http://informahealthcare.com/bmk
ISSN: 1354-750X (print), 1366-5804 (electronic)

Biomarkers

Biomarkers, 2013; 18(6): 502–508

DOI: 10.3109/1354750X.2013.816777

informa healthcare

RESEARCH ARTICLE

A comparative analysis of total serum miRNA profiles identifies novel signature that is highly indicative of metastatic melanoma: a pilot study

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Abstract

Context: Quantification of circulating microRNAs (miRNAs) has recently become feasible and reliable, with most efforts focusing on miRNAs overexpressed by cancer cells.
Objective: Identification of a characteristic circulating miRNAs profile in melanoma patients.
Methods: We conducted a pilot study comprised of unbiased qPCR comparison of serum miRNA profiles between metastatic melanoma patients and healthy donors.
Results: Loss of two normal serum-miRNAs, miR-29c and miR-324-3p, is highly indicative of metastatic melanoma. Hierarchical clustering analysis supported the results and clearly distinguished melanoma patients from healthy donors, metastatic colon and renal cancer patients.
Discussion and conclusions: This approach is independent of tumor heterogeneity and is expected to have superior biomarker performances.

Keywords

Biomarkers, circulating microRNAs, diagnostic, melanoma, serum

History

Received 7 May 2013
Revised 11 June 2013
Accepted 14 June 2013
Published online 29 July 2013

Introduction

Melanoma is a cancer that develops from melanocytes located predominantly in the skin, but also found in the eyes, ears, gastrointestinal tract, and oral and genital mucous membranes (Cummins et al., 2006). While melanoma accounts for nearly 4% of all skin cancers, it causes 75% of skin cancer-related deaths worldwide and is considered to be the most common fatal malignancy of young adults (Gloster & Brodland, 1996). The incidence of melanoma has almost tripled over the last 30 years and around 130 000 new cases of melanoma are diagnosed each year in the Western world (Howlader et al., 2011). The prognosis of patients with melanoma is strongly determined by early detection, as thin local tumors are highly curable by surgical resection, while metastatic disease carry an ominous prognosis of 14% for 5 years (Miller & Mihm, 2006) necessitating early diagnosis and better diagnostic tools.

Serum tests are widely used in diagnostic medicine, mainly due to simple accessibility and preservation of serum samples. Accordingly, the use of serum tumor markers has been integrated in clinical management of some malignant diseases, such as PSA in prostate cancer (Lee et al., 1994). Serum markers for melanoma have been described before, such as LDH and S100 (Banfalvi et al., 2002; Deichmann et al., 1999, 2004). However, serum markers of melanoma lack specificity and in many cases they remain within the normal limit, especially in early stages, which precludes their use for early detection of malignant melanoma (Cohen et al., 1998; Ikuta et al., 2005; Molina et al., 2002; Mouawad et al., 2010). Therefore, the development of novel biomarkers for this tumor is urgently needed.

MicroRNAs (miRNAs) are small, non-coding, 19–22 nucleotide long RNA molecules, which function as specific post-transcriptional regulators of gene expression by inhibiting protein translation, leading targeted mRNA to degradation, or both (Bartel, 2009; Ma & Weinberg, 2008). miRNAs are involved in the regulation of many biological processes, such as embryonic development, cell differentiation, cell cycle, apoptosis and angiogenesis (reviewed in (Garzon et al., 2009)). They are also directly implicated in cancer development, progression and metastasis *in-vitro*, *in-vivo* and reported even in patients (Calin & Croce, 2006; Ma & Weinberg, 2008). Melanoma development is controlled by a variety of miRNAs (Bar-Eli, 2011), as well as regulation of the malignant phenotype (Greenberg et al., 2011a). We have recently postulated that UV-induced mutations in the 3′UTR of genes cause a unique global reduction in gene regulation by miRNAs (Greenberg et al., 2011b).

