

# Unraveling the Role of microRNA and isomiRNA Networks in Multiple Primary Melanoma Pathogenesis

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## Research

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# Abstract

**Background** Malignant cutaneous melanoma (CM) is a potentially lethal form of skin cancer whose worldwide incidence has been constantly increasing over the past decades. During their lifetime about 8% of patients with CM will develop multiple primary melanomas (MPM). Patients affected by MPM could have a genetically determined susceptibility, though germline mutations in hereditary melanoma genes are rarely detected.

**Methods** To better characterize the biology of this subset of melanomas, we explored the miRNome of 24 single and multiple primary melanomas, including multiple tumors from the same patient, using a smallRNA sequencing approach and bioinformatic detection of miRNA isoforms. The differential expression of specific miRNAs/isomiRs was obtained using quantitative PCR.

**Results** From a supervised analysis, 22 miRNAs were differentially expressed in MPM compared to single CM, including key miRNAs involved in epithelial-mesenchymal transition (EMT). Moreover, the first and second melanoma from the same patient presented a different miRNA profile. Ten miRNAs, including miR-25-3p, 149-5p, 92b-3p, 211-5p, 125a-5p, 125b-5p, 205-5p, 200b-3p, 21-5p and 146a-5p, were further validated in a larger cohort of single and multiple melanoma samples (N=47). Overall, the Pathway Enrichment Analysis revealed a more differentiated and less invasive status of MPMs. Analyzing our smallRNA seq data, we detected a panel of melanoma-specific miRNA isoforms (isomiRs), which were validated in The Cancer Genome Atlas SKCM cohort. Specifically, we identified hsa-miR-125a-5p|0|2 isoform as 10-fold over-represented in melanoma and differentially expressed in MPMs. IsomiR-specific target analysis revealed that the miRNA shortening confers a novel pattern of target gene regulations, including genes implicated in melanocyte differentiation and cell adhesion.

**Conclusions** Overall we provided a comprehensive characterization of the miRNA/isomiRNA regulatory network of multiple primary melanomas, highlighting mechanisms of tumor development and correlating miRNA expression with MPM clinical characteristics.

## Background

Melanoma is a malignant tumor that develops from transformed melanocytes. The incidence of cutaneous melanoma (CM) has been rising constantly in the past several decades, reaching an age-standardized rate of 7, 22 and 70 per 100,000 persons among fair-skinned population in Northern Europe, North America and Australia continent respectively (source: IARC 2018).

CM accounts for 3–5% of all skin cancers, determining up to 65% of the deaths<sup>19</sup>. The pathogenesis of CM is complex and poorly understood. Risk factors include genetic, environmental and phenotypic factors such as ultraviolet (UV) exposure, fair phototypes, multiple dysplastic nevi and a positive family/personal history of CM<sup>12</sup>.

Regarding genetic factors, somatic mutations of *BRAF* gene have been found in almost 40–50% of sporadic CMs located in body sites with intermittent UV exposure; 15–20% of the other cases are associated to *NRAS* mutations and correlated with chronic UV exposure<sup>24</sup>. A small portion of melanomas occurs in acral or mucosal locations and a subset of them are related to *KIT* and *GNAQ* mutations<sup>7</sup>. These findings have brought important therapeutic implications and changed the management of CM patients with the development of specific target therapies. Germline mutations instead, can be found in multiple or familial cases of CM. The most frequently described germline mutation is in *CDKN2A* (cyclin-dependent kinase inhibitor 2A) gene occurring in 8–15% of subjects diagnosed with multiple primary melanomas (MPMs) without familial history and up to 40% of patients with hereditary CM<sup>6,25,40,44</sup>. Mutations in other susceptibility genes such as *CDK4* (cyclin-dependent kinase 4), *MITF* (microphthalmia-associated transcription factor) and *POT1* (protection of telomeres 1) are less frequently detected<sup>4,14</sup>. During their lifetime about 8% of patients with cutaneous melanoma will develop multiple primary melanomas, usually at a young age and within 3 years from the first tumor/diagnosis<sup>18</sup>.

The occurrence of MPMs in the same patient is thought to be related to a personal genetic susceptibility in association with environmental factors. These patients may represent a model of high-risk CM occurrence. As a matter of fact, it is estimated that a personal history of CM is a strong risk factor for the development of a subsequent primary CM<sup>18,43</sup>. The excision of a prior CM determines a risk up to 8.5% to develop another CM and the frequency of MPMs is reported to be between 0.2 and 10%<sup>13,25,29</sup>. The above-reported rates may underestimate the lifetime rates due to limited series of patients and different follow-up periods. Variability may also arise due to differences in environmental factors such as ultraviolet radiation exposure across geographical regions. Among the cases of MPMs, 13–40% of patients are diagnosed with synchronous lesions (i.e. a subsequent primary CM diagnosed within 3 months from the prior diagnosis), while the remainder develop metachronous lesions<sup>1,29,41</sup>. The risk of a subsequent CM is highest in the first year following the diagnosis of the primary CM; however, this risk remains increased for at least 20 years<sup>1</sup>. Despite the increased risk of multiple tumor development, there is still an open debate about the prognosis of MPM patients. On one hand, Doubrovsky et al. observe a favorable prognosis in patients with MPM<sup>16</sup>, but a worse prognosis for these patients was recently reported by El Sharouni and colleagues<sup>17</sup>.

Moreover, the frequency of germline mutations in melanoma susceptibility genes (*CDKN2A*, *CDK4*, *MITF*, *POT1/ACD/TERF2IP*, *TERT*, *BAP1*) is lower than expected in MPM patients<sup>5,6,9,25</sup>. Therefore, a better characterization of MPM pathogenesis and biological features is of the utmost importance.

The dysregulation of small noncoding RNAs, specifically microRNAs (miRNAs, 18–22 nucleotides in length), plays a significant role in tumorigenesis, including melanoma onset and progression<sup>46</sup>. MiRNAs regulate multiple and specific target genes, determining an oncogenic or tumor-suppressive function, being implicated in the proliferation, apoptosis and tumor progression. Moreover, miRNA global expression profile faithfully reflects the overall expression profile of normal and pathological cells and

tissues, with the advantage to be feasible also from formalin-fixed and paraffin-embedded (FFPE) tissues.

In this study, we investigated the global miRNA and isomiRNA expression profile of multiple primary melanomas using an unbiased smallRNA sequencing approach. A comparison of familial/non familial MPM vs. single primary melanoma miRNome was established in order to investigate the possible similarities. Moreover, the evolution of MPM miRNA profile was assessed matching multiple tumors from the same patient.

## Methods

### Clinical samples

A retrospective series of 47 samples from 29 patients was collected. Patients were selected among those referring to the melanoma center of the Dermatology Unit at Bologna University Hospital. The study was approved by Comitato Etico Indipendente di Area Vasta Emilia Centro - CE-AVEC, Emilia-Romagna Region (number 417/2018/Sper/AOUBo). Before study entry, all the patients provided written and voluntary informed consent for inclusion, collection and use of clinical-pathological data and samples and data privacy.

The specimens were classified into three groups: benign nevi, single primary cutaneous melanoma (CM) and multiple primary melanoma (MPM). Group 1 (n = 3), benign nevi of 3 patients with no prior diagnosis of CM or non-melanoma skin cancer and follow up of at least 10 years. Group 2 (n = 35), MPM samples from 17 patients with prior diagnosis of  $\geq 2$  CMs. 3 out of 17 patients had positive family history of CM (FAM). MPM patients were tested for CDKN2A, MITF and CDK4 genetic alterations and only 1 patient had a mutation in CDKN2A gene (c.249C > A p.His83Gln). Group 3 (n = 9), 9 samples from CM patients with no history of prior CMs and a follow-up of at least 10 years.

Tumor and nevi samples were formalin-fixed and paraffin-embedded (FFPE). For each sample, 5/6 tissue sections on glass slides were obtained. One section was stained with hematoxylin-eosin (HE) and examined by an expert pathologist to select the tumor/nevus area, which was grossly dissected before RNA extraction.

### RNA extraction

RNA was isolated from 10  $\mu$ m-thick FFPE sections using miRNeasy FFPE kit (Qiagen) according to the manufacturer's instructions. Deparaffinization was performed with xylene followed by an ethanol wash. RNA was eluted in 30  $\mu$ L of RNase-free water and quantified by absorbance at 260 and 280 nm.

### SmallRNA sequencing

We analyzed 3 benign nevi, 4 single CM, 17 multiple primary or familial melanomas from 8 different patients. The 24 smallRNA libraries were generated using TruSeq Small RNA Library PrepKit v2 (Illumina,

RS-200-0012/24/36/48), according to manufacturer's indications. Briefly, 35 ng of purified RNA was linked to RNA 3' and 5' adapters, converted into cDNA, and amplified using Illumina primers containing unique indexes for each sample. High Sensitivity DNA kit was adopted for libraries quantification using Agilent Bioanalyzer (Agilent Technologies, California, USA5067-4626) and the 24 DNA libraries were combined in equal amount to generate a libraries pool.

Pooled libraries underwent to size selection employing magnetic beads (Agencourt) and amplicons with a length in the 130–160 bp range, were recovered.

