Identification of plasma microRNAs as new potential biomarkers with high diagnostic power in human cutaneous melanoma

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Short title

MicroRNAs and melanoma

Novelty and Impact

Melanoma is a devastating disease with few therapeutic options in the advanced stage and with the urgent need of reliable biomarkers for early detection. In this context, circulating miRNAs are raising great interest.

In current pilot study we identified in plasma melanoma patients five circulating miRNAs, three of which detected for the first time as circulating in this type of cancer. In particular, miR-149-3p, miR-150-3p and miR-193a-3p signature showed high sensitivity and specificity in discriminating between healthy subjects and affected individuals, making it suitable to be used as biomarker in melanoma diagnosis.

Abstract

Melanoma is a devastating disease with few therapeutic options in the advanced stage and with the urgent need of reliable biomarkers for early detection. In this context, circulating miRNAs are raising great interest as diagnostic biomarkers. We analyzed the expression profiles of 21-selected microRNAs (miRNAs) in plasma sample from melanoma patients and healthy donors to identify potential diagnostic biomarkers. Data analysis was performed using Global Mean Normalization (GMN) and NormFinder algorithm. Linear regression followed by Receiver Operating Characteristic (ROC) analyses was carried out to evaluate whether selected plasma miRNAs were able to discriminate between cases and controls. We found 5 miRNAs that were differently expressed among cases and controls after Bonferroni correction for multiple testing. Specifically, miR-15b-5p miR-149-3p and miR-150-5p were up-regulated in plasma of melanoma patients compared to healthy controls, while miR-193a-3p and miR-524-5p were down-regulated. ROC analyses of these selected miRNAs provided area under the ROC curve values ranging from 0.80 to 0.95. Diagnostic value of miRNAs is improved when considering the combination of miR-149-3p, miR-150-5p and miR-193a-3p. The triple classifier had a high capacity to discriminate between melanoma patients and healthy controls, making it suitable to be used in early melanoma diagnosis.

Introduction

MicroRNAs (miRNAs) are small, non-coding, single-stranded RNAs produced by eukaryotic cells that play a key role in regulating the expression of hundreds of target genes(1,2). MiRNAs are involved in numerous processes including proliferation, differentiation and apoptosis and their altered expression and function have been linked to the development and progression of various types of cancers(3-9). In particular, miRNAs may act as oncomirs or tumor suppressor molecules since their expression in tumor cells can be up- or down-regulated compared with normal tissues(10). MiRNAs can be released into circulation as a consequence of tumor cell death or, in some cases, actively secreted by viable tumor cells. Moreover, miRNAs might also derive from blood and/or endothelial cells (11). MiRNAs display several properties that make them potentially valuable biomarkers including stability in body fluids, resistance to endogenous RNase activity, prolonged life at room temperature and resistance to multiple freeze-thaw cycles (12). A growing body of evidence shows a different pattern of expression of circulating miRNAs between cancer patients and healthy donors (11,13-19), only a limited number of studies have been focused on melanoma.

Melanoma is the fourth and sixth most common malignancy in men and women, respectively. Disease incidence is increasing by 3% every year and melanoma accounts for 75% of skin cancer deaths. The 5-year survival rate critically depends on disease stage and it has been estimated to be around 95% for stage I and 15 to 20% for stage IV (20). Although several circulating diagnostic and prognostic biomarkers have been investigated in melanoma patients, the only one routinely used in clinical setting is lactate dehydrogenase (LDH)(21). Serum LDH has a significant prognostic value, only in the advanced disease (stage IV) (21). At present, no circulating marker is available to help clinicians in detecting melanoma in early stage when survival is at the highest rate.

Many recent papers reported circulating miRNAs as being dysregulated in melanoma cancer patients with respect healthy subjects (Carpi et al., 2016). Nevertheless, further studies are urgently needed to better define a more clear role of specific miRNAs in melanoma diagnosis and prognosis.

