Study of Circulating MicroRNA-125b Levels in Serum Exosomes in Advanced Melanoma

Estibaliz Alegre, PhD; Miguel F. Sanmamed, PhD; Carmen Rodriguez, BSc; Omar Carranza, MD; Salvador Martín-Algarra, PhD; Álvaro González, PhD

• ***Context.***—**Malignant melanoma is an aggressive tumor that produces exosomes, which contain microRNAs (miRNAs) that could be of utility in following tumoral cell dysregulation. MicroR-125b is a miRNA whose down-regulation seems to be implicated in melanoma progression.**

Objective.—**To analyze miR-125b levels in serum, and in exosomes obtained from serum, from patients with advanced melanoma.**

Design.—**Serum samples were obtained from 21 patients with advanced melanoma, from 16 disease-free patients with melanoma, and from 19 healthy volunteers. Exosomes were isolated from serum by precipitation, and miR-16 and miR-125b levels were quantified by real-time polymerase chain reaction.**

Results.—**MicroR-16, but not miR-125b, was detected in all samples, and miR-16 levels were significantly higher in serum than they were in exosomes. MicroR-16 expression levels did not differ significantly between the 2 groups (patients with melanoma and healthy donors). There was a significant relationship between miR-125b and miR-16 levels in exosomes. Additionally, miR-125b levels in exosomes were significantly lower in patients with melanoma compared with disease-free patients with melanoma and healthy controls.**

Conclusions.—**Exosomes can provide a suitable material to measure circulating miRNA in melanoma, and miR-16 can be used as an endogenous normalizer. Lower levels of miR-125b in exosomes obtained from serum are associated with advanced melanoma disease, probably reflecting the tumoral cell dysregulation.**

(*Arch Pathol Lab Med.* 2014;138:828–832; doi: 10.5858/arpa.2013-0134-OA)

Advanced melanoma is among the most aggressive and treatment-resistant human cancers. For decades, chemotherapy was the backbone of systemic treatment in cases of advanced melanoma with poor outcomes.[1] When detected in early stages, melanoma has a good prognosis, but when the diagnosis occurs after the tumor has already metastasized, the survival rate is poor.[1,2] Recently, new therapeutic treatments have greatly improved patient survival.[1,3]

Melanoma cells produce exosomes, which are microvesicles derived from the cellular endosomal membrane and secreted into surrounding media. Tumor-derived exosomes are abundant in body fluids from patients with cancer,[4] and they seem to be important in the development of metastases.[5] In addition, exosome analysis may provide a dynamic approach for detecting cellular changes during tumor progression.[6]

MicroRNAs (miRNAs) are small, noncoding RNAs of about 20 to 24 nucleotides that can interact with seed sequences in messenger RNAs (mRNAs), down-regulating their translation or even inducing mRNA degradation.[7] MicroRNAs are important gene-expression regulators, and more than one-third of the cellular transcriptome may be under miRNA control.[8] MicroRNA can be dysregulated in tumors, compared with normal tissues,[9] and there is a different miRNA expression profile associated with melanoma progression.[10] For example, miR-125b has been found to be down-regulated in melanoma biopsies, compared with normal melanocytes.[11,12] That dysregulation could be implicated in tumor spreading[11–13] because miR-125b controls melanoma progression by direct suppression of c-Jun protein expression.[11]

Cells can release miRNA to the medium and are detected in several body fluids, including blood,[14,15] saliva,[15] and effusions,[16] where they are stable and resistant to degradation by RNAases.[14] The pattern of expression or serum levels of different miRNAs have been found to be altered in cancer,[14,17] and the miRNAs could be potential, noninvasive biomarkers that reflect disease status and progression.[18]

Circulating miRNAs are mainly included in exosomes,[15] although there are other carriers, such as Argonaute2 protein[19] or the high-density lipoproteins.[20] Because melanoma-derived exosomes have characteristic miRNAs and proteomic profiles compared with exosomes from normal

Accepted for publication July 12, 2013.

From the Laboratory of Biochemistry (Drs Alegre and González and Ms Rodriguez) and the Department of Medical Oncology (Drs Sanmamed and Martín-Algarra and Dr Carranza), University Clinic of Navarra, Pamplona, Navarra, Spain.