Finally, 20pM of pooled libraries, quantified using the HS-DNA Kit (Agilent) were denatured, neutralized and combined with a Phix control library (standard library normalizator). A 1.8 pM final concentration of pooled libraries (obtained by dilution with a dedicated buffer as described in Illumina protocol guidelines) was obtained and sequenced using NextSeq 500/550 High Output Kit v2 (75 cycles) (Illumina, FC-404-2005) on the Illumina NextSeq500 platform.

Raw base-call data were demultiplexed using Illumina BaseSpace Sequence Hub and converted to FASTQ format. After a quality check with FastQC tool, the adapter sequences were trimmed using Cutadapt, which was also used to remove sequences shorter than 16 nucleotides and longer than 30 nucleotides. Reads were mapped using the STAR algorithm. Only reads that mapped unambiguously to the genome (at least 16 nucleotides aligned, with a 10% mismatch allowed) were used for the analyses. The reference genome consisted in human miRNA sequences from the miRbase 21 database. Raw counts from mapped reads were obtained using the htseq-count script from the HTSeq tools<sup>3</sup>. Counts were normalized using DESeq2 bioconductor package<sup>37</sup>. NGS raw data (FASTQ format) are available through European Nucleotide Archive with the following accession number: PRJEB35819.

## Quantitative PCR

### miRCURY LNA Assay

RNA from 47 samples was converted to cDNA using miRCURY LNA RT kit (Qiagen, cat. no. 339340). Reverse transcription (RT) reaction was performed as follows: 2 µL miRCURY RT Reaction Buffer, 4.5 µL RNase-free water, 1 µL miRCURY RT Enzyme Mix, 0.5 µL UniSp6 spike-in, and 2 µL template RNA (5 ng/µL). RT cycling protocol consisted in 60 min at 42 °C, 5 min at 95 °C, and cooling at 4 °C. cDNA samples were stored at -20 °C. RT-qPCR was performed using miRCURY LNA SYBER Green PCR kit (Qiagen, cat. no. 339346) and primers from miRCURY LNA miRNA PCR Assays (Qiagen, cat. no. 339306): miR-21-5p (cat. no. YP00204230), miR-25-3p (cat. no. YP00204361), miR-125b-5p (cat. no. YP00205713), miR-146a-5p (cat. no. YP00204688), miR-205-5p (cat. no. YP00204487), miR-149-5p (cat. no. YP00204321), miR-92b-3p (cat. no. YP00204384), miR-200b-3p (cat. no. YP00206071), miR-211-5p (cat. no. YP00204009), miR-16-5p (cat. no. YP00205702), miR-125a-5p (cat. no. YP00204339), SNORD44 (cat. no. YP00203902). For each miRNA target, cDNA was diluted at the ratio of 1:80 with the exception of miR-92b-3p, for which cDNA was diluted 1:4. Cycling program consisted in: 10 min at 95 °C and 2-step cycling (40 cycles) of denaturation (10 s at 95 °C), and combined annealing/extension (60 s at 60 °C). Raw Cq values were

obtained from BioRad CFX software. Interplate calibrators were used to standardize miRNA Cq values across plates. miR-16-5p was selected as reference gene due to its stability across samples in NGS experiment. The calculation of relative expression was performed using  $2^{-\Delta Ct}$  methods.

## **miSCRIPT Assay (miR-125a-5p isoforms quantification)**

RNA from 39 samples was reverse transcribed using miSCRIPT HiSpec Buffer from miSCRIPT II RT kit (Qiagen, cat. no. 218161) according to the manufacturer's instructions. Specifically, RT reaction was prepared in a total reaction volume of 10  $\mu$ L with 2  $\mu$ L miScript HiSpec Buffer, 1  $\mu$ L miScript Nucleics Mix, 4  $\mu$ L RNase-free water, 1  $\mu$ L miScript Reverse Transcriptase Mix, 2  $\mu$ L template RNA (5 ng/ $\mu$ L). RT cycling protocol consisted in 60 min at 37 °C, 5 min at 95 °C, and cooling at 4 °C. cDNA samples were stored at 20 °C. RT-qPCR was performed using miSCRIPT SYBER Green PCR kit (Qiagen, cod. no. 218073) and primers from miSCRIPT Primer Assays (Qiagen, cat. no. 218300): miR-125a-5p (cod. no. MS00003423), RNU6 (cod. no. MS00033740). Reaction mix was prepared with 10  $\mu$ L QuantiTect SYBR Green PCR Master Mix, 2  $\mu$ L miScript Universal Primer, 2  $\mu$ L miScript target Primer, 3  $\mu$ L RNase-free water, 3  $\mu$ L cDNA template (1:40).

Cycling program consisted in: 15 min at 95 °C, and 3-step cycling (40 cycles) of denaturation (15 s at 94 °C), annealing (30 s at 55 °C) and extension (30 s at 70 °C). Raw Cq values were obtained from BioRad CFX software. Small nuclear RNA U6 (RNU6) was used as reference gene. The calculation of relative expression was performed using  $2^{-\Delta Ct}$  methods.

## **Quantification of isomiRs**

IsomiRs were identified in our NGS dataset of 24 samples as described in Loher et al. <sup>36</sup>. Briefly, sequence reads were quality trimmed using the cutadapt tool, and mapped unambiguously using SHRIMP2 (PMID: 21278192) to the human genome assembly GRCh38. During the mapping, no insertions or deletions, and at most one mismatch was permitted. IsomiRs were identified as done previously <sup>36, 52</sup>.

For TCGA isomiR analysis, short RNA-seq Aligned BAM files were downloaded from the Genomic Data Commons Data Portal (<https://portal.gdc.cancer.gov/>) for all 32 cancer types. IsomiR profiles were generated using the same approach as described in Loher et al. <sup>36</sup>

To simplify the labeling of the isomiRs, we used the annotation system developed by Loher et al. This nomenclature specifies the name of the canonical miRNA, the start site (5' end) of the isomiR compared to the canonical miRNA sequence in miRBase, the end-site (3' end) and the eventual insertion of uracil. In particular, to annotate the start and end site of an isomiR, a negative (-) or positive sign (+) followed by the number of nucleotides is used to indicate how many nucleotides the isomiR terminus has, when compared to the canonical miRNA sequence. Zero indicate the same terminus of the canonical miRNA sequence.

We quantified isomiR abundances in reads per million (RPM). Only reads that passed quality trimming and filtering and could be aligned exactly to miRNA arms were used in the denominator of this

calculation.

IsomiR targets were predicted using the RNA22 algorithm<sup>35</sup> and targets were allowed to be present in the 5'UTR, CDS, and 3'UTR of the candidate mRNA. We selected only those targets that had a p-value < 0.01 and a predicted binding energy < -16 while also allowing G:U wobbles and bulges within the seed region.

## Statistical analysis

Normalized sequencing data were imported and analyzed in Genespring GX software (Agilent Technologies). Differentially expressed miRNAs were identified using a fold change > 1.5 filter and moderated t-test (FDR 5% with Benjamini-Hochberg correction) in CM vs. MPM comparison and fold-change > 1.2 and paired t-test (p < 0.05) in 1st vs. 2nd MPM comparison. Cluster Analysis, was performed using Manhattan correlation as a similarity measure. Principal Component Analysis was performed on 24 samples using all human miRNAs detected by NGS analysis (n = 1629).

Graphpad Prism 6 (GraphPad Software) was used for statistical analyses. Group comparison was performed using unpaired t-test, when data had a normal distribution, with or without Welch's correction according to the significance of the variance test. Data that did not present a normal distribution were compared using Mann-Whitney non-parametric test.

Association of gene expression with overall survival in TCGA SKCM cohort, was obtained using OncoInC website (<http://www.oncolnc.org>), logrank test was used to calculate the p-value.

## Pathway Analysis

Pathway and network analysis of differentially expressed miRNAs, miR-125a-5p isoforms and their targets was investigated using the web-based software MetaCore (GeneGo, Thomson Reuters). A p value of 0.05 was used as a cut off to determine significant enrichment.

## Results

### Patient Characteristics

Demographic, clinical and pathological features of 29 patients are summarized in Table 1. A total number of 16 males and 13 females were included, with a mean age at first diagnosis of 59 years for single primary melanomas and 53 years for multiple primary melanomas. Nine patients had single cutaneous melanoma and 10 years of follow up; 17 developed more than one primary melanoma in an average time of 33 months (range 3–98). MPM patients were tested for germline genetic alterations in CDKN2A, CDK4 and MITF gene<sup>15</sup> and only one patient was found to have a germline CDKN2A mutation (c.249C > A p.His83Gln exon 2) of unknown clinical significance. Melanoma specimens were examined by two dermatopathologists.