Recent studies have shown the capability of miRNAs expression profiles to clearly distinguish between normal and

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neoplastic tissues (Calin & Croce, 2006). These results led to the common approach of searching in the serum for miRNAs that were up-regulated in the tumors. The presence of miRNAs circulating in a cell-free form was recently demonstrated in the blood (Chim et al., 2008; Hunter et al., 2008; Mitchell et al., 2008), most probably in exosomes which protect them from degradation by RNase (Chim et al., 2008; Mitchell et al., 2008). MicroRNA signatures in blood are similar in men and women, as well as individuals of different age (Hunter et al., 2008). Thus, altered circulating miRNAs patterns could potentially become novel biomarkers for cancer. Indeed, some of these tumor up-regulated derived miRNAs have been demonstrated to reach the serum and serve as diagnostic and/or prognostic markers (Wulfken et al., 2011). However, this approach could be limited by size of the tumor, its location, differently expressed miRNAs and it does not take into account the host response or the ability to look at circulating miRNAs that are lost.

Here we conducted a pilot study demonstrating a proof of concept for using the total serum miRNA profile to differentiate malignant melanoma patients from healthy controls, with no restrictions to tumor-derived miRNAs. Moreover, this strategy allowed us to find a specific signature that differentiates melanoma patients from healthy controls as well as from colon and renal cancers.

Materials and methods

Serum samples

Sera samples were obtained from healthy controls, metastatic melanoma, renal and colon cancer patients (IRB approval #8920-11 and IMoH #3518/2004) for this study. All serum samples from patients were obtained prior to initiation of treatments. Serum samples were aliquoted and preserved in deep freezing. The serum samples of the colon and renal cancer patients were obtained from the Sheba Tissue Banks.

Study design

This pilot study was comprised of three steps: (1) Retrospective complete screen of miRNAs comparing four young healthy male controls (ages 30–36) and 7 age- and gender-matched metastatic melanoma patients. This highlighted a shortlist of differentially expressed miRNAs (p value <0.05); (2) Expansion of the population size of both the melanoma patients and the healthy controls to 28 and 10, respectively. This step included samples of both males and females and a broader age range (ages 22–71). Only the differential miRNAs were tested and greater statistical stringency was applied (p value <0.01); (3) Addition of age- and gender-matched metastatic colon (20 patients) and metastatic renal carcinoma patients (23 patients) and increasing the healthy population size to 20.

RNA extraction

RNA was extracted using the miRNeasy mini kit (Qiagen, Valencia, CA), with an exception of adding MS2 carrier RNA (Roche, Basel, Switzerland) to the QIAzol mix following kit adjustments according to Exiqon's protocol for miRNA extraction from serum (Andreasen et al., 2010). RNA quality and concentration were determined using the Bioanalyzer 2100 (Agilent, Santa Clara, CA) and the Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA). RNA samples were stored at −80 °C.

Quantitative PCR

First strand synthesis was executed using Universal cDNA synthesis kit (Exiqon, Vedbaek, Denmark), according to the manufacturer's guidelines. In detail, the RT master mix was prepared, placed on ice and then 9 µl were dispensed into nuclease free tubes. One microliter of template RNA was added to each tube followed by a very gentle vortex or pipetting to ensure that all reagents were thoroughly mixed. After mixing, the mix was spun down and incubated for 60 min at 42 °C in the PCR thermocycler followed by heating inactivation of the reverse transcriptase for 5 min at 95 °C. The reaction was then immediately cooled to 4 °C and stored at −20 °C.

Full panels consisted of 742 miRNAs (Exiqon, Vedbaek, Denmark). Real time PCR mix was prepared by mixing 1:1 the SYBR® Green master mix with the nuclease free water diluted cDNA. For detection of specific miRNAs, the SYBR® Green master mix, Universal RT, 25 ml kit (Exiqon) were used. PCR master mix (5µl/reaction of SYBR® Green master mix and 1 µl/reaction of PCR primer mix) was prepared, placed on ice and then dispensed into the wells. 4 µl of diluted cDNA template was then added to each well. The reaction mix was mixed by gentle pipetting to ensure that all reagents were mixed thoroughly. After mixing, the plate was sealed with optical sealing as recommended by the manufacturer. The plate was spun down in a centrifuge and placed in the LC480 qPCR machine (Roche, Mannheim, Germany) for detection according to the manufacturer guidelines, followed by melting curve analysis at the end of the run.