In the current pilot study, the expression profiles of 21 miRNAs selected from literature for their role in melanoma progression were analysed in plasma samples collected from both melanoma patients, at different disease stages, and healthy controls. The data obtained allowed the identification of some plasma microRNAs with high sensitivity and specificity as potential diagnostic biomarkers in melanoma patients worthy of being validated in a large prospective clinical trial.

Materials and methods

Study participants

Control blood samples from 32 healthy volunteers with similar age and sex distribution were recruited from the Blood Donor Center of the University Hospital of Pisa. Healthy subjects who had taken systemic drugs in the week prior to enrolment and those affected by other medical conditions were excluded by sample collection.

Patient recruitment was carried out at the Department of Oncology of the University Hospital of Pisa (Pisa, Italy). Ten AJCC stage I-II, ten stage III and ten stage IV (https://cancerstaging.org/) subsequent patients entered this study. 2.5 ml of blood were drawn from each patient, collected in BD Vacutest Kima tubes containing EDTA and centrifuged at 4 °C for 10 min at 1900 ×g for 1 h. Plasma supernatant was then aliquoted into 1.5 ml DNA LoBind tubes (Eppendorf AG, Germany) and stored at -80 °C until analysis.

The timing of blood collection in patients was: i) within a month following the surgical removal of the lesion and the availability of the pathology report for patients with stages I-II melanoma; ii) within a month following the surgical removal of the sentinel lymph node and

the availability of the pathology report for patients with stages III melanoma; iii) within a month following histologically proven melanoma metastasis for patients with stage IV disease.

The study protocol was approved by local Ethics Committee and conducted in accordance to the principles of the Declaration of Helsinki. Signed informed consent was obtained from every participant.

Selection criteria for miRNAs panel

For the purpose of this analysis, 21 miRNAs were selected from the literature according to at least two of the following properties: dysregulation in the presence of BRAF mutations, negative correlation with metastatic melanoma patient survival and potential involvement in drug resistance phenotype (please refer to Supplementary material). Among all miRNAs selected, 11 have already been individuated as circulating in plasma melanoma patients and the others had only been identified as dysregulated in melanoma cells or tissue samples but they were not yet detected in plasma.

miRNA extraction and real-time PCR analyses

All 21 selected miRNAs were isolated from plasma samples (200 µl) by miRNeasy Serum/Plasma kit (Qiagen, Valencia, CA, USA) following manufacturer's instructions and eluted with 14 µl RNase-free water. Sample concentration was measured by Qubit® microRNA HS Assay (Thermo Fisher Scientific, MA, USA). Reverse transcription was carried out using miRCURY LNATM Universal RT microRNA PCR system (Exiqon, Denmark) according to the manufacturer's instructions. cDNA synthesis was conducted by incubation at 42 °C for 60 min and 95 °C for 5 min. Prior to quantitative real-time PCR (RT-qPCR) reactions being performed, cDNA was diluted 1:40. RT-qPCRs were run on a 7900HT thermocycler (Applied Biosystems) using the miRCURY LNA Universal RT microRNA PCR

assays with the thermal-cycling parameters recommended by Exiqon. Raw Cq values were calculated, as recommended by Exiqon, using the RQ manager software v1.2.1 (ABI) with manual settings for threshold and baseline.

Data analysis and statistics

Data analysis was carried out by GenEx software (MultiD Analyses). Since there is no validated reference miRNA for miRNA analysis in serum/plasma, we used two different methods for data normalization: Global Mean Normalization (GMN) and NormFinder model. The first method was based on the normalization of each single miRNA expression value over the mean expression of all analyzed miRNAs (i.e., difference between single Cq and global mean Cq values), assuming that the latter is constant when the same amount of total RNA from patients and healthy controls is analysed. Such a strategy is likely to reduce technical variations and provide a more accurate assessment of biological changes than using endogenous small nuclear RNAs such as U6 (22). The second method data were normalised according to miRNA expression stability using the algorithm NormFinder.