The authors have no relevant financial interest in the products or companies described in this article.

All authors concur with the submission and the funding for the study ("Fondo de Investigación Sanitaria" grant PI11/02119). The Ethical Committee approval was received for the studies with human samples.

Reprints: Álvaro González, PhD, Laboratory of Biochemistry, University Clinic of Navarra, Avd Pio XII 36, Pamplona 31008, Spain (e-mail: agonzaleh@unav.es).

melanocytes,[21] the miRNA levels in these vesicles could be a useful biomarker for these tumors.

Although exosomes obtained from serum (srm-exosomes) seem to be suitable specimens for measuring circulating miRNA from patients with melanoma, most studies addressing the measurement of miRNA circulating levels are performed on total serum.[22,23] To our knowledge, no studies have addressed the differences between the miRNA analyses using total serum and those using extracted srm-exosomes. For that reason, the aim of this study was to compare miRNA levels obtained from serum and those obtained from srm-exosomes in patients with melanoma. We analyzed miR-125b, a miRNA whose circulating levels have been analyzed in several types of cancer,[24,25] but not in melanoma, and miR-16, a miRNA frequently used as an internal normalizer in blood.[26–29]

MATERIALS AND METHODS

Peripheral blood samples were obtained (after informed consent was received) from 21 patients with advanced melanoma (mean age, 53 years; males, 52% [11 of 21]; patients with metastases, 71% [15 of 21]), from 16 disease-free patients with melanoma (mean age, SD = 14, 54 years; males, 50% [8 of 16]), and from 19 age- and sex-matched healthy volunteers used as a control group. Blood samples were centrifuged, and serum was isolated and kept at –80°C until analysis. The study was approved by the University Clinic of Navarra (Pamplona, Navarra, Spain) Ethical Committee.

Exosome Isolation

The srm-exosomes were obtained from 200 μL of serum with ExoQuick precipitation solution (System Biosciences, Mountain View, California) according to manufacturer's instructions. Exosome concentration was estimated indirectly through protein concentration measured with a Nanodrop spectrophotometer (Thermo Scientifics, Wilmington, Delaware).

miRNA Extraction and Quantification

MicroRNA expression analysis was performed in serum samples (200 μL) and in exosome samples previously obtained from 200 μL of serum. In both types of samples, we analyzed the expression of miRNA-16 and miRNA-125b, along with the expression of miR-54 from *Caenorhabditis elegans* (cel–miR-54), an exogenous miRNA that was spiked in all samples to be used as an internal standard. For that, 25 fmol of this oligonucleotide, purchased from Sigma (St. Louis, Missouri), was added to each serum or srm-exosome sample before total RNA extraction with TRIzol (guanidinium thiocyanate-phenol-chloroform extraction, Invitrogen, Carlsbad, California). Extracted RNA was resuspended with 50 μL of diethylpyrocarbonate-treated water (H_2O-DEPC), and the concentration was measured by spectrophotometry with a Nanodrop (Thermo Scientifics). Each miRNA was then specifically retrotranscribed from 1.67 μL of resuspended total RNA using the TaqMan MicroRNA Reverse Transcription kit (Applied Biosystems, Foster City, California) and specific retrotranscription primers for each miRNA from the TaqMan microRNA assays kit (Applied Biosystems).[30] The complementary DNA obtained was then preamplificated by polymerase chain reaction (PCR) with TaqMan Preamp Master Mix (Applied Biosystems) before amplification with real-time PCR and TaqMan Universal PCR Master Mix No AmpErase UNG (Applied Biosystems). Both preamplification and real-time PCR were performed with specific amplification primers for each miRNA, also contained in TaqMan microRNA assays kit. Real-time PCR was performed in triplicate for each sample and each miRNA.

Statistical Analysis

Raw amplification data of real-time PCR were expressed as C_T (threshold cycle) and processed as follows: Mean C_T was calculated from triplicates of each sample and each miRNA amplification (C_T-miR). Normalization factor for each sample was calculated as described by Kroh et al[30]: The median of the normalizer C_T values (C_T–cel–miR-54 or C_T–miR-16) from all samples was obtained and subtracted to respective normalizer of each sample. To calculate the normalized C_T (NC_T-miR), the normalization factor was subtracted from each C_T-miR in each sample.