Table 1  
Patient characteristics

	Benign Nevi (BN)	Cutaneous Melanoma (CM)	Multiple Primary Melanoma (MPM)		
			MPM 1st	MPM 2nd	MPM 3rd
Gender (n;%)					
<b>Male</b>	0 (0%)	3 (33.3%)	13 (76.5%)		
<b>Female</b>	3 (100%)	6 (66.7%)	4 (23.5%)		
<b>Total</b>	3	9	17		
Histology (n;%)					
<b>Compound melanocytic nevus</b>	2 (66.7%)	-	-	-	-
<b>Dermal nevus</b>	1 (33.3%)	-	-	-	-
<b>Superficial spreading melanoma</b>	-	4 (44.5%)	15 (88.2%)	16 (94.1%)	1 (100%)
<b>Superficial spreading melanoma with vertical growth phase</b>	-	3 (33.3%)	1 (5.9%)	-	-
<b>Nodular melanoma</b>	-	2 (22.2%)	1 (5.9%)	-	-
<b>Nevus-associated melanoma</b>	-	0	-	1 (5.9%)	-
Age at first diagnosis (n;%)					
<b>&lt; 50</b>	-	7 (77.8%)	8 (47%)		
<b>≥ 50</b>	-	2 (22.2%)	9 (53%)		
<b>Mean (range)</b>	-	59 (29–85)	53 (30–80)		
Localization (n;%)					
<b>Trunk</b>	3 (100%)	6 (66.7%)	13 (76.5%)	14 (82.4%)	1 (100%)
<b>Limbs</b>	-	2 (22.2%)	3 (17.6%)	3 (17.6%)	-
<b>Head and neck</b>	-	1 (11.1%)	1 (5.9%)	-	-
Breslow thickness (n;%)					
<b>&lt; 0.8 mm</b>	-	1 (11.1%)	12 (70.6%)	17 (100%)	1 (100%)

	Benign Nevi (BN)	Cutaneous Melanoma (CM)	Multiple Primary Melanoma (MPM)		
			MPM 1st	MPM 2nd	MPM 3rd
<b>≥ 0.8 mm</b>	-	8 (88.9%)	5 (29.4%)	0 (0%)	-
Family history of melanoma (n;%)					
<b>Yes</b>	-	-	3 (17.6%)		
<b>No</b>	-	-	14 (82.4%)		

### The microRNA profile of multiple primary melanoma

The global miRNA profile of 17 multiple primary melanomas, obtained from 8 patients, was analyzed using a smallRNA sequencing approach. For each MPM patient we analyzed the first and second primary tumor, and for one case also a third one. Three patients had a family history of melanoma. We compared the global miRNA profile of MPM toward that of 4 single melanomas and 3 benign nevi. From the smallRNA sequencing data, we identified 1629 mature miRNAs expressed in melanoma and nevus cells. The unsupervised Principal Component Analysis (PCA) of all miRNAs and all samples (n = 24) revealed that familial and non-familial multiple primary melanomas have a greatly overlapping miRNA profile (Fig. 1A), which is different from single cutaneous melanoma (CM) and benign nevi (BN). Indeed, a statistical comparison between familial and non-familial MPMs did not provide any significant result. Therefore, we considered familial and non-familial melanomas as a unique group in all subsequent analyses. From the PCA we can already observe that MPMs displayed a miRNA profile more similar to benign nevi than CMs. When we compared multiple and single melanoma tumors, we obtained a markedly different miRNA expression profile and a list of 22 miRNAs differentially expressed (adjusted p < 0.05, Table 2), which are represented with a Volcano plot in Fig. 1B. Cluster analysis of these samples based on the expression of the 22 differentially expressed miRNAs confirmed the separation between single and multiple tumors Fig. 1C. The MPM group was constituted by the paired first and second tumors (and one additional tumor in one case) developed by the same patient over the years (Table 1). Comparing the miRNA profile of these two groups using a paired statistical analysis, miRNAs that characterize the second tumors, usually thinner and less aggressive than the first melanoma given their early diagnosis, were identified. Despite the similarities between the two matching MPMs, a variation in miRNA expression was observed (Fig. 1B). Specifically, thirty-seven miRNAs were differentially expressed between the first and second MPM (paired t-test, p < 0.05, Table 3) and a significant separation was obtained applying the cluster analysis (Fig. 1D).

Table 2

– List of microRNAs differentially expressed in multiple vs. primary melanoma

<b>microRNA</b>	<b>adjusted p-value</b>	<b>Regulation MPM vs. CM</b>	<b>Fold change</b>
hsa-miR-25-3p	0.0084	down	-2.2
hsa-miR-3614-5p	0.0135	down	-3.2
hsa-let-7i-5p	0.0153	down	-2.7
hsa-miR-181a-3p	0.0153	down	-2.7
hsa-miR-21-3p	0.0153	down	-4.7
hsa-miR-584-5p	0.0167	down	-6.3
hsa-miR-149-5p	0.0205	up	3.2
hsa-miR-21-5p	0.0205	down	-4.3
hsa-miR-29a-3p	0.0205	down	-1.7
hsa-miR-146a-5p	0.0250	down	-4.8
hsa-miR-651-5p	0.0275	down	-1.7
hsa-miR-125b-2-3p	0.0314	up	2.1
hsa-miR-15a-5p	0.0314	down	-2.3
hsa-miR-99a-5p	0.0314	up	2.4
hsa-miR-941	0.0318	down	-1.8
hsa-miR-340-5p	0.0341	down	-2.4
hsa-miR-532-5p	0.0341	down	-2.1
hsa-miR-205-5p	0.0344	up	2.1
hsa-miR-106b-3p	0.0347	down	-2.3
hsa-miR-181c-5p	0.0360	down	-2.0
hsa-miR-125b-5p	0.0398	up	2.4
hsa-miR-132-3p	0.0450	down	-1.8

Table 3

List of microRNAs differentially expressed in paired multiple melanomas from the same patient

<b>microRNA</b>	<b>p-value (paired)</b>	<b>Regulation MPM vs. CM</b>	<b>Fold change</b>
hsa-let-7d-3p	0.0174	down	-1.8
hsa-let-7e-5p	0.0153	down	-2.0
hsa-miR-1226-3p	0.0345	down	-3.1
hsa-miR-1248	0.0423	up	3.4
hsa-miR-1249	0.0338	down	-1.8
hsa-miR-125a-5p	0.0063	down	-1.9
hsa-miR-1269b	0.0254	down	-2.0
hsa-miR-145-3p	0.0313	down	-1.7
hsa-miR-149-5p	0.0157	down	-1.7
hsa-miR-200b-3p	0.0134	down	-1.8
hsa-miR-223-3p	0.0458	up	1.8
hsa-miR-224-5p	0.0135	down	-1.8
hsa-miR-2392	0.0362	up	2.3
hsa-miR-320b	0.0243	down	-2.0
hsa-miR-328-3p	0.0103	down	-1.9
hsa-miR-330-5p	0.0060	down	-1.5
hsa-miR-3607-5p	0.0308	up	3.1
hsa-miR-3609	0.0028	up	2.5
hsa-miR-365a-5p	0.0398	down	-1.6
hsa-miR-375	0.0166	down	-1.8
hsa-miR-4286	0.0071	up	1.9
hsa-miR-433-3p	0.0019	down	-2.2
hsa-miR-4423-3p	0.0090	down	-2.0
hsa-miR-4466	0.0190	up	2.0
hsa-miR-505-3p	0.0194	down	-1.8
hsa-miR-6511b-3p	0.0299	down	-2.0

microRNA	p-value (paired)	Regulation MPM vs. CM	Fold change
hsa-miR-671-3p	0.0431	down	-1.6
hsa-miR-7641	0.0129	up	2.1
hsa-miR-8058	0.0334	down	-2.2
hsa-miR-877-5p	0.0160	down	-2.1
hsa-miR-887-3p	0.0441	up	2.0
hsa-miR-92a-1-5p	0.0464	down	-1.6
hsa-miR-92b-3p	0.0100	down	-1.6
hsa-miR-96-5p	0.0062	down	-2.6
hsa-miR-98-3p	0.0246	down	-1.8
hsa-miR-99a-3p	0.0342	up	1.6
hsa-miR-99b-5p	0.0054	down	-1.6

### Validation of microRNA differential expression in single and multiple primary melanomas and paired primary tumors from the same patient

Nine miRNAs were selected for an independent technical validation using quantitative RT-PCR in 47 novel samples including BN, CM and 1st and 2nd MPM. Specifically, we included the miRNAs differentially expressed between CM and MPM (miR-21-5p, miR-25-3p, miR-125b-5p, miR-146a-5p, miR-205-5p, miR-149-5p) and others between the first (MPM 1st ) and second (MPM 2nd ) melanoma within the same MPM patient (miR-149-5p, miR-92b-3p, miR-200b-3p, miR-125a-5p).

According to the smallRNA NGS results, an upregulated expression of miR-21-5p, miR-25-5p, miR-146a-5p, and a downregulated expression of miR-125b-5p, miR-149-5p and miR-205-5p in CM compared to MPM were expected. In MPM samples, all selected miRNAs are upregulated in the MPM 2nd compared to MPM 1st. In the validation experiment, we included also miR-21-5p, considering that it is a melanocyte specific miRNA, whose genetic locus is located inside melastatin gene and whose expression is particularly high in nevi. The expression of this miRNA was higher in BN, with borderline statistical significance when compared to CM or MPM in our NGS data.

The validation was performed in a cohort of 29 patients, as described in Table 1, using miR-16-5p as a reference gene due to its invariant expression in NGS data. Expression distributions of selected miRNAs in benign nevi, cutaneous melanoma and multiple primary melanoma samples are represented in Fig. 2. The significant upregulation of miR-21-5p in CM vs. BN and MPM was confirmed. Moreover, a statistically significant miR-25-3p downregulation in CM and MPM compared to BN was observed. miR-146a-5p resulted downregulated in MPM compared to both BN and CM. For miR-125b-5p, a similar expression level in CM and BN, and a trend toward increased expression in MPM was obtained. A significant

upregulation of miR-200b-3p and miR-205-5p was observed in MPM. A similar trend can be observed for miR-149-5p. As expected, the melanocyte specific, MITF-regulated miR-211-5p is progressively downregulated in multiple and single melanomas (Fig. 2).