Primers list

hsa-mir-150 (Exiqon, cat #204660), hsa-mir-451 (Exiqon, cat #204734), hsa-mir-29a (Exiqon, cat #204698), hsa miR 320a (Exiqon, cat #204154), hsa-mir-342-3p (Exiqon, cat #204511), hsa-mir-324-3p (Exiqon, cat #204303), hsa-mir-29c (Exiqon, cat #204729), hsa-mir-503 (Exiqon, cat #204334), hsa-mir-197 (Exiqon, cat #204380), hsa-mir-140-3p (Exiqon, cat #204304), hsa-mir-339-3p (Exiqon, cat #204160), hsa-mir-20a (Exiqon, cat #204052), hsa-mir-374a (Exiqon, cat #204758), hsa-mir-301a (Exiqon, cat #204687).

Data analysis

Data analysis was performed using the Genex pro software (MultiD Analyses AB, Göteborg, Sweden). Cutoff levels for significance were determined as at least 1.5-fold ratio between tested samples and the lowest read of the negative control samples as the limit for expression range. Suitable normalizing gene was found by applying the NormFinder (Andersen et al., 2004) and Genorm (Vandesompele et al., 2002) algorithms on the whole miRanome panel results. Both algorithms found miR-320a as the best normalizer, hence, all genes were normalized with this miRNA of choice. Hierarchical clustering feature was carried out using the dendrogram feature of the Genex software, using the Ward's algorithm and Euclidean method for distance measurements.

Quantitative differential expression of miRNAs between patients and healthy donors was calculated as $2^{-\Delta\Delta Cp}$.

Statistical analysis (Mann–Whitney/*t*-test) and dendrograms were performed using Genex pro software.

Results

Identification of a circulating oligonucleotide signature in metastatic melanoma patients

The initial screen of 754 miRNAs for identification of differentially expressed miRNAs included a group of healthy donors ($n = 4$, males, age 30–36) and a group of Stage IV melanoma patients ($n = 7$, males, age 22–41) (Table 1). We hypothesized that comparing homogenous groups of young adults with no confounding factors such as background illnesses or permanent drug treatments would yield refined patterns. This screen yielded 12 differentially expressed miRNAs (Table 2, p value < 0.05). Normalizing miRNA was chosen by applying the Normfinder and the Genorm algorithms (Andersen et al., 2004; Vandesompele et al., 2002) on the panels' results. Both algorithms agreed on miR-320a as the best choice for normalization of miRNA levels (data not shown).

In order to validate the initial screen results, the differentially expressed miRNAs were further tested by individual qPCR assays in cohorts of 10 healthy donors and 28 stage IV melanoma patients. These groups were heterogeneous and included males and females, as well as an age range up to 71 (Table 1). None of the samples was hemolytic. The statistical significance of two of the circulating oligonucleotides further improved by at least 10-fold: hsa-miR-29c and hsa-miR-324-3p (Table 2). Interestingly, all these oligonucleotides were found in higher amounts in the healthy donors as compared to the melanoma patients: 2.8-fold and 1.7-fold for hsa-miR-29c, and hsa-miR-324-3p, respectively. The differences among 9 of the other miRNAs were found to be totally insignificant and hence did not qualify for further analyses. Although hsa-miR-451 still displayed a statistically significant difference among these cohorts, it was not further tested due to the increment in the p value by more than 10-fold (Table 2).

The identified circulating miRNA signature differentiates melanoma patients from healthy controls and other malignancies

Next, the specificity and ability of the suggested melanoma serum signature (hsa-miR-29c and hsa-miR-324-3p) to distinguish between melanoma and different cancer patients, were tested. For that purpose, the population size of the healthy control group was increased to $n = 20$ and two other groups of metastatic colon and renal carcinoma patients were added ($n = 20$ and $n = 23$, respectively). The circulating oligonucleotides were quantified with qPCR, as described above.