Statistical analysis of C_T and NC_T was performed using nonparametric tests with SPSS (IBM, Armonk, New York) program Version 15.0 for Windows. MicroRNA levels were compared between patients with melanoma and healthy control patients using the Mann-Whitney *U* test, and comparison between miRNA levels in serum samples and srm-exosome samples from the same patient was performed with the Wilcoxon test. Correlation analysis was performed using the Spearman test. A $P < .05$ was considered significant.

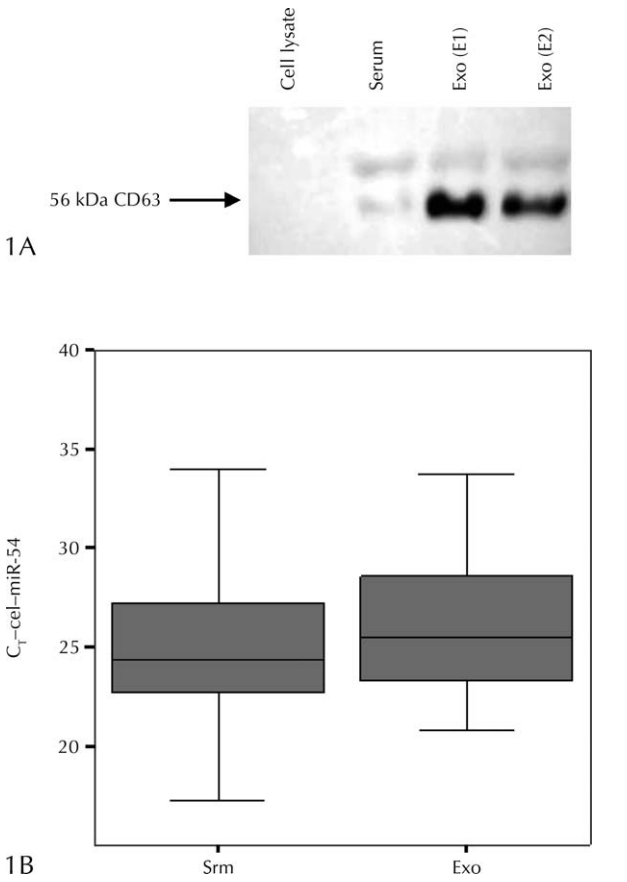

Figure 1. *A, Representative example of the immunoblot for CD63 detection in exosomes obtained by precipitation from serum samples (E1 and E2). Negative controls were a lysate of melanoma M8 cell line and a serum sample. Equal quantities of protein were loaded in each lane. B, Threshold cycle (C_T) values for spiked microRNA-54 from* Caenorhabditis elegans *(cel–miR-54) in serum (Srm) and in exosomes obtained from serum (Exo) samples. Statistical analysis was done using the Wilcoxon test.*

RESULTS

Isolation of Exosomes and miRNA Analysis in Serum and Exosomes

Exosome purification by precipitation using ExoQuick was confirmed by Western blot using anti-CD63, which is considered a marker of exosomes and, as expected, we observed the predicted band at 56 kDa (Figure 1, A). First, we checked whether the protocol for exosome isolation could interfere with the RNA extraction and miRNA