The differential expression of miR-149-5p, miR-92b-3p, miR-200b-3p, miR-205-5p between paired first and second melanomas from the same patient is represented in Fig. 3. A significant upregulation for miR-149-5p, miR-92b-3p, miR-205-5p and miR-200b-3p in MPM 2nd tumor was confirmed in this larger group of samples.

### **Functional annotation of multiple primary melanoma miRNA signature**

The list of 22 miRNAs differentially expressed in multiple vs. single melanomas, was uploaded in Metacore software (Clarivate Analytics) to identify both the pathways that are significantly regulated by these miRNAs (**Supplementary Table 1, Additional file 1**) and the most significant miRNAs/targets networks (**Supplementary Fig. 1A, Additional file 2**).

Multiple primary melanomas were found to have a higher expression of miR-200 family, miR-205-5p and miR-149-5p compared to single CM and even nevi (Fig. 2 and Fig. 4). These microRNAs target ZEB1/TCF8 and ZEB2/SIP1 genes, and by doing so they inhibit the epithelial–mesenchymal transition (EMT) pathway. This pathway therefore appears to be specifically activated in single melanomas (Fig. 4).

From MetaCore network analysis, three hub genes (TLR4, ITGA6 and BTG2) were identified as targeted by multiple miRNAs, either up- or down-regulated in multiple melanomas. When we assessed the association of TLR4, ITGA6 and BTG2 gene expression with melanoma prognosis, we observed that their higher expression (median cutoff) was significantly associated with a worse overall survival in TCGA SKCM cohort of 458 samples (**Supplementary Fig. 1B, Additional file 2**).

### **IsomiRNA analysis revealed that miR-125a-5p isoforms are dysregulated in multiple primary melanoma**

Interestingly, miR-125a-5p differential expression in MPM was not confirmed by qPCR technology and we wondered about a possible explanation. We observed that the reads generated by the smallRNA sequencing experiment and attributed to mature miR-125a-5p following the standard matching pipeline were actually shorter by 1, or most frequently 2 nucleotides (lack of GA at the 3' end) in all samples (**Supplementary Fig. 2A, Additional file 3**). Although miRBase database reports a unique mature sequence for each miRNA, the so called canonical form, many evidences from deep sequencing experiments suggest that miRNAs have frequent modifications in length and sequence in human tissues. These miRNA isoforms are called isomiRs <sup>11</sup>.

We analyzed the isomiR expression level in all single and multiple primary CMs from our NGS experiments. We found 90 miRNAs with sequence and length heterogeneity, generating 324 different isomiRs, and 40 canonical microRNAs without any isomiR. In addition, we found 40 isomiRs named “orphan”, because their canonical miRNA sequences could not be detected. For each isomiR, we

calculated the average expression in melanoma samples and the ratio between each isomiR and its canonical miRNA. Finally, we obtained a panel of 17 miRNAs whose isoforms are 3- to 10-fold more abundant in melanoma than their canonical form (Table 4). Among them, hsa-miR-125a-5p|0|-2 isoform was differentially expressed in multiple vs. single primary melanomas and between the first and second tumor of the same patient (paired t-test  $P = 0.0006$ ). Unusually, miR-125a-5p canonical and 3' shorter isoforms show an opposite expression trend in nevi, single and multiple primary melanomas (Fig. 5A).

Table 4  
IsomiRs most represented in melanoma

<b>IsomiR</b>	<b>Type</b>	<b>IsomiR expression (mean)</b>	<b>Canonical miRNA expression (mean)</b>	<b>Ratio isomiR/canonical miRNA</b>
hsa-miR-141-3p 0 -1	end-site isomiR	200.33	8.65	23.15
hsa-miR-222-3p 0 +3	end-site isomiR	154.64	9.05	17.08
hsa-miR-30a-5p 0 +2	end-site isomiR	211.95	13.33	15.90
hsa-miR-125a-5p 0 -2	end-site isomiR	765.69	56.46	13.56
hsa-miR-30d-5p 0 +2	end-site isomiR	416.87	41.32	10.09
hsa-miR-10b-5p 0 -1	end-site isomiR	2950.88	357.98	8.24
hsa-miR-27a-3p 0 -1	end-site isomiR	167.78	30.55	5.49
hsa-miR-30a-5p 0 -2	end-site isomiR	72.12	13.33	5.41
hsa-miR-222-3p 0 +4	end-site isomiR	46.98	9.05	5.19
hsa-miR-19b-3p 0 -1	end-site isomiR	52.06	11.59	4.49
hsa-miR-30c-5p 0 +1	end-site isomiR	151.53	34.28	4.42
hsa-miR-26b-5p 0 +1	end-site isomiR	172.47	39.32	4.39
hsa-miR-222-3p 0 +2	end-site isomiR	36.83	9.05	4.07
hsa-miR-10a-5p 0 -1	end-site isomiR	636.58	184.52	3.45
hsa-miR-30a-5p 0 +1	end-site isomiR	45.68	13.33	3.43
hsa-miR-30d-5p 0 +1	end-site isomiR	140.07	41.32	3.39
hsa-miR-200b-3p 0 +1	end-site isomiR	44.86	13.26	3.38

We studied two different technical approaches for miRNA quantification based on qPCR (miRCURY LNA and miSCRIPT, both by Qiagen), to selectively quantify miR-125a-5p isoforms in all samples and validate the NGS data. Specifically, we used miR-125a-5p miRCURY LNA assay (Exiqon/Qiagen) for the quantification of the canonical, 24nt-long isoform (**Supplementary Fig. 2B, Additional file 3**). Results revealed a lack of variation of this mature isoform between single and multiple melanomas, and a higher expression in the first vs. second melanoma (Fig. 5B,C). To quantify the miR-125a-5p 22nt-long isoform, we selected the miSCRIPT assay by Qiagen. The assay can quantify both the long and short isoforms of miR-125a-5p due to the use of a universal 3' primer for miRNA amplification. Given the high predominance of the short isoform in our NGS data, we assumed this assay could provide a bona fide quantification of the short 22nt-long isoform (**Supplementary Fig. 2B, Additional file 3**). As expected, an increase in miR-125a-5p levels in MPMs vs. CMs and in the second tumor from the same patient was observed (Fig. 5B,C). We examined the expression of hsa-miR-125a-5p|0|-2 and 0|0 (WT) isoforms across TCGA tumor types and discovered an overall higher expression of the shorter form in human cancers and a specifically altered ratio of the two forms in SKCM (cutaneous melanoma cohort), which shows the largest variation (Fig. 6).

### miR-125a-5p 3' isomiR regulate novel target genes and pathways

We run a bioinformatics analysis to predict the impact of miR-125a-5p 3' isomiR generation in target gene binding. We used RNA22 algorithm to obtain the list of putative pairing sites for miR-125a-5p canonical form (WT, N = 1342) and its 3' isoform miR-125a-5p|0|-2 (ISO, N = 971) (**Supplementary Table 2, Additional file 4**). The predicted target genes were submitted for functional annotation to MetaCore website. The shorter miR-125a-5p isoform lose the ability to bind a fraction of canonical miR-125a-5p|0|0 targets. Significantly enriched pathways and networks were identified for common and specific targets of canonical miR-125a-5p canonical miR-125a-5p|0|0 and its 3' isomiR miR-125a-5p|0|-2 (**Supplementary Table 3, Additional file 5**) revealing a loss of targeting for miR-125a-5p|0|-2 of genes involved in nervous system development, neurogenesis and neuronal differentiation. IsomiR miR-125a-5p|0|-2 no longer targets key genes involved in cell adhesion and migration (Ephrin receptors, Netrin 1) or intracellular signaling (PIK3C2B).

## Discussion

The risk of melanoma development is influenced by environmental and genetic factors. Families with history of melanoma could have a germline mutation that confers hereditary susceptibility, and this is particularly demonstrated in families where more members develop multiple primary melanomas. In 1968, Lynch and Krush described the familial atypical multiple mole-melanoma (FAMMM syndrome) which encompasses an association between pancreatic cancer, multiple nevi, and melanoma<sup>38</sup>. In the 70 s Clark described a similar phenotype, the B-K mole syndrome, consisting of familial melanoma in the setting of numerous atypical nevi. In the early 1990's, germline mutations in the cell cycle gene, p16 (CDKN2A), were reported among a subset of FAMMM kindreds. Nowadays, most studies report a very low prevalence of CDKN2A /CDK4 in familial or multiple melanoma patients, especially in the Southern

Europe countries<sup>9</sup>. Though MPM patients often report similar sun exposure experiences, the high percentage of atypical nevi in these patients and their family members, the frequent family history of melanoma, as well as the early onset of melanoma (young age) suggest that predisposing factors for the development of multiple melanomas are involved. Regardless of family history, they are reported also cases of multiple primary melanoma in individuals without familial history of melanoma. In these cases, germline mutations in melanoma predisposing genes are rarely detected. Therefore, it is evident that some other genetic or epigenetic factor is active in multiple primary melanoma to fuel multiple events of melanocytic transformation.