Importantly, both hsa-miR-29c and hsa-miR-324-3p consistently differentiated between melanoma patients and healthy donors (Figure 1). The statistical significance of these differences became even more prominent, reaching p values $<1 \times 10^{-7}$ for both miR-29c and miR-324-3p (Figure 1). Moreover, miR-29c clearly distinguished melanoma patients from renal and colon cancer patients (ΔCp of 6 and 5, respectively; p value $< 1 \times 10^{-8}$), but much less between these two malignancies ((ΔCp of 0.74; p.value $= 0.005$). miR-324-3p distinguished the melanoma patients from the renal cancer patients (p value $= 0.001$) but not from the colon cancer patients (p value $= 0.25$) (Figure 1). Moreover, miR-324-3p did not distinguish between the renal and colon cancer patients (p value $= 0.28$).

Dot plotting of normalized Cp values for both miR-29c and miR-324-3p shows the unique cluster of melanoma patients, which is distinct from healthy donors (Figure 2A) and the other malignancies (Figure 2B). Hierarchical clustering based on normalized Cp values of miR-29c and miR-324-3p presented a clear segregation of the melanoma patients from all other groups (healthy, colon cancer and renal cancer), implying that the melanoma signature is specific (Figure 3). Only two of the 20 healthy controls were clustered with the ''melanoma cluster'' and only two melanoma patients were not clustered with the ''melanoma cluster'' (Figure 3). As expected, the combined measurement of miR-29c and miR-324-3p could not efficiently distinguish between the colon cancer patients and the renal cancer patients (Figure 3). However, it should be noted that the combined measurement of miR-29c and miR-324-3p displayed a good segregation between the renal and the colon cancer patients from the healthy/melanoma patients (Figure 3).

Discussion

The common strategy for the screening of circulating miRNAs is to compare between malignant tissues and their normal counterparts. This comparison allows the identification of up-regulated, cancer derived miRNAs and increases the possibility of finding these miRNAs in the individual's serum as well (Kanemaru et al., 2011; Morimura et al., 2011; Wang & Gu, 2011; Wulfken et al., 2011). Since tumors dynamically interact with their microenvironment (e.g. adjacent tissues and immune system cells) we hypothesized that the presence of malignant cells might differentially alter the circulating miRNAs expression profile in the serum, which may not be confined only to tumor-derived miRNAs, but to host-derived miRNAs as well. For that purpose, we conducted a pilot study, in which unbiased analysis of the entire spectrum of circulating miRNAs of healthy and melanoma patients revealed differential expression and provided the basis for the identification of a unique, highly significant, signature comprised of the *loss* of miR-29c and miR-324-3p (p value $< 1 \times 10^{-7}$). These results support our hypothesis that a significant loss of circulating oligonucleotides observed in healthy donors, could be indicative of disease and cannot be identified by searching only for miRNAs that are up-regulated in the cancer tissue. Although results of our expansion cohort support this observation, further studies with larger populations are required. The expression levels of these circulating miRNAs in earlier stages of melanoma, such as regional Stage III or even local stages I–II, should be thoroughly tested in future studies to evaluate potential clinical use for early diagnosis.

It was previously suggested that miRNAs are present in the serum within exosomes (Gallo et al., 2012). Recent papers

Table 1. Clinical data.