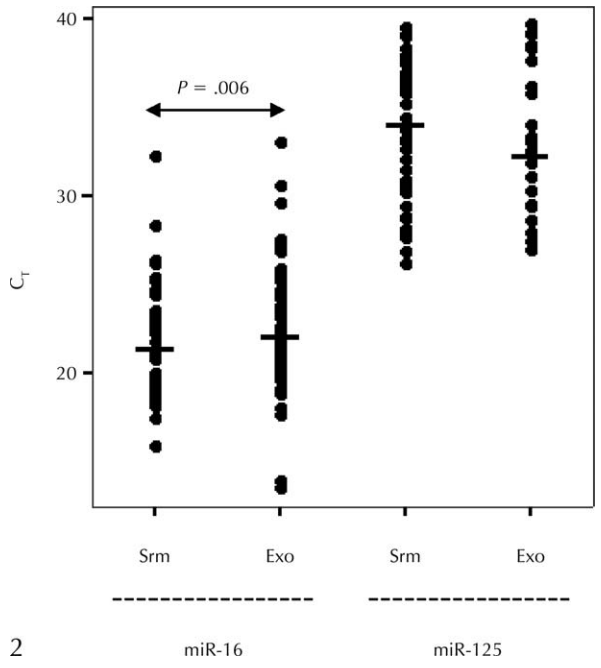

Figure 2. *Threshold cycle (C_T) values for circulating microRNA-16 (miR-16) and miR-125b in serum (Srm) and in exosomes obtained from serum (Exo) samples. Lines indicate medians. Statistical analysis was done using the Wilcoxon test.*

quantification. To achieve that, we compared the amplification of cel–miR-54 spiked in the same quantities to the serum and to the isolated srm-exosomes. We did not observe significant differences in C_T–cel–miR-54 between serum and srm-exosomes ($P = .07$; Figure 1, B), which indicated that the exosome isolation by this method, using a commercial precipitation reagent, did not affect the latter miRNA extraction and quantification.

Analysis of Circulating miR-16 in Serum and Exosomes

Initially, we analyzed the presence of circulating miR-16 in srm-exosomes and serum. MicroR-16 was observed in all serum and srm-exosome samples, with a significant relationship in miR-16 levels between srm-exosomes and serum ($r = 0.612$; $P < .001$). However, C_T–miR-16 values were significantly lower in serum than they were in srm-exosomes (serum median C_T–miR-16, 21.3; interquartile range [IQR], 19.2–23.3; srm-exosomes median C_T–miR-16, 22; IQR, 20.7–25.2; $P = .006$) (Figure 2). Additionally, miR-16 expression levels both in serum and in srm-exosomes from patients with advanced melanoma were very similar to those observed in serum or srm-exosomes from disease-free patients with melanoma and healthy donors (Table). Furthermore, there was no statistical difference in miR-16 levels between controls and disease-free patients with melanoma.

Some authors have added cel–miR-54 as an external normalizer to correct for intra-assay and interassay differences in miRNA extraction efficiency.[14,19] To confirm that our results were not due to technical biases, we normalized miR-16 with spiked cel–miR-54. We confirmed the significant relationship between $N_{54}C_T$–miR-16 values in srm-exosomes and in serum ($r = 0.543$; $P < .001$) and that $N_{54}C_T$–miR-16 was significantly lower in serum than it was in srm-exosome ($P = .01$). In addition, there were no

Levels (Median and Interquartile Range [IQR]) of miR-125b and miR-16 in Serum and Exosomes Obtained From Serum (Srm-Exosomes) in Healthy Control Patients (n = 19), Disease-Free Patients With Melanoma (n = 16), and Patients With Advanced Melanoma (n = 21)

Patients	Serum, Median (IQR)	Srm-Exosomes, Median (IQR)
C_T–miR-16		
Control	22.4 (19.2–23.9)	21.2 (20.2–25.3)
Melanoma		
Disease free	19.9 (18.5–23.0)	21.7 (20.2–25.5)
Advanced	21.1 (19.4–23.1)	22.3 (20.9–24.6)
N_{54}–C_T–miR-16		
Control	22.4 (19.5–24.9)	23.1 (21.7–24.6)
Melanoma		
Disease free	21.9 (20.8–23.2)	23.7 (22.7–24.5)
Advanced	22.0 (20.3–24.5)	23.1 (21.5–24.0)
C_T–miR-125b		
Control	32.8 (28.7–35.7)	30.2 (29.0–31.6)
Melanoma		
Disease free	33.3 (29.3–36.8)	32.1 (31.0–35.7)
Advanced	35.2 (32.8–37.2)	37.6 (31.3–39.1)[a]
N_{16}–C_T–miR-125		
Control	34.0 (30.8–37.1)	32.5 (28.1–33.6)
Melanoma		
Disease free	32.9 (29.9–34.3)	32.6 (30.3–33.2)
Advanced	35.0 (33.3–35.9)	35.4 (33.2–36.3)[b,c]

[a] $P = .04$ compared with control group.
[b] $P = .01$ compared with control group.
[c] $P = .02$ compared with disease-free patients with melanoma.

statistical differences in $N_{54}C_T$–miR-16 values between control patients and patients with melanoma (Table).