In this study, we provide the first comprehensive molecular characterization of MPMs by assessing their miRNome with a smallRNA sequencing approach. The global microRNA expression reflects the mRNA expression of cells and tissues, with the advantage of being assessable in FFPE tissues. This analysis revealed a specific expression pattern of multiple melanoma tumors when compared to single cutaneous melanoma. MPM miRNome is more similar to benign nevi, thus suggesting a less aggressive and more differentiated phenotype. We validated a panel of microRNAs in additional samples, including also multiple tumors from the same patient, obtaining a panel of microRNAs differentially expressed in tumors from the same patient.

Prognosis of patients with multiple primary CM is an old, but still open, question<sup>27</sup>. Many studies have attempted to address this issue and results are still controversial, with studies stating that developing multiple melanomas is associated with worse prognosis or the opposite<sup>27,48,54</sup>. Recently, Grossman et al. (2018) revealed the potential reasons for these controversies by analyzing with proper multivariate statistical analysis the Surveillance, Epidemiology, and End Results (SEER) data using a single matching method<sup>21</sup> and demonstrating no substantial difference among single and multiple melanoma patients.

We provide here evidence that MPMs, from a biological point of view, have a less invasive phenotype as pointed out by the main regulatory pathways activated in these tumors, thus providing further elements of discussion to support MPM less aggressive evolution. It is worth mentioning that microRNAs known to inhibit epithelial-mesenchymal transition (e.g. miR-200 family, miR-205, miR-149) are more expressed in multiple primary melanoma compared to single melanoma. Tumor cells promote EMT to escape from the microenvironment and migrate to a new location to develop metastasis<sup>22</sup>. The acquisition of a mesenchymal phenotype promotes the production of extracellular matrix proteins, the resistance to apoptosis, the invasiveness and the migration<sup>30</sup>. EMT results from the loss of cell-to cell junctions, induced by the loss of E-cadherin; the process is mediated by transcription factors, including SNAIL, SLUG, SIP1, and E2A, and affected by regulatory proteins such as TGF $\beta$ , EGF, PDGF, ERK/MAPK, PI3K/AKT, SMADS, RHOB,  $\beta$ -catenin, LEF, RAS, C-FOS, integrins  $\beta$ 4 and integrin  $\alpha$ 5<sup>53</sup>., EMT has been reported in melanoma cells, despite their origin from neural crest-derived melanocytes. In fact, EMT promotes the metastatic phenotype of malignant melanocytes<sup>28</sup>. Moreover, melanocytes express E-cadherin, which mediates the adhesion between melanocytes and keratinocytes<sup>51</sup>. Many studies described the loss of E-cadherin in melanoma<sup>32,49</sup>, and CDH1 ectopic expression was associated with

the downregulation of adhesion receptors, such as MCAM/MUC18 and  $\beta$ 3 integrin subunit, resulting in suppression of melanoma cells invasion<sup>26</sup>. Hao et al. observed a switch from E-cadherin to N-cadherin expression in melanoma progression, a process regulated by PI3K and PTEN through TWIST and SNAIL<sup>23</sup>.

Consistently, we examined the main cellular hubs regulated by MPM specific miRNAs and discovered that they are centered in TLR4, ITGA6 and BTG2 proteins. MicroRNAs regulating these hubs are mostly downregulated in MPMs and high expression of these three genes is associated with a more favorable prognosis in TCGA SKCM cohort.

Integrin  $\alpha$ 6 (ITGA6), also known as CD49f, is a transmembrane glycoprotein adhesion receptor that mediates cell-matrix and cell-cell interactions. ITGA6 was identified and described as an important stem cell biomarker. Indeed, it is the only common gene expressed in embryonic stem cells, neural stem cells and hematopoietic stem cells<sup>28, 45</sup>. It is also expressed in more than 30 stem cell populations, including cancer stem cells<sup>31</sup>. ITGA6 can combine with other integrins such as integrin beta 1 and integrin beta 4 to form respectively integrin VLA-6 and TSP180. The role of ITGA6 in melanoma is not clear but our observation point toward its upregulation in MPMs upon miR-25 and 29 downregulation.

BTG2 is part of the anti-proliferative BTG/TOB family and its expression is p53-dependent<sup>47</sup>. This protein is involved in several cellular processes, including cell cycle regulation, DNA damage repair, cell differentiation, proliferation and apoptosis. However, its role is often cell-type dependent<sup>39</sup>. In fact, BTG2 inhibits proliferation and migration, acting as a tumor suppressor protein, in gastric cancer cells<sup>59</sup> and in lung cancer cells<sup>57</sup>, while in bladder cancer it promotes cancer cell migration<sup>55</sup>. In B16 melanoma cells it was shown that miR-21 promotes a metastatic behavior through the downregulation of many tumor suppressor proteins, including PTEN, PDCD4 and BTG2<sup>58</sup>. In MPMs, we observe the downregulation of several miRNAs targeting BTG2, including miR-21-5p, 146a-5p, 132-3p, 15a-5p. Therefore, an upregulation of BTG2 is to be expected.

Toll-like Receptor 4 (TLR4) belongs to TLR family and plays an important role in inflammation and cancer. TLR4 protein is expressed at very low levels in melanoma cells in vivo (Human protein atlas) but its activation has been reported to promote an inflammatory microenvironment and tumor progression in vitro<sup>20</sup>. In addition, TLR4 is associated with induction of proliferation and migration of melanoma cells<sup>50</sup>. TLR4 plays an important role in melanoma also because it interacts with TRIM44, a negative prognostic factor in melanoma. In particular, TRIM44 binds and stabilizes TLR4 leading to the activation of AKT/mTOR signaling, which results in EMT promotion<sup>56</sup>. This biological role for TLR4 in melanoma is partially in contrast with our observation of a better survival in melanoma patients with higher TLR4 levels.

Finally, we extended our molecular investigation to miRNA isoforms that were most abundant in our samples. According to the recent observation that miRNA isoforms can discriminate human cancers<sup>52</sup>,

we detected a relevant number of miRNA variants in our dataset of single and multiple melanomas. A specific isoform of miR-125a-5p, lacking 2 nucleotides at the 3'end, was detected as differentially expressed in MPMs. This isoform is highly abundant in melanoma, as we confirmed by analyzing its levels across 32 tumor types from TCGA database; and the ratio between miR-125a-5p isoform and canonical form is the broader in TCGA SKCM tumors (range 0.1–1100 times) and 2–6 logs more abundant in nevi and melanomas in our study. Moreover, we detected a specific dysregulation of the isoform, but not the canonical form, in multiple melanomas. Bioinformatic analyses revealed that miR-125a-5p shorted isoform loses the ability to target and regulate a group of genes specifically involved in cell adhesion and cell differentiation. Particularly relevant seems to be the lack of regulation of genes involved in neuronal differentiation. Indeed, miR-125a is the human ortholog of lin-4, the very first miRNA identified in *C. Elegans* in 1993<sup>34</sup>. In mammals, miR-125 is expressed in embryonic stem cells and promote cell differentiation. Specifically, miR-125 has a specific role in adult nervous system development and neuronal differentiation<sup>10,33</sup>. The imbalance between major miR-125 isoforms in melanocytes could reflect a major role for miR-125 in melanocyte development and differentiation from the neural crest<sup>42</sup>, differentiating this lineage from other common ancestor cells. A role that is consequently reflected in melanoma development and progression.

## Conclusions

Overall, we provide here a comprehensive characterization of microRNA/isomiRNA dysregulation and regulatory network in single and multiple primary melanomas. The pattern of miRNA alterations supports a less aggressive phenotype of multiple primary melanomas, whilst isomiR-125a-5p levels proved to be enriched in melanoma and differentially expressed in MPMs, thus confirming the relevance of small non-coding RNA alterations in this fascinating - but poorly studied - melanoma subtype. Our observations about non-random dysregulation of specific miRNA isoforms in melanoma pose the basis for additional functional studies.

## List Of Abbreviations

cutaneous melanoma (CM)

multiple primary melanomas (MPM)

benign nevi (BN)

miRNA isoforms (isomiRs)

ultraviolet (UV)

*CDKN2A* (cyclin-dependent kinase inhibitor 2A)

*CDK4* (cyclin-dependent kinase 4)

*MITF* (microphthalmia-associated transcription factor)

*POT1* (protection of telomeres 1)

microRNAs (miRNAs)

formalin-fixed and paraffin-embedded (FFPE)

hematoxylin-eosin (HE)

Reverse transcription (RT)

Small nuclear RNA U6 (RNU6)

reads per million (RPM)

Principal Component Analysis (PCA)

epithelial–mesenchymal transition (EMT)

Toll-like Receptor 4 (TLR4)

## **Declarations**

### **Ethics approval and consent to participate**

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was approved by the Ethics Committee Center Emilia-Romagna Region – Italy (417/2018/Sper/AOUBo).

### **Consent for publication**

All authors of the manuscript have read and agreed to its content.

### **Availability of data and material**

Next Generation Sequencing raw data (FASTQ format) are available through European Nucleotide Archive (ENA) with the following accession number: PRJEB35819.