Statistical analysis was carried out using the Wilcoxon test (non parametric) followed by Bonferroni correction for multiple comparisons. Linear regression followed by Receiver Operating Characteristic (ROC) analyses was carried out to evaluate whether selected miRNAs were able to discriminate between cases and controls. To this aim, the optimal cut-off value for sensitivity and specificity was determined based on the highest Youden's Index in ROC curve analysis. ROC curves were drawn using GraphPad Prism 6 and the area under the ROC curve (AUC) was calculated to evaluate the specificity and sensitivity of selected miRNAs.

Fundings

Results

Characteristics of study population

From January 2015 to February 2016 Blood samples were collected from 30 consecutive melanoma patients in different disease stages. Patients' characteristics, including sex, age and other clinical information are reported in Table I. The median age of the melanoma patient and healthy volunteers was 63 and 57 years, respectively.

Expression levels of miRNAs in patients and healthy controls

Data normalization by GMN method provided 13 out of the 21 miRNAs significantly dysregulated ($p < 0.05$, ANOVA) (Table II). The NormFinder algorithm identified 8 out of 21 miRNAs as normalizers and the subsequent comparison provided 7 significantly dysregulated miRNAs ($p < 0.05$) (Table II). Only 5 miRNAs resulted significantly ($p < 0.01$) dysregulated with both methods of data analysis after the Bonferroni correction for multiple tests (Figure 1). Specifically, circulating miR-15b-5p, miR-149-3p and miR-150-5p were significantly up-regulated, and miR-193a-3p and miR-524-5p significantly down-regulated in plasma of melanoma patients compared to healthy controls. These miRNAs were selected for a further statistical analysis to evaluate their diagnostic power.

As opposed to the others, miR-200c-3p was not dysregulated in plasma melanoma patients compared to healthy donors, but its expression was significantly down-regulated in different stages of the disease. Particularly, while the expression of miR-200c-3p in plasma of stage I-II melanoma patients is not different from healthy donors, its expression in plasma of stage III-IV melanoma patients was significantly down-regulated compared to that of stage I-II melanoma patients ($p=0.001$) and of healthy donors($p=0.00004$) (Figure 2). Diagnostic power of miR-200c-3p was not considerate since its value is not different in plasma of melanoma patients compared to healthy donors.

No significant miRNA dysregulation due to gender or age was observed in the study subjects (data not shown).

Diagnostic value of plasma miR-150-5p, miR-149-3p, miR-193a-3p, miR-15b-5p and miR-524-5p in melanoma patients

The area under the ROC curve (AUC) for each of these five miRNAs ranged from 0.80 to 0.95 (Table III). Although high AUC values were attributed to each miRNA, miR-150-5p, miR-149-3p and miR-193a-3p gave the best performance in terms of diagnostic power. Noteworthy, diagnostic power was further improved when considering miR-150-5p, miR193a-3p and miR-149-3p signature. The triple classifier has increased AUC, sensitivity, and specificity (Table III) with an accuracy of 91%.

Discussion

The incidence of melanoma is increasing all over the world. This disease is curable when detected in early stages and it might be deadly when diagnosed in advanced stages (20). Although lately new and very effective therapies came in clinical use, mortality rate of stage IV melanoma patients it is still high (20). Since melanoma must be treated in early stages to maximise the chances of patient survival, the ability to identify early signs of melanoma progression would be very valuable in clinical setting. To date no reliable biomarker has been identified as sensitive or specific enough to be beneficial for early detection of melanoma (all stages). Reliable circulating biomarker(s) to detect melanoma with distant or regional spread prior to clinical evidence of metastasis could improve treatment and outcome for melanoma patients. Stability, sensitivity and specificity together with resistance to degradation are the main characteristics to look for a potential circulating biomarker.

The potential diagnostic role of plasma miRNAs in melanoma has been investigated in a few studies (22, 31) with different results due mainly to differences in analytical methods that lead to the identification of different miRNA panels with diagnostic value. A major source of difference in analytic methods is the normalisation in RT-qPCR. Up to now, there is no validate normalizator for the analysis of circulating miRNA expression levels. Therefore, we used different methods for normalisation (Global mean Normalisation and NormFinder), not involving the use of a single molecule as a control, given the great biological variability of expression of circulating miRNAs. Indeed, we have not used miR-16, as in many studies conducted by others, since this miRNA is particularly susceptible to haemolysis (Marabita et al., 2016).