These data implied that miR-16 can be used as an endogenous normalizer, although the normalization with miR-16 is different for serum and srm-exosomes and are not interchangeable.

Analysis of Circulating miR-125b in Serum and Exosomes

MicroR-125b was detected in 85.4% of the samples. To investigate whether the expression levels depended on the type of sample analyzed, we compared the level of miR-125b expression in serum and exosomes. MicroR-125b was less-frequently observed in srm-exosomes (56% of samples) than it was in total serum (75% of samples). However, when expressed, there was no difference in the expression levels between serum and srm-exosomes from the same patient (serum median C_T–miR-125b, 34.0; IQR, 30.2–36.9; srm-exosomes median C_T–miR-125b: 32.2; IQR, 29.6–37.2; $P = .34$; Figure 2). In addition, there was a relationship between the miR-125b and miR-16 levels in srm-exosomes ($r = 0.45$; $P = .02$), but not in serum ($P = .97$).

We observed that the levels of miR-125b in srm-exosomes were significantly lower in patients with advanced melanoma than they were in healthy controls ($P = .04$) (Table; Figure 3, A). Furthermore, after normalizing with miR-16, srm-exosome miR-125b levels were significantly lower in patients with advanced melanoma than they were in melanoma-free patients ($P = .02$) and controls ($P = .01$) (Figure 3, B). On the other hand, there was no statistical difference in the miR-125b levels between patients and controls when analyzing serum samples ($P = .78$).

We can conclude that the level of expression of miR-125b in srm-exosomes from patients with melanoma was lower

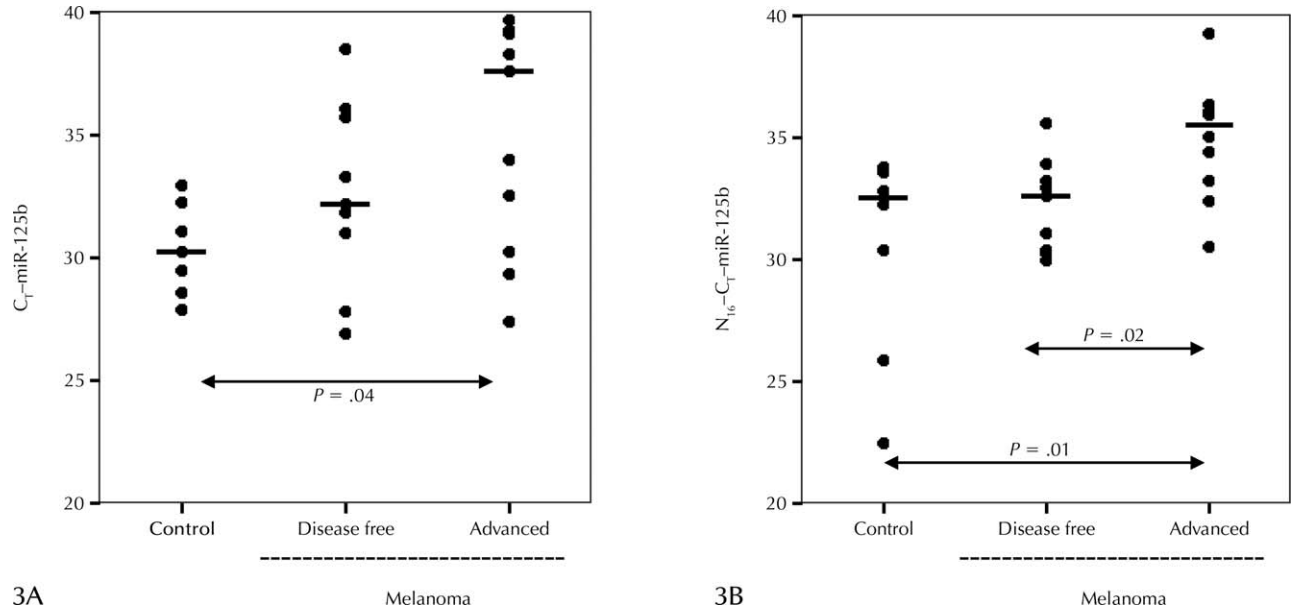

Figure 3. *Threshold cycle (C_T) values for circulating microRNA-125b (miRNA-125b) (A) nonnormalized and (B) normalized with miR-16 in srm-exosome samples taken from healthy controls, disease-free patients with melanoma, and patients with advanced melanoma. Lines indicate medians. Statistical analysis was done using the Mann-Whitney* U *test.*

than that from disease-free patients with melanoma and healthy controls.