#	Sample ID	Sex	Age	Cancer type	Stage
1	9	m	55	Melanoma	IV
2	23	m	34	Melanoma	IV
3	31	m	50	Melanoma	IV
4	34	m	36	Melanoma	IV
5	39	m	36	Melanoma	IV
6	42	m	41	Melanoma	IV
7	47	m	60	Melanoma	IV
8	48	m	22	Melanoma	IV
9	51	f	41	Melanoma	IV
10	52	m	45	Melanoma	IV
11	53	m	57	Melanoma	IV
12	54	m	66	Melanoma	IV
13	57	m	71	Melanoma	IV
14	62	m	65	Melanoma	IV
15	63	m	52	Melanoma	IV
16	65	m	58	Melanoma	IV
17	66	m	41	Melanoma	IV
18	72	m	49	Melanoma	IV
19	73	m	25	Melanoma	IV
20	74	m	68	Melanoma	IV
21	77	f	60	Melanoma	IV
22	84	m	55	Melanoma	IV
23	85	f	54	Melanoma	IV
24	87	m	33	Melanoma	IV
25	91	f	56	Melanoma	IV
26	94	f	27	Melanoma	IV
27	96	f	57	Melanoma	IV
28	MA	m	55	Melanoma	IV
29	10R	m	68	Renal carcinoma	FUHRMAN'S NUCLEAR GRADE 3.
30	11R	f	50	Renal carcinoma	FUHRMAN GRADE 1–2.
31	12R	f	51	Renal carcinoma	FUHRMAN NUCLEAR GRADE 1–2. PT1a, NX, MX.
32	13R	f	54	Renal carcinoma	FUHRMAN'S GRADE 1–2.
33	14R	f	55	Renal carcinoma	FURHMAN'S GRADE 1. T1A.NX.MX.
34	15R	f	67	Renal carcinoma	FUHRMAIN'S GRADE 1–2
35	16R	f	67	Renal carcinoma	Fuhrman nuclear grade 2. pT3a, N0, Mx.
36	17R	f	74	Renal carcinoma	FUHRMAN GRADE 3
37	18R	f	74	Renal carcinoma	FUHRMAN CYTOLOGICAL GRADE 2–3
38	19R	f	75	Renal carcinoma	FUHRMAN'S NUCLEAR GRADE 2–3. PT3B NO MX
39	1R	m	39	Renal carcinoma	FUHRMAN GRADE 4. PT1B NX MX.
40	20R	f	75	Renal carcinoma	FUHRMANS HISTOLOGICAL GRADE 2.
41	21R	m	60	Renal carcinoma	metastases.
42	22R	m	64	Renal carcinoma	TCC. GLEASON GRADE 3+3(6)/10. T4A. NX. MX.
43	23R	m	65	Renal carcinoma	HIGH GRADE
44	2R	m	43	Renal carcinoma	FUHRMAN GRADE 2. PT1A, NX, MX.
45	3R	m	44	Renal carcinoma	NUCLEAR GRADE III
46	4R	m	56	Renal carcinoma	FUHRMAN NUCLEAR GRADE I–II. PT1B, NX, MX.
47	5R	m	57	Renal carcinoma	FUHRMAN'S GRADE 3.
48	6R	m	57	Renal carcinoma	HISTOLOGIC GRADE I.
49	7R	m	58	Renal carcinoma	FUHRMAN GRADE II–III
50	8R	m	60	Renal carcinoma	FUHRMAN GRAED G1
51	9R	m	60	Renal carcinoma	FUHRMAN'S NUCLEAR GRADE 2
52	10c	m	63	Colon cancer	metastases.
53	11c	f	24	Colon cancer	MODERATELY TO POORLY DIFFERENTIATED. 3/37 LN are positive.
54	12c	f	56	Colon cancer	metastases.
55	13c	f	59	Colon cancer	PT3, NO, MX.
56	14c	f	59	Colon cancer	METASTATIC TUMOR
57	15c	f	63	Colon cancer	METASTATIC TUMOR
58	16c	f	63	Colon cancer	T3.N1.M1.
59	17c	f	65	Colon cancer	MODERATELY TO POORLY DIFFERENTIATED AC.
60	18c	f	65	Colon cancer	moderately differentiated
61	19c	f	69	Colon cancer	T2 N0 Mx.
62	20c	f	70	Colon cancer	UNDIFFERENTIATED
63	1c	m	41	Colon cancer	METASTATIC TUMOR
64	2c	m	42	Colon cancer	metastases.
65	3c	m	46	Colon cancer	Low-grade. pT3.pN1a.pM1
66	4c	m	47	Colon cancer	METASTATIC TUMOR
67	5c	m	48	Colon cancer	
68	6c	m	51	Colon cancer	METASTATIC TUMOR
69	7c	m	58	Colon cancer	T4.N1.MX
70	8c	m	59	Colon cancer	PT3.NO.MX
71	9c	m	62	Colon cancer	MODERATELY TO POORLY DIFFERENTIATED AC.