### **Competing interests**

The authors declare no competing interests

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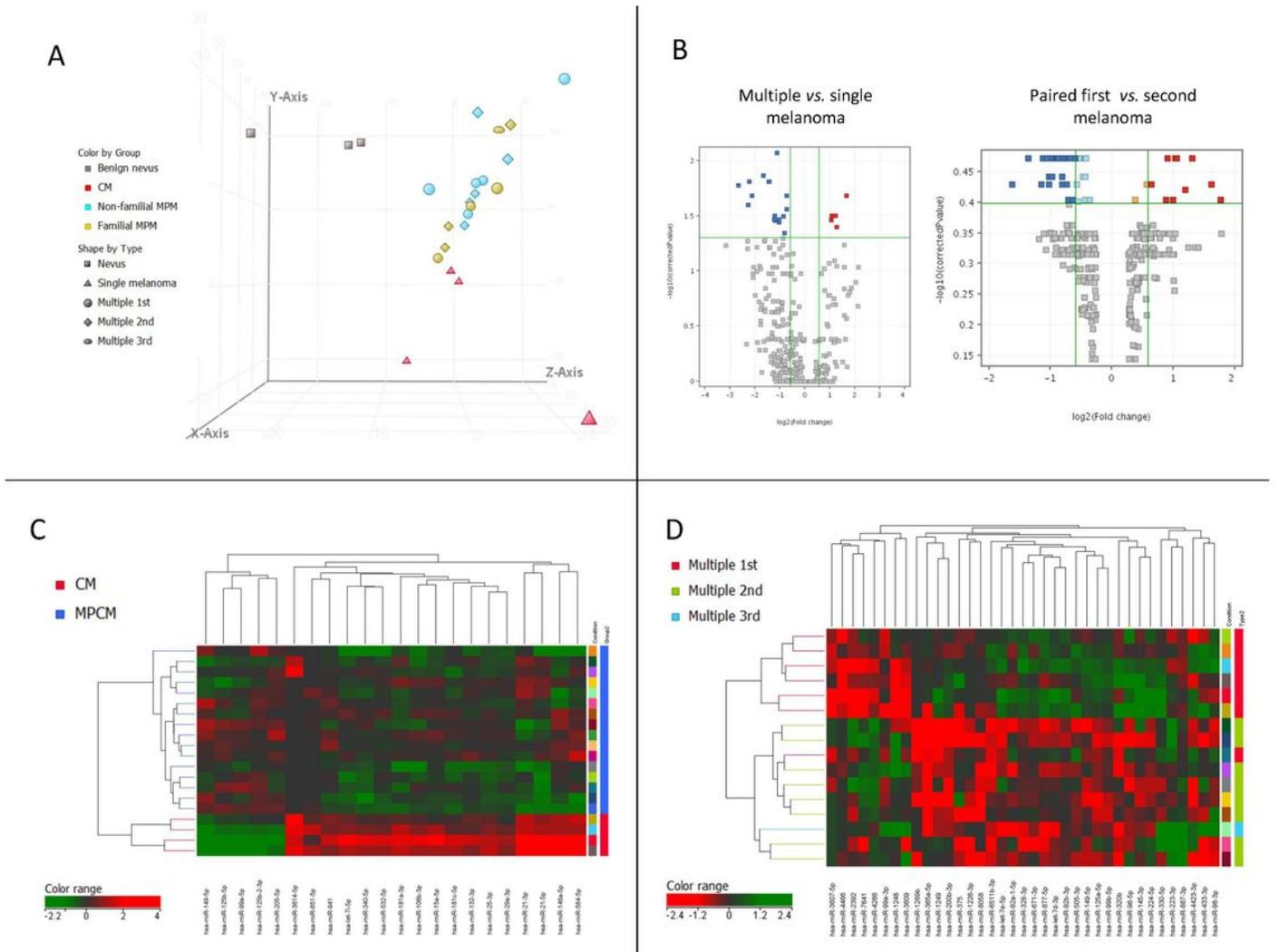
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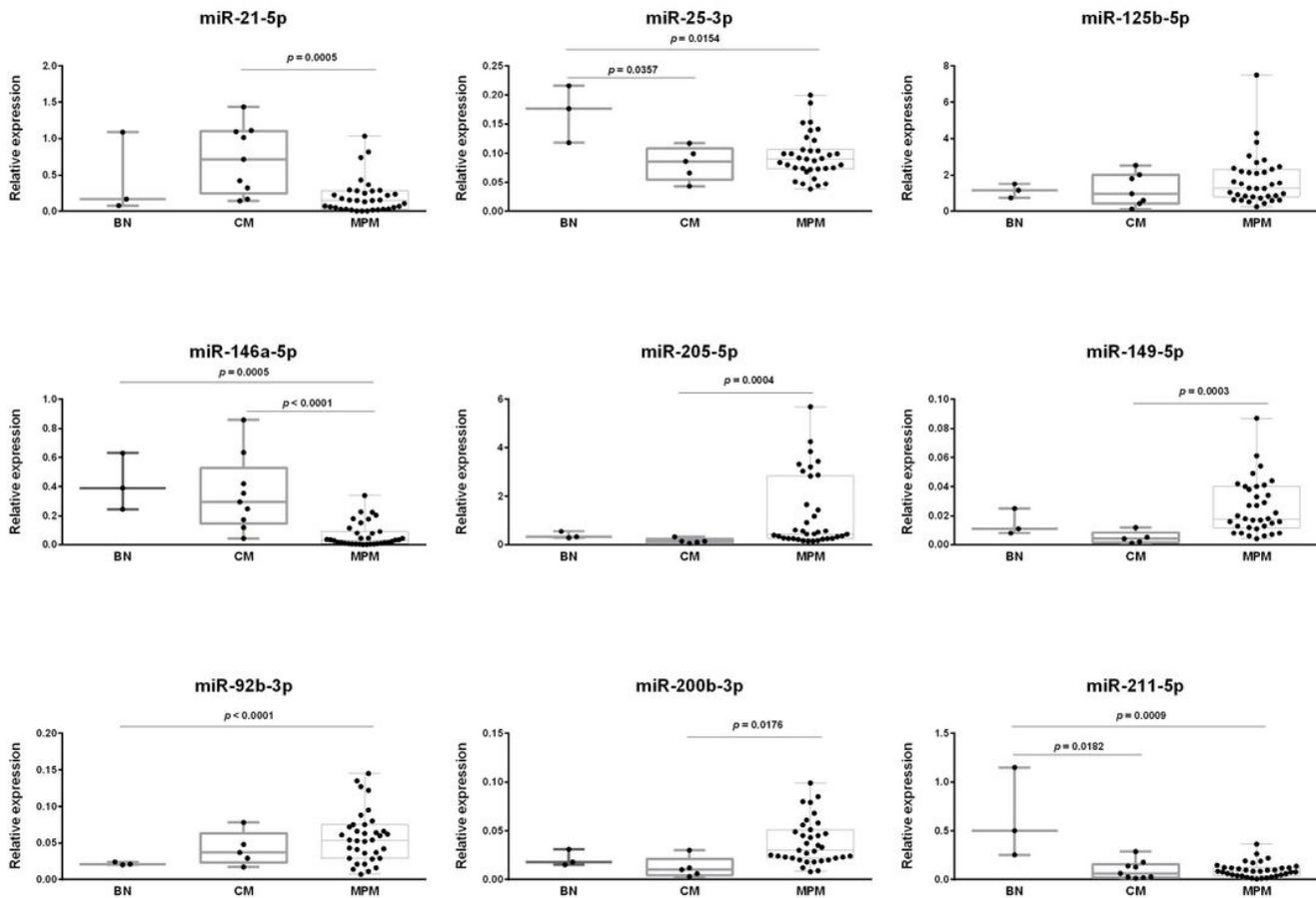
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## Figures



