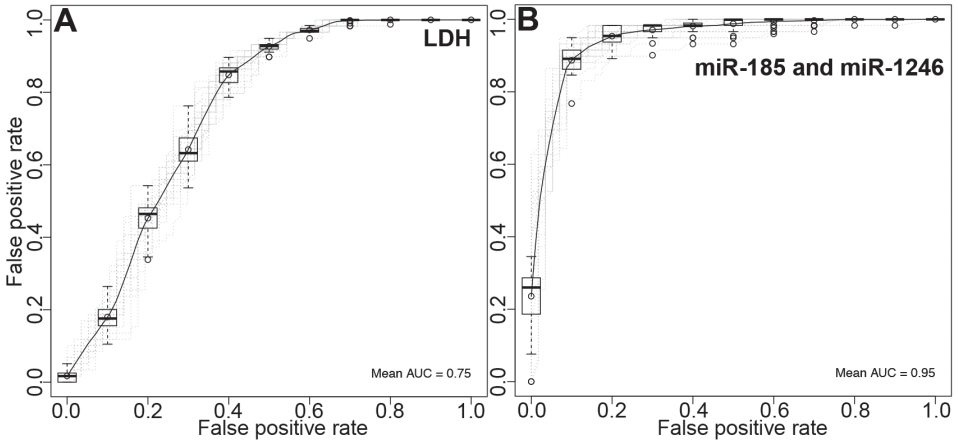

Fig. 1. Cross-validation receiver operating characteristic (ROC) curves of lactate dehydrogenase (LDH) (A) and the (miR-185+miR-1246) model (B) on both the training and validation cohorts. ROC curves were obtained through a cross-validation procedure. A curve was obtained for each cross-validation block and then a mean curve was generated. The box-plots allow visualization of the variations around this mean curve at a specified false-positive rate. AUC: Area under the curve.

Bivariate analysis suggested that miR-20a, miR-152, miR-2110 and miR-185 expression might be associated with a protective effect on survival; however, after adjusting for possible confounding factors in a multivariate Cox analysis, the protective effect on survival disappeared. The 4 miRNAs displayed only a non-significant tendency to higher survival when expressed. Due to a lack of statistical power, a prognostic value of miRNA expression may have been missed by the present study. Greenberg et al. (24) have reported that miR-185 inhibits melanoma cell proliferation and invasion and has a suppressive effect on tumour growth. miR-185 has also been shown to suppress proliferation and tumour growth in other cancers, such as non-small cell lung cancer, and ovarian and breast cancers (25, 26). Further studies are needed on larger cohorts of AJCC IIIa-b stage patients after complete resection of the tumour, in order to assess the prognostic value of miRNA expression.

Other limitations of our study must be taken into account when interpreting the results. First, there were significant differences in age between patients and controls in both cohorts. The impact of age on miRNA is matter of debate. Some authors have reported that age has no impact on the expression of miRNAs (20), while others have found that miRNAs (27) and, notably, several circulating miRNAs (28–30) are affected by age difference, but none of our miRNAs of interest. In our study, control samples were obtained from volunteer blood donors. As a consequence, age-matched controls were not available. To control for this result, we evaluated the correlation between expression of the miRNAs of interest and the age of the controls and patients. As shown in Fig. S5[1], the Spearman's correlation coefficients are low, indicating that age did not result in any significant variations in miRNA expression within the different populations (separately for patients and donors) and analysed an isolated set of age-homogeneous individuals within the 2 populations. Secondly, there was a bias with regard to the presence of BRAF mutations in the patients from both cohorts. Preliminary analyses found no evidence that the BRAF mutation had an impact on the plasma level of the 4 miRNAs of interest, but additional research on matched cohorts with a higher number of patients is necessary to confirm this.

During the course of this study, circulating miRNA signatures for cutaneous melanoma were published. Kanemaru's team reported an increase in miR-221 in melanoma patients vs. healthy donors (31) and, more specifically, that miR-221 could be used for detection of early, but not metastatic, melanoma (32). This team also showed that a miRNA profile (miR-9, miR-145, miR-150, miR-155, miR-203 and miR-205) could discriminate a small cohort of 11 patients with metastatic melanoma vs. 16 patients with primary melanoma (32). In 2013, Greenberg et al. (33) reported, after an initial screening, that miR-29c and miR-324 serum signature differentiated 28 metastatic patients and 10 healthy donors. Interestingly, in our screening step by microarray analysis we also observed a decrease in miR-29c between metastatic patients and healthy donors (Table SI[1]), who were excluded from our subsequent analysis because we selected only the overexpressed miRNAs. Lastly, miR-125b levels in serum exosomes were also reported to be reduced in metastatic melanoma with a threshold cycle value > 37 (34).

Our comparison of the miRNA expression pattern in plasma and tissues provides evidence to support the use of circulating miRNAs as reliable biomarkers. Expression of miR-338-3p was higher in both plasma and tumour tissue from patients with melanoma. It has been shown previously that miR-338-3p is overexpressed in melanomas from patients with short survival times (35). The rate of plasma miR-1246 expression was significantly higher in the plasma of patients with melanoma than in healthy donors; however, the ratio of miR-1246 expression between tumour and peritumoural tissues was lower. Similarly, high levels of serum miR-1246 have previously been shown to allow patients with oesophageal squamous cell carcinoma to be distinguished from healthy controls. miR-1246 was not reported to be up-regulated in the tissue samples. The authors proposed that release of miR-1246 is selective and independent of tissue miRNA abundance (36). miR-1246 may also be produced by the tumour microenvironment or circulating immune cells. miR-1246 has been shown to be expressed by natural killer (NK) cells and is down-regulated after activation by interleukin (IL)-2, IL-15 or IL-21 (37). Previous research into metastatic melanomas found that infiltrating NK cells were primarily localized in the fibroblast-rich peritumoural zone (38).

In conclusion, this study shows that co-detection of miR-185 and miR-1246 in plasma allows accurate differentiation of patients with metastatic melanoma from healthy individuals. As no specific biomarker of tumour load or residual disease exists, this miRNA profile may be used as a biomarker for monitoring melanoma patients with a high risk of recurrence. We hypothesize that the co-detection of miR-185 and miR-1246 may allow early diagnosis of disease recurrence or the identification of a patient subpopulation requiring adjuvant therapy.

AKNOWLEDGEMENTS

The authors would like to thank Dr Marie Brulliard (Genclis, Vandoeuvre-lès-Nancy, France) for providing helpful expertise in statistical analysis. The authors also thank Dr Jean-José Maoret and Frederic Martin (GeT-TQ platform, Genopole, Toulouse, France) for their excellent technical advice and support.

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