Cancer patients' clinical data including sex, age, disease and disease staging.

Table 2. Statistical score of candidate melanoma signature serum miRNAs.

miRNA	*p* Value-initial screen	*p* Value-validation step	Validation ΔCp-healthy	Validation ΔCp-melanoma
hsa-miR-150	0.0006	0.0905	N/A	N/A
hsa-miR-451	0.0009	0.0125	N/A	N/A
hsa-miR-342-3p	0.0019	0.2519	N/A	N/A
hsa-miR-324-3p	0.0109	0.0007	4.4	5.2
hsa-miR-29c	0.0114	0.0001	3.4	4.9
hsa-miR-197	0.0128	0.8241	N/A	N/A
hsa-miR-140-3p	0.0150	0.6200	N/A	N/A
hsa-miR-29a	0.0207	0.0577	N/A	N/A
hsa-miR-503	0.0313	0.4622	N/A	N/A
hsa-miR-339-3p	0.0346	0.8508	N/A	N/A
hsa-miR-20a*	0.0375	0.1368	N/A	N/A
hsa-miR-374a	0.0431	0.1043	N/A	N/A

Initial screen step and validation step mean ΔCp and *p* values of melanoma versus healthy control serum miRNAs.

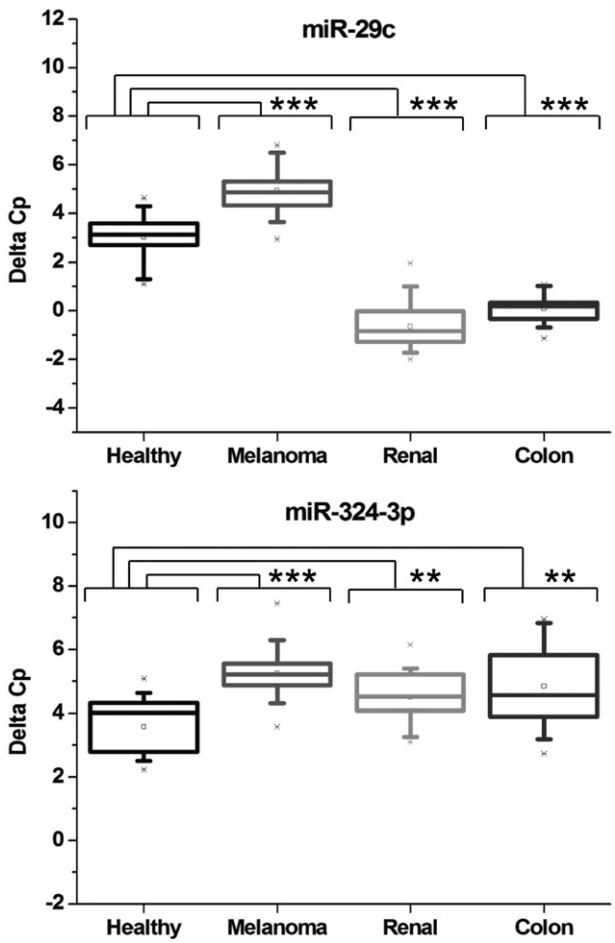

Figure 1. Individual serum miRNAs differentiate melanoma patients. Serum miR-324-3p and miR-29c specificity and ability to distinguish between melanoma ($n = 28$), healthy ($n = 20$), renal ($n = 23$) and colon ($n = 20$) cancers was tested by qPCR. ** denotes $p < 0.001$, *** denotes $p < 0.0001$.