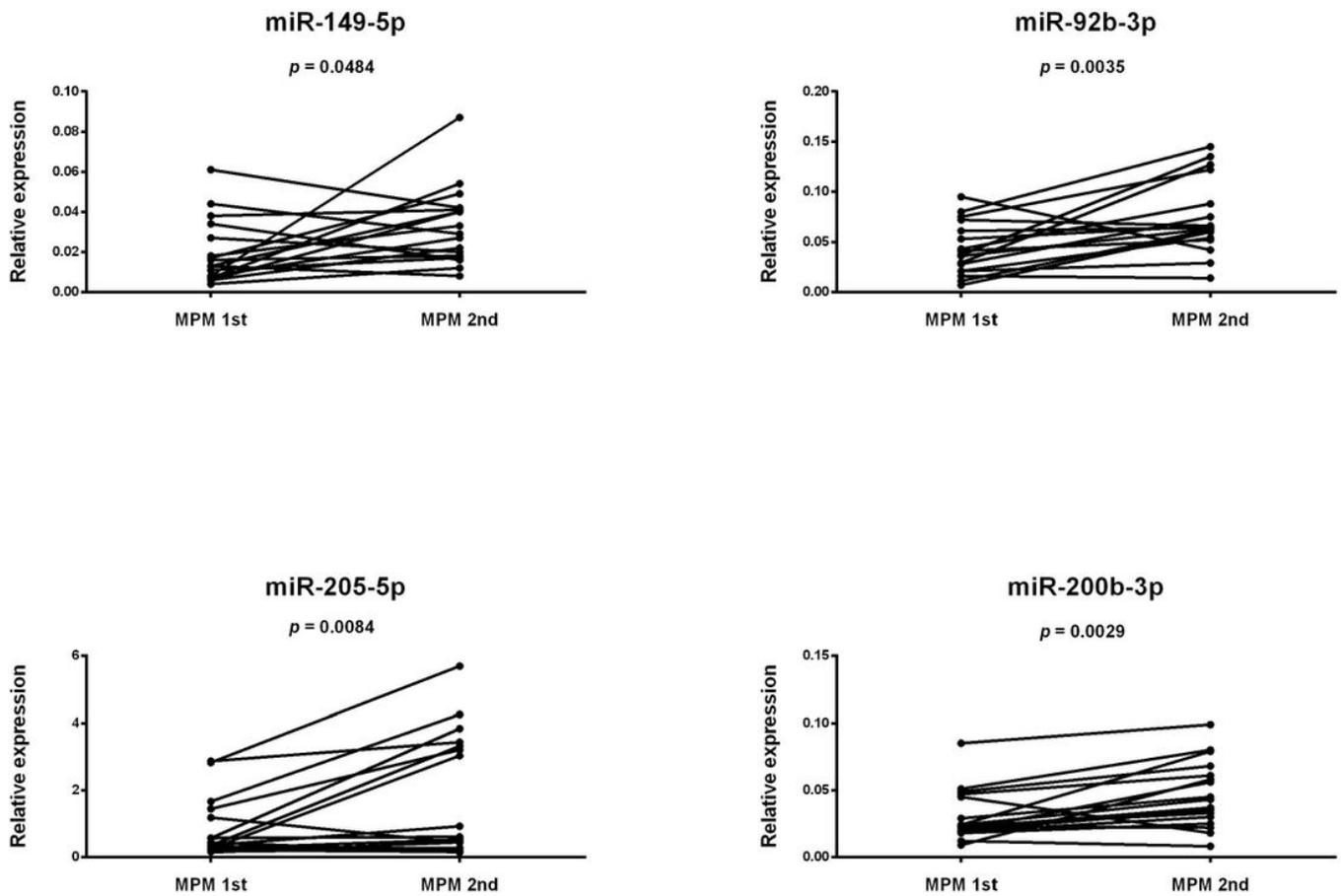
**Figure 1**

Principal Component Analysis (PCA) of samples analyzed by smallRNA sequencing. A) Familial (yellow) and non-familial (cyan) multiple primary melanomas display a similar microRNA profile, which is different from single cutaneous melanoma (red) and benign nevi (grey). B) Heatmap of multiple and single melanoma based on the expression of 22 differentially expressed miRNAs (moderated t-test, adjusted  $p < 0.05$ ). Red and green color represent the increased or reduced expression across samples. C) Heatmap of the first and second melanomas from the same patient based on the expression of 37 differentially expressed miRNAs (paired t-test,  $p < 0.05$ ). Red and green color represent the increased or reduced expression across samples. D) Volcano plot showing the differentially expressed miRNAs at the selected p-value and fold change combinations.



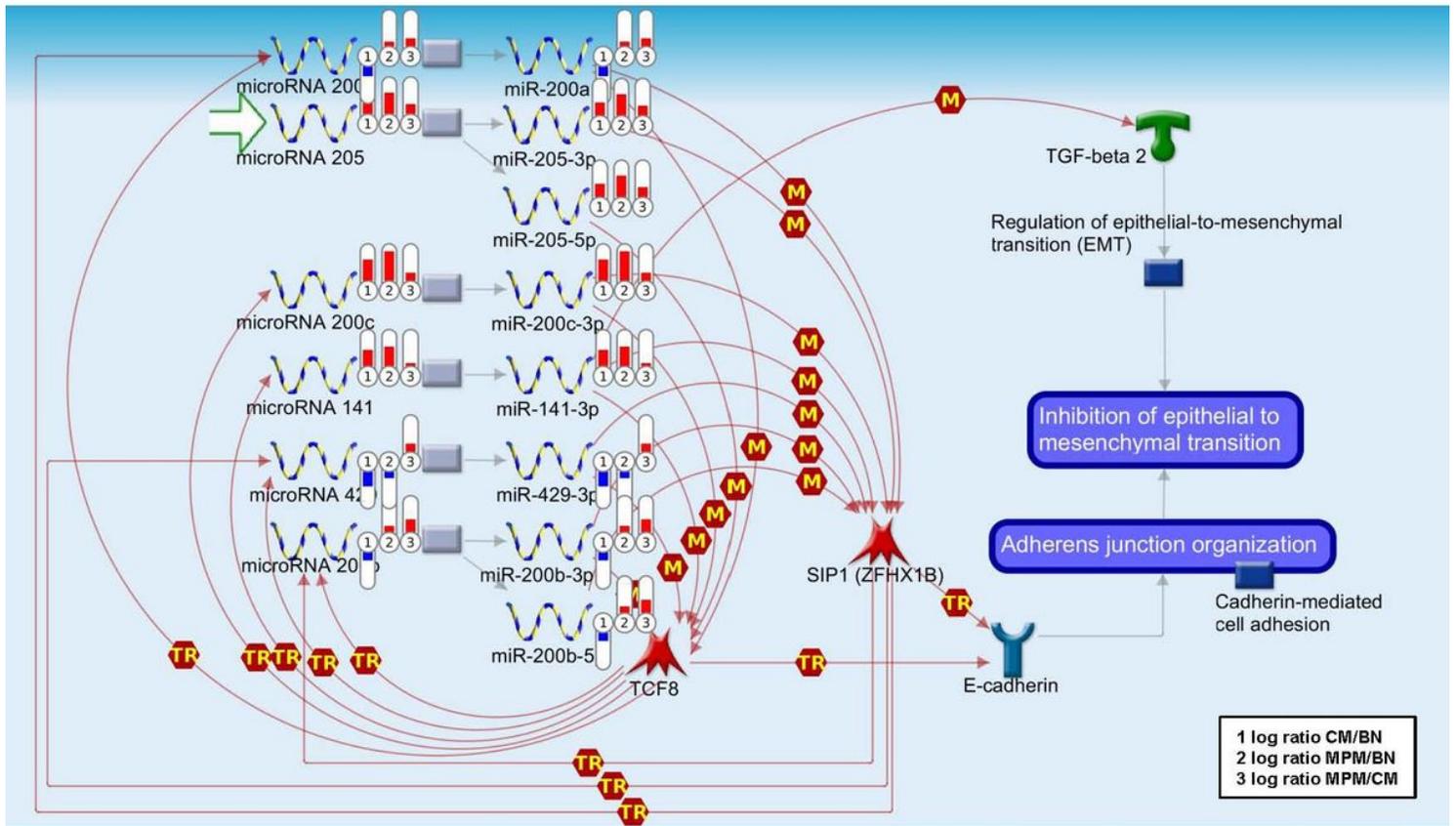
**Figure 2**

Differential microRNA expression in benign nevi (BN), cutaneous melanoma (CM) and in multiple primary melanoma (MPM). Dot plot representation of 9 microRNAs differentially expressed in single and multiple primary melanomas ( $P < 0.05$ ). MPM shows higher expression levels of miR-205-5p, miR-200b-3p, miR-149-5p compared to CM, and higher expression of miR-92b-3p compared to BN. MPM downregulates miR-21-5p compared to CM and miR-146a-5p compared to CM and BN. BN upregulates miR-25-3p and miR-211-5p compared to CM and MPM. Each miRNA was tested in triplicate by quantitative RT-PCR. Relative miRNA expression was normalized on invariant miR-16-5p.