COMMENT

Exosomes are actively secreted by tumoral cells, and particularly, melanoma cells seem to produce a great quantity of these microvesicles,[21] which are known to transport different miRNAs.[15] Plasma can also contain miRNA, derived from platelets,[27] and cell disruption during clotting can release miRNA as well.[27] In fact, erythrocyte disruption can be a source of miR-16,[27] and although we have not detected visible hemolysis in any of our samples, other sources of miR-16 can be present in serum, apart to exosomes. For example, Argonaut2 is an important carrier for miR-16.[19] Both miR-125b and miR-16 are released in exosomes,[21] but the different sources and carriers of released miRNAs could be the reason for the different levels of miR-16 and miR-125b expression observed in serum and srm-exosomes.[19] The method of exosome isolation did not interfere with the miRNA analysis,[31] as we have shown with cel–miR-54 analysis. Also, the positive relationship between mR-125b and miR-16 in srm-exosomes, but not in serum, points to a similar source for the miRNA released in these microvesicles.

The optimal internal standard should not only correct technical differences in the analysis but also neutralize possible preanalytic variations. Although cel–miR-54 can correct the technical differences,[14,19,27] it cannot solve other variability sources, such as different exosome release, which is shown to be increased in blood from patients with advanced stages of cancer.

A prerequisite for using a miRNA as endogen normalizer is that it be ubiquitously expressed and at stable levels. The selection of an internal normalizer is a very challenging issue when analyzing circulating miRNA. Probably the most widely used endogenous normalizer is miR-16 because it is quite stable,[14,26,27] produced ubiquitously, and present in blood.[28] Apart from miR-16, some other miRNAs have been proposed. Small, nuclear RNA U6 has also been used as a normalizer in cells,[32] but the expression in serum is very low or even absent, so its use is limited to certain types of samples.[26,32] We have detected miR-16 expression in all srm-exosomes and serum samples without differences between levels in patients and in controls. Considering the differences in the type of specimen, miR-16 could be used separately as an internal standard in the analysis of miRNA in either serum or srm-exosomes from patients with melanoma.

Other authors have studied miR-125b in serum from patients with other cancers,[24,25] but this is the first study, to our knowledge, to address the analysis of this miRNA in serum from patients with melanoma, with a special focus in srm-exosomes. We have observed that the levels of miR-125b were lower in exosomes, but not in whole serum, in patients with advanced melanoma compared with melanoma free patients and controls. The differences described here could be because we measured the expression in srm-exosomes, avoiding other potential sources of circulating miRNAs, such as cellular disruption. Recent reports suggest that miRNA melanoma cell-derived exosomes have a close relationship with their originating melanoma cells,[21] and particularly, miR-125b is one of the miRNA more dysregulated in exosomes from the melanoma compared with normal melanocyte cell lines.[21] Although we did not observe miR-125b expression in exosomes from all patients studied, our data suggest that its measurement in exosomes could be useful in analyzing the miR-125b down-regulation in melanoma. Also, and of importance, we have shown that exosomes could provide different information related to circulating miRNA than that provided by total serum analysis, and those data would probably be more accurate.[10,14] Certainly, circulating miRNAs in exosomes could represent the biology within tumor, although further studies should be performed to develop that hypothesis.