provide evidence that these exosomes can be internalized by various cells, including cancer cells, while strikingly, the internalized miRNAs retain their function and exert it on the host cell (Lim et al., 2011). Logically, these reports imply that circulating miRNAs within exosomes can mediate remote

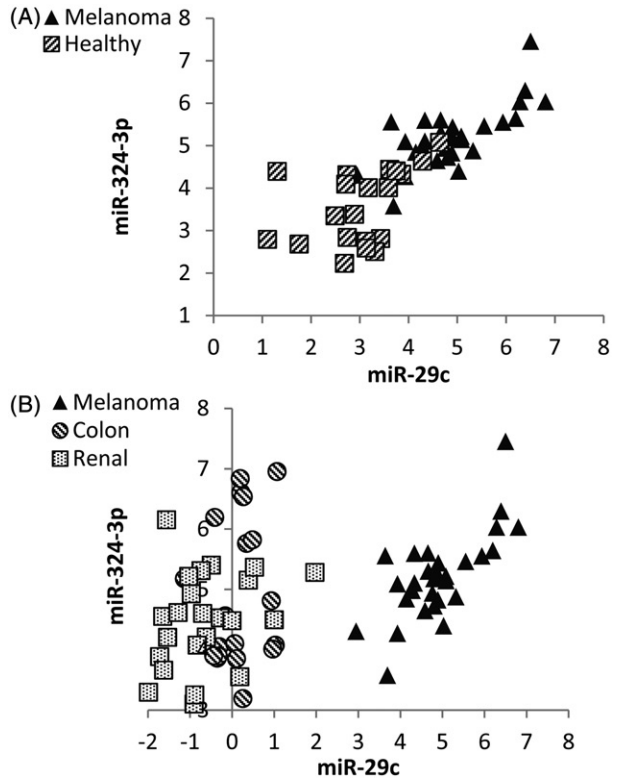

Figure 2. The combination of miR-29c and miR-324-3p can diagnose melanoma. (A) Dot plot of serum miR-29c (X axis) and miR-324-3p (Y axis) normalized Cp values for the melanoma patients versus the healthy controls; (B) Dot plot of serum miR-29c (X axis) and miR-324-3p (Y axis) normalized Cp values for the melanoma patients versus the renal and colon cancer patients.

effects on various cells, comparable to cytokines or hormones. This notion could imply that the significant loss of miR-29c and miR-324-3p in melanoma occurs due to a biological reasoning, which reflects the interaction between the host and the disease. Therefore, our approach might offer at least two new advantages over the dogmatic search after miRNAs overexpressed by cancer cells: (1) not all melanomas express the same miRNA profile and therefore patients could be missed resulting in false negatives. On the other hand, hosts tend to respond with certain convergent responses against diseases in a broader sense. Detection of such generic elements in the response to melanoma might minimize false negatives due to tumor heterogeneity; (2) the findings might lead to the discovery of novel mechanisms of pathogenesis and therapeutic derivatives.

A recent study showed a significant decrease in miR-29c expression in stage III/IV compared to stage I/II melanoma tumors. The downregulation of miR-29c in melanoma cells was found to be associated with hypermethylation of tumor-related genes and poor prognosis in cutaneous melanoma (Nguyen et al., 2011). Nevertheless, it is still unclear how tumor downregulation of miR-29c along disease progression is linked to the decreased serum concentrations of miR-29c in melanoma patients as compared to healthy individuals. This association could point on a novel role of circulating miR-29c in host response to the presence of melanoma cells, and its disappearance from the serum should be further studied as part of the pathogenesis of disease. Unlike miR-29c, there are hardly any studies on the role or involvement of