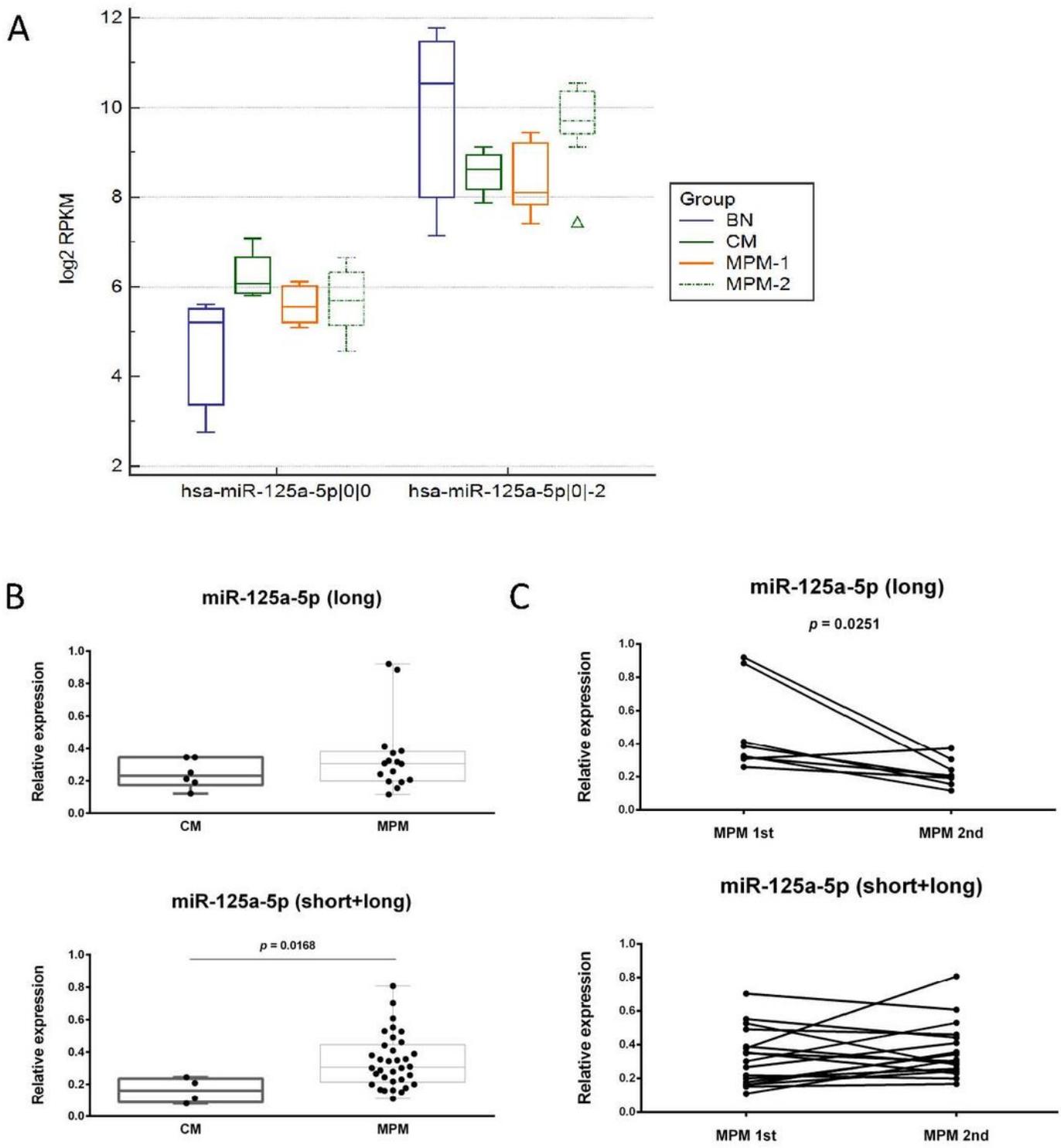
**Figure 3**

Differential microRNA expression in 1st vs. 2nd melanoma from the same patient. Before-after plot showing the paired expression of 4 selected microRNAs in 17 multiple primary melanoma (MPM) patients. miR-92b-3p, miR-205-5p, miR-200b-5p and miR-149-5p are significantly downregulated in the 1st melanoma compared to the 2nd melanoma. Each miRNA was tested in triplicate by quantitative RT-PCR. Relative miRNA expression was normalized on invariant miR-16-5p. Paired P-value is reported.



**Figure 4**

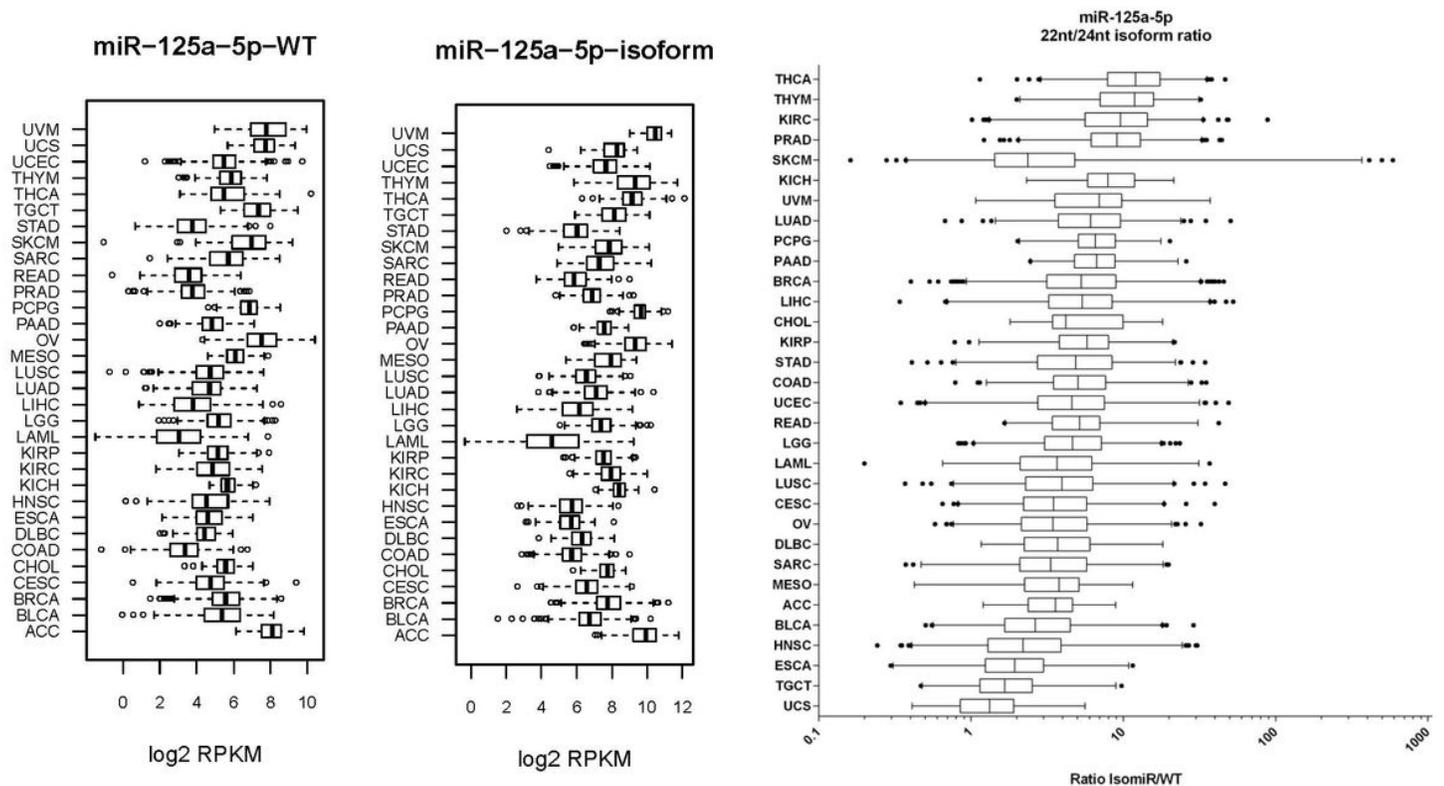
MetaCore pathway analysis showing the involvement of differently expressed miRNAs in epithelial-mesenchymal transition (EMT). EMT pathway representation with regulating miRNAs. Log ratio of miRNA expression level in CM/BN (1), MPM/BN (2), MPM/CM (3) is visualized on the maps as a thermometer-like figure. Upward thermometers have red color and indicate upregulated signals and downward (blue) ones indicate downregulated expression level of specific microRNAs. MPM showed higher expression levels of microRNAs involved in the inhibition of epithelial-mesenchymal transition (EMT), including miR-205-5p and miR-200b-3p. (BN, benign nevi; CM, cutaneous melanoma; MPM multiple primary melanoma).



**Figure 5**

Comparison of canonical and isomiR miR-125a-5p expression trends in benign nevi (BN), cutaneous melanoma (CM) and multiple primary melanoma (MPM). A. Box-plot graph of canonical miR-125a-5p (hsa-miR-125a-5p|0|0) showing a lower expression level and opposite expression trend in BN, CM and MPM if compared to its shorter isomiR (hsa-miR-125a-5p|0|-2). B. Dot plot representation of canonical miR-125a-5p expression assessed with miRCURY LNA assay, which is specific for the canonical form,

and overall expression of all miR-125a-5p isoforms, detected using miSCRIPT assay. Results show that the combined expression of miR-125a-5p isoforms of levels is higher in MPM compared to CM. C. Before-after plot of canonical miR-125a-5p expression (miRCURY LNA assay) and all miR-125a-5p isoforms (miSCRIPT assay) shows an opposite trend in 1st and 2nd melanoma from the same patient.



**Figure 6**

Canonical (wild-type) and isomiR analysis of miR-125a-5p across 32 TCGA tumor types. miR-125a is most expressed in many cancer types. Box plots of canonical (WT) and shorter isoform of miR-125a-5p show variable expression levels, represented here as log<sub>2</sub> RPKM data, across 32 different cancer type. miR-125a-5p isoform is most abundant in many cancer types, and shows a specifically high canonical/isoform ratio in melanoma (SKCM) group. TCGA abbreviations: ACC, adrenocortical carcinoma; BLCA, bladder urothelial carcinoma; BRCA, breast invasive carcinoma; CESC, cervical squamous cell carcinoma and endocervical adenocarcinoma; CHOL, cholangiocarcinoma; COAD, colon adenocarcinoma; DLBC, lymphoid neoplasm diffuse large B-cell lymphom; ESCA, esophageal carcinoma; HNSC, head and neck squamous cell carcinoma; KICH, kidney chromophobe; KIRC, kidney renal clear cell carcinoma; KIRP, kidney renal papillary cell carcinoma; LAML, acute myeloid leukemia; LGG, brain lower grade glioma; LIHC, liver hepatocellular carcinoma; LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma; MESO, mesothelioma; OV, ovarian serous cystadenocarcinoma; PAAD, pancreatic adenocarcinoma; PCPG, pheochromocytoma and paraganglioma; PRAD, prostate adenocarcinoma; READ, rectum adenocarcinoma; SARC, sarcoma; SKCM, skin cutaneous melanoma; STAD, stomach adenocarcinoma; TGCT, testicular germ cell tumors; THCA, thyroid carcinoma; THYM,

thymoma; UCEC, uterine corpus endometrial carcinoma; UCS, uterine carcinosarcoma; UVM, uveal melanoma.

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