In the current pilot study, we identified five miRNAs with promising diagnostic power in plasma of melanoma patients at different stage of disease. Three, miR-149-3p, miR-150-5p and miR-15b-5p, were significantly up-regulated, while two, miR-193a-3p and miR-524-5p were significantly down-regulated in plasma of patients compared to healthy controls. To our knowledge, the present paper is the first to report on dysregulation of miR-149-3p, miR-524-5p and miR-193a-3p in plasma samples of melanoma patients.

Moreover, the expression of miR-200c-3p in plasma of stage I-II melanoma patients is not different from healthy donors, while its expression in plasma of stage III-IV melanoma patients was significantly down-regulated compared to both stage I-II melanoma patients (p=0.001) and healthy donors(p=0.00004). Therefore, miR-200c-3p is not suitable as diagnostic biomarker but instead it could be studied as a prognostic biomarker.

While we found significant levels of circulating miR-149-3p from plasma samples of melanoma patients compared to healthy controls, Pfeffer and co-workers (23) found higher miR-149-5p levels in peripheral exosomes extracted from plasma samples of metastatic melanoma patients compared to healthy controls. This suggests that miR-149 -3p and -5p species may have a similar functional role as oncomirs in human melanoma. Mature

miRNA-3p and -5p are generated from the same pre-miRNA precursor but more often one of them is degraded, although co-existence of these two species has also been reported (24). The reason of this apparent discrepancy is currently unclear. It has been hypothesized that the main (-3p) and the passenger (-5p) strands might influence the expression of different targets and in some cases they can regulate invasiveness of cancer cells (25).

Concerning miR-149-5p, several lines of evidence suggest that in some cancer types other than melanoma, it may function as a tumor suppressor. For example, miR-149-5p expression was decreased in gastric cancer cell lines and in clinical specimens compared to normal counterparts (26). Furthermore, low miR-149-5p expression levels were correlated with lymphnode or distant metastasis and advanced disease stages in colorectal (27) and breast cancers (28). In the present work, high levels of miR-149-3p have been detected in plasma of melanoma patients according to the work of Jin, L. *et al.* which described miR-149-3p in melanoma cells and found that it induced a p53-dependent survival by increasing the expression of the anti-apoptotic Mcl-1 protein. The same author described elevated expression of miR-149-3p in fresh human metastatic melanoma isolates (29).

In the current study, plasma miR-150-5p was up-regulated in plasma of patients compared to healthy controls. MiR-150 was also found to be overexpressed in formalin-fixed paraffin-embedded melanoma metastases and in primary melanoma compared with nevi (30). In line with the hypothesis of its oncogenic role, high levels of circulating miR-150 were also detected in melanoma patients with high risk of recurrence (31). Preclinical evidence showed that tumor growth and metastasis were reduced in immunodeficient mice injected with miR-150−/− NK cells, compared to non injected mice (32). Noteworthy, miR-150 has been reported to increase tumor immunoresistance by post-transcriptionally down-regulating perforin-1 in mouse natural killer cells (32). Although these findings suggest an oncogenic role for miR-150 in human melanoma, some evidence seems to go in the opposite direction. For instance, miR-150-5p up-regulation was found both in serum and tissues

derived from melanoma patients with longer disease free survival after resection of the metastatic lymph node disease (30,33). Recently published evidence on the ability of miR-150-5p to suppress glioma cell proliferation and migration by targeting membrane-type-1 matrix metalloproteinase (34), reinforces the tumor suppressor role of miR-150-5p. These apparent discrepancies may be due to the complex functional role of miR-150-5p in the tumor biology. Indeed, this specific miRNA is expressed in the lymph nodes, spleen, and thymus and it is highly up-regulated during lymphocyte maturation and down-regulated during the activation of mature B and T cells (35). Thus, elevated circulating expression of miR-150 could also be the consequence of its role in the modulation of T-cell response. In line with these findings, in vivo studies show that miR-150 is released in the external environment after T cell activation and this may cause an increase of its blood levels during immune system stimulation (36). Further experiments aimed at clarifying the precise role of miR-150 in human melanoma immunity are warranted.