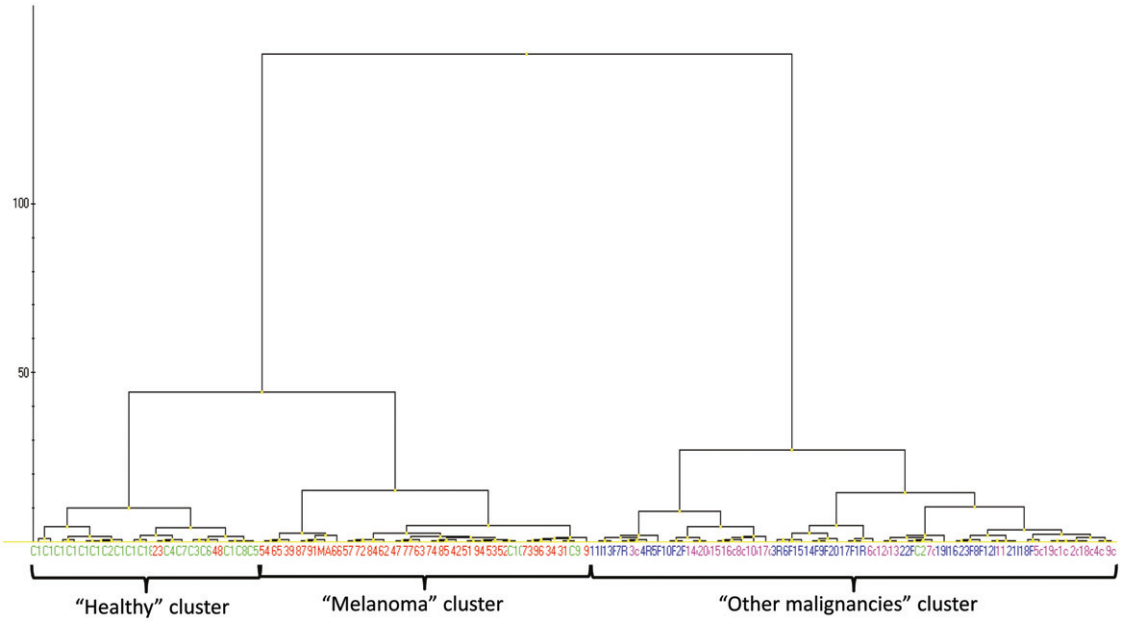

Figure 3. Hierarchical clustering analysis. Hierarchical clustering analysis based on normalized Cp values of both miR-29c and miR-324-3p.

miR-324-3p in cancer in general or specifically in melanoma. A recently published paper might reveal an apparent link between this miRNA and breast cancer (Hu et al., 2012), demonstrating overexpression of serum miR-324-3p in breast cancer patients as compared to healthy controls. It is known that the expression of some miRNAs is cancer-specific and might confer differential functional effects (Ivanov et al., 2010; Valastyan et al., 2009). It is, therefore, not necessarily surprising that circulating, as well as tumoral, miR-29c is lost in melanoma patients, but on the other hand its amount is substantially increased in colon and renal cancer patients, as compared to the same healthy controls (Figure 2).

Conclusion

To the best of our knowledge, this is the first report demonstrating decreased expression of circulating miR-29c and miR-324-3p in melanoma patients' sera. Taking into consideration the limitations of the small cohorts used in this pilot study, this work highlights a proof of concept regarding the possibility to use serum specific miRNAs signature, which are not derived from cancer over-expressed miRNAs in general, and in metastatic melanoma in particular. In order to ratify the use of circulating miR-29c and miR-324-3p loss as a potential biomarker for malignant melanoma there is a clear need to increase the sample size. Validation of this approach and identification of additional signatures in other malignancies is clearly of interest. Finally, this approach should be tested in earlier stages of disease, in order to determine the feasibility of this approach as a screening tool.

Acknowledgements

The authors would like to thank Mr. Michael Aronson and the Lemelbaum family for their general support, as well as to Mr. Roi Nave for his efforts. This work was performed in partial fulfillment of the requirements for a PhD degree of Eyal Greenberg, Sackler Faculty of medicine, Tel Aviv University, Israel.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

This work was supported by the Ella Institute of Melanoma and the Flight Attendants Medical Research Institute (FAMRI).

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