Our results agree with previous reports indicating that there is a down-regulation of miR-125b in metastatic

melanoma.[11,13] This miRNA has also been shown to be down-regulated in ovarian and thyroid cancers.[33,34] However, elevated serum levels of miR-125b has been observed in patients with non–small cell lung cancer associated with poorer prognosis,[24] and in patients with breast cancer associated with chemotherapeutic resistance.[25] Those data are not conflicting with ours because miR-125b targets many different genes, such as *E2F3* in breast cancer cells.[25]

MiR-125b inhibition in melanoma cells decreases apoptosis[11,35] by a mechanism that involves a posttranscriptional regulation of the tumor promoter c-jun gene.[11] The decrease of miR-125b observed in melanoma can augment c-jun mRNA translation and affect indirectly target genes, particularly causing the repression of tumor suppressor gene expression and function and the induction of cyclin D1 transcription.[11]

CONCLUSIONS

In summary, we suggest that srm-exosomes are a more-accurate material than serum for measuring circulating miRNA levels in melanoma and that the method of exosome isolation used here does not affect miRNA analysis. MicroR-16 can be used as an endogenous normalizer provided it can be quantified in all samples analyzed and does not change with the disease. In addition, miR-125b levels can help discriminate between patients with advanced melanoma and disease-free patients with melanoma or healthy controls. Probably, the miR-125b lower levels in advanced disease reflect its down-regulation in melanoma cells, favoring tumoral spreading.

We thank Beatriz Mateos for her technical assistance.

References

1. Gray-Schopfer V, Wellbrock C, Marais R. Melanoma biology and new targeted therapy. *Nature*. 2007;445(7130):851–857.

2. Dummer R, Hauschild A, Guggenheim M, Jost L, Pentheroudakis G; ESMO Guidelines Working Group. Melanoma: ESMO clinical practice guidelines for diagnosis, treatment and follow-up. *Ann Oncol*. 2010;21(suppl 5):v194–v197.

3. Hodi FS, O'Day SJ, McDermott DF, et al. Improved survival with ipilimumab in patients with metastatic melanoma. *N Engl J Med*. 2010;363(8):711–723.

4. Valenti R, Huber V, Iero M, Filipazzi P, Parmiani G, Rivoltini L. Tumor-released microvesicles as vehicles of immunosuppression. *Cancer Res*. 2007;67(7):2912–2915.

5. Peinado H, Aleckovic M, Lavotshkin S, et al. Melanoma exosomes educate bone marrow progenitor cells toward a pro-metastatic phenotype through MET. *Nat Med*. 2012;18(6):883–891.

6. Logozzi M, De Milito A, Lugini L, et al. High levels of exosomes expressing CD63 and caveolin-1 in plasma of melanoma patients. *PLoS One*. 2009;4(4):e5219.

7. He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation. *Nat Rev Genet*. 2004;5(7):522–531.

8. Bartel DP. MicroRNAs: target recognition and regulatory functions. *Cell*. 2009;136(2):215–233.

9. Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. *Nature*. 2005;435(7043):834–838.

10. Mueller D, Rehli M, Bosserhoff A. miRNA expression profiling in melanocytes and melanoma cell lines reveals miRNAs associated with formation and progression of malignant melanoma. *J Invest Dermatol*. 2009;129(7):1740–1751.

11. Kappelmann M, Kuphal S, Meister G, Vardimon L, Bosserhoff A-K. MicroRNA miR-125b controls melanoma progression by direct regulation of c-Jun protein expression. *Oncogene*. 2013;32(24):2984–2991.

12. Chan E, Patel R, Nallur S, et al. MicroRNA signatures differentiate melanoma subtypes. *Cell Cycle*. 2011;10(11):1845–1852.

13. Glud M, Rossing M, Hother C, et al. Downregulation of miR-125b in metastatic cutaneous malignant melanoma. *Melanoma Res*. 2010;20(6):479–484.

14. Mitchell PS, Parkin RK, Kroh EM, et al. Circulating microRNAs as stable blood-based markers for cancer detection. *Proc Natl Acad Sci U S A*. 2008;105(30):10513–10518.

15. Gallo A, Tandon M, Alevizos I, Illei GG. The majority of microRNAs detectable in serum and saliva is concentrated in exosomes. *PLoS One*. 2012;7(3):e30679. doi:10.1371/journal.pone.0030679.

16. Xie L, Chen X, Wang L, et al. Cell-free miRNAs may indicate diagnosis and docetaxel sensitivity of tumor cells in malignant effusions. *BMC Cancer*. 2010;10(1):591.