The third circulating miRNA found up-regulated in the current study is miR-15b-5p, whose levels were significantly higher in plasma samples from melanoma patients than healthy donors, with a trend for a more pronounced increase in advanced stage patients (data not shown). Our results are consistent with data of Fleming et colleagues showing that circulating miR-15b levels were increased compared to the levels found at diagnosis in melanoma patients whose disease recurred compared to disease free patients (37). Finally, evidence showing miR-15b-5p over-expression in melanoma tissues compared to melanocytic nevi suggests a potential oncogenic role for this specific miRNA (38). In line with this notion, the same authors clearly demonstrated that up-regulation of miR-15b-5p is responsible for increasing cell proliferation and decreasing apoptosis in melanoma cell lines (38).

Mir-193a-3p is a member of the miR-193 family whose expression was linked to BRAF mutation status in melanoma tissues (39). Mir-193a-3p was found to be significantly up-

regulated in both tissue and blood samples of colorectal cancer patients and there was an increasing trend of expression of circulating miR-193a-3p in advanced stages colon cancer patients compared to healthy individuals (40). Up-regulation of miR-193 was also found in the high-grade prostate tumors (Gleason score ≥ 8) (41) as well as in serum samples obtained from patients with esophageal squamous cell carcinoma (42). In apparent contrast with these findings but in agreement with those of the current study, in lung and oral squamous cell carcinoma (43,44), acute myelogenous leukemia (45), breast cancer (46) and Wilms tumor (47), circulating miR-193a-3p levels were found to be reduced in plasma of patients compared to healthy controls. The report that methylation is responsible for miR-193a down-regulation (44,45,48) reinforces the notion that miR-193a-3p might have a role as tumor suppressor in a variety of human cancers, including melanoma.

The functional role of miR-524-5p in cancer development and progression is largely unknown. A possible tumor suppressor role for miR-524-5p may be in line with findings of the current study showing how circulating miR-524-5p levels was significantly lower in plasma of patients compared to healthy controls. Preclinical data demonstrating that miR-524-5p overexpression inhibited melanoma cell proliferation and migration in human melanoma cell lines (49) are likely to support this notion.

Although not significantly down-regulated in plasma of all melanoma patients compared to healthy controls, miR-200c-3p showed different levels of expression in plasma of patients with advanced melanoma stages compared to those detected in patients with early stages of the disease and healthy individuals. This suggests that mir-200c-3p can undergo a process of dynamic change at different tumor stages. In agreement with this hypothesis are findings by Xu and colleagues (50) who observed that miR-200c-3p was down-regulated in metastatic melanoma compared with benign melanocytic nevi. Noteworthy, several lines of evidence support the hypothesis that miR-200c may play a role in epithelial-mesenchymal transition by modulating expression of E-cadherin (17). Therefore, up-regulation of

circulating miR-200c-3p in patients with early melanoma stages may correlate with the presence of an epithelial phenotype in the majority of melanoma cells, whereas down-regulation of circulating miR-200c-3p in patients with melanoma in advanced stage may indicate the predominance of the mesenchymal cell phenotype with increased capacity of tissue invasion and distant metastasis. Such a biological behaviour suggests that miR-200c-3p could be an interesting biomarker of prognosis in melanoma patients. Finally, miR-200c have been reported to be under-expressed in melanoma tissues collected from patients considered non-responders to BRAF-inhibitors compared to responders (17), suggesting a possible involvement of miR-200c in drug resistance phenotype.

Compared to Stark et al. (2015), we were able to identify the signature of three different miRNAs with comparable diagnostic sensitivity and specificity to the MELmiR-7. Moreover, although limited by the small sample size, our findings are to be considered reliable since we utilised two different normalization methods compared to the one chosen by Stark et colleagues.