17. Lodes MJ, Caraballo M, Suciu D, Munro S, Kumar A, Anderson B. Detection of Cancer with Serum miRNAs on an Oligonucleotide Microarray. *PLoS One*. 2009;4(7):e6229. doi: 10.1371/journal.pone.0006229.

18. Brase JC, Johannes M, Schlomm T, et al. Circulating miRNAs are correlated with tumor progression in prostate cancer. *Int J Cancer*. 2011;128(3):608–616.

19. Arroyo JD, Chevillet JR, Kroh EM, et al. Argonaute2 complexes carry a population of circulating microRNAs independent of vesicles in human plasma. *Proc Natl Acad Sci U S A*. 2011;108(12):5003–5008.

20. Vickers KC, Palmisano BT, Shoucri BM, Shamburek RD, Remaley AT. MicroRNAs are transported in plasma and delivered to recipient cells by high-density lipoproteins. *Nat Cell Biol*. 2011;13(4):423–433.

21. Xiao D, Ohlendorf J, Chen Y, et al. Identifying mRNA, MicroRNA and protein profiles of melanoma exosomes. *PLoS One*. 2012;7(10):e46874. doi: 10.1371/journal.pone.0046874.

22. Kanemaru H, Fukushima S, Yamashita J, et al. The circulating microRNA-221 level in patients with malignant melanoma as a new tumor marker. *J Dermatol Sci*. 2011;61(3):187–193.

23. Friedman EB, Shang S, de Miera EV, et al. Serum microRNAs as biomarkers for recurrence in melanoma. *J Transl Med*. 2012;10:155.

24. Yuxia M, Zhennan T, Wei Z. Circulating miR-125b is a novel biomarker for screening non-small-cell lung cancer and predicts poor prognosis. *J Cancer Res Clin Oncol*. 2012;138(12):2045–2050.

25. Wang H, Tan G, Dong L, et al. Circulating MiR-125b as a marker predicting chemoresistance in breast cancer. *PLoS One*. 2012;7(4):e34210. doi: 10.1371/journal.pone.0034210.

26. Song J, Bai Z, Han W, et al. Identification of Suitable Reference Genes for qPCR Analysis of serum microRNA in gastric cancer patients. *Dig Dis Sci*. 2012;57(4):897–904.

27. McDonald JS, Milosevic D, Reddi HV, Grebe SK, Algeciras-Schimnich A. Analysis of circulating microRNA: preanalytical and analytical challenges. *Clin Chem*. 2011;57(6):833–840.

28. Chim SSC, Shing TKF, Hung ECW, et al. Detection and characterization of placental microRNAs in maternal plasma. *Clin Chem*. 2008;54(3):482–490.

29. Miyachi M, Tsuchiya K, Yoshida H, et al. Circulating muscle-specific microRNA, miR-206, as a potential diagnostic marker for rhabdomyosarcoma. *Biochem Biophys Res Commun*. 2010;400(1):89–93.

30. Kroh EM, Parkin RK, Mitchell PS, Tewari M. Analysis of circulating microRNA biomarkers in plasma and serum using quantitative reverse transcription-PCR (qRT-PCR). *Methods*. 2010;50(4):298–301.

31. Taylor DD, Zacharias W, Gercel-Taylor C. Exosome isolation for proteomic analyses and RNA profiling. *Methods Mol Biol*. 2011;728:235–246.

32. Spinetti G, Fortunato O, Caporali A, et al. MicroRNA-15a and MicroRNA-16 impair human circulating proangiogenic cell functions and are increased in the proangiogenic cells and serum of patients with critical limb ischemia. *Circ Res*. 2013;112(2):335–346.

33. Nam EJ, Yoon H, Kim SW, et al. MicroRNA expression profiles in serous ovarian carcinoma. *Clin Cancer Res*. 2008;14(9):2690–2695.

34. Visone R, Pallante P, Vecchione A, et al. Specific microRNAs are downregulated in human thyroid anaplastic carcinomas. *Oncogene*. 2007;26(54):7590–7595.

35. Glud M, Manfé V, Biskup E, et al. MicroRNA miR-125b induces senescence in human melanoma cells. *Melanoma Res*. 2011;21(3):253–256.