In conclusion, our pilot study identified five circulating miRNAs, of which three detected for the first time in plasma of melanoma patients. MiR-149-3p, miR-150-5p and miR-193a-3p either alone or as a signature are suitable to be considered as potential diagnostic biomarkers in human melanoma and worthy to be validated in further prospective clinical trials.

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Tables

Table I. Clinical characteristics of study subjects.

Variable	n, %
Sex	
Male	56.6
Female	43.3
Stage	
I-II	46.6
III-IV	53.3
M staging	
1a	45.5
1b	27.2
1c	27.2
Breslow thickness	
Stage I-II	
<0,75 mm	50.0
>0,75 mm	50.0
Stage III-IV	
<0,75 mm	18.1
>0,75 mm	82.8
Clark level	
Stage I-II	
I-II-III	49.4
IV-V	50.6
Stage III-IV	
I-II-III	90.2
IV-V	9.8
Ulceration	
Present	36.7
Absent	63.3
Growth phase	
Radial	81.5

Vertical	19.5
LDH levels	
Normal	100.0
Elevated	0.0
Patient condition	
Alive without disease	83.3
Alive with disease	8.3
Dead	8.3

Table II. MiRNAs found differentially expressed in melanoma patients compared to healthy subjects.

GMN			NormFinder		
miRNA	*p*-value	Gene regulation	miRNA	*p*-value	Gene regulation
miR-149-3p	$1.3×10^{-8}$	↑	miR-150-5p	$2.8×10^{-12}$	↑
miR-193a-3p	$1.3×10^{-6}$	↓	miR-149-3p	$3.4×10^{-12}$	↑
miR-150-5p	$1×10^{-5}$	↑	miR-193a-3p	$1.3×10^{-6}$	↓
miR-524-5p	$1×10^{-5}$	↓	miR-15b-5p	$1.3×10^{-5}$	↑
miR-200c-3p	$1×10^{-4}$	↓	miR-524-5p	$2.4×10^{-5}$	↓
miR-15b-5p	$2.3×10^{-4}$	↑	miR-200c-3p	$9.5×10^{-3}$	↓
miR-425	$9.5×10^{-4}$	↓	miR-145-5p	$3.1×10^{-2}$	↑
let-7-5p	$2.2×10^{-3}$	↓			
miR-1250-5p	$6.1×10^{-3}$	↓			
miR-4270	$6.4×10^{-3}$	↓			
miR-338-3p	$1.2×10^{-2}$	↓			
miR-9-5p	$2.3×10^{-2}$	↓			
miR-106-5p	$4.4×10^{-2}$	↓			

Data were normalized to mean expression value of all expressed microRNAs and with NormFinder. Significant *p*-values (<0.05) were obtained by Wilcoxon test. GMN: Global Mean Normalization. ↑ :up-regulated; ↓ :down-regulated

Table III. The area under the ROC curve (AUC) for single miRNAs and for triple classifier with corresponding sensitivity and sensibility (level of significance, p< 0.0001)

miRNA	AUC	95% C. I.	Sensitivity (%)	Sensibility (%)
miR-149-3p	0.95	0.86-0.98	93.3	87.5
miR-150-5p	0.94	0.86-0.97	96.7	70.0
miR-193a-3p	0.84	0.69-0.91	76.7	62.5
miR-15b-5p	0.80	0.68-0.92	90.0	77.4
miR-524-5p	0.80	0.68-0.92	90.0	68.8
Triple classifier	0.97	0.91-0.981	94.8	83.9

AUC: area under the curve; Triple classifier: miR-149-3p + miR-150-5p + miR-193a-3p

Figure legends

Figure 1. Expression levels of the five selected miRNAs in plasma samples from melanoma patients (MP) and healthy subjects (C). p-value is reported in Table II.

Figure 2. Expression levels of miR-200c-3p in plasma samples from melanoma patients at different stages and healthy subjects (C). eMP: early melanoma patients (stage I-II); aMP: advanced melanoma patients (stage III-IV). **$p < 0.01$.