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## Identification of a novel microRNA cluster *miR-193b-365* in Multiple Myeloma

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### Abstract

There is accumulating evidence that the patterns of altered expression of microRNAs (miRNAs) correlate with distinct types of hematological malignancies. There is also emerging evidence for such miRNAs expression in multiple myeloma (MM), but the identity of the miRNAs that are abnormally expressed in this disease has not been elucidated. In the current study we examined the miRNAs perturbed in CD138 positive MM cell lines and CD138 positive primary MM cells, using microarray analysis and quantitative real-time PCR. The expression levels in these cells were compared to the expression levels in CD138 positive plasma cells isolated from bone marrows of healthy donors. Our data demonstrate that multiple new miRNAs are commonly up-regulated and down-regulated including a new miRNA cluster, *miR-193b-365*, composed of two paralogs that were significantly up-regulated in MM. The organization and the sequences of the new *miR-193b-365* cluster are highly conserved among vertebrates. Given that dysregulation of *miR-193b-365* cluster has not been identified in other hematological malignancies examined to date our findings suggest that *miR-193b-365* cluster is part of the unique miRNA signature in MM.

### Keywords

Multiple Myeloma; microRNA; *miR-193b-365* cluster

### Introduction

Multiple myeloma (MM) is characterized by proliferation of malignant plasma cells in the bone marrow, secretion of monoclonal protein in the blood and/or urine and skeletal destruction with osteolytic lesions [1]. In MM patients the number of plasma cells are elevated compared to the numbers in normal bone marrow and can be identified by their expression of syndecan-1 (CD138) surface protein. Although increased plasma cell content signifies a common feature in myeloma, the overall biology of MM is quite complex. Therefore elucidation of regulatory circuits altered in myeloma such as microRNAs (miRNAs) will allow us to gain a better understanding into the biology of MM.

miRNAs are recently discovered class of 19–25 nucleotides of small non-coding RNA molecules that are evolutionally conserved and play important regulatory functions by targeting mRNAs for transcriptional or translational repression [2]. Studies in model organisms have revealed that miRNAs are involved in the control of cellular processes, such as differentiation,

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development, cell growth, and apoptosis, as well as in various human diseases including cancer [3,4]. While several miRNAs have been reported to show differential expression pattern in hematological malignancies and solid tumors very little is known with respect to specific miRNAs that are perturbed in MM. In a recent study it was shown that several miRNA clusters were perturbed in myeloma using relatively small number (345) of capture probes [3–6]. In the current study using the new locked nucleic acid technology comprising 757 capture probes and quantitative real-time PCR (Q-PCR) we have carried out a comprehensive analysis of miRNAs that are perturbed in MM. These studies allowed us to identify *miR-193b-365* as a highly up-regulated cluster in MM. In addition using the same array technology we identified several new commonly up-regulated miRNAs and a smaller set of commonly down-regulated miRNAs in myeloma plasma cells that has not been recognized in previous studies.

## MATERIALS AND METHODS

**Patient specimens, Cell lines and Cell culture**—For microarray and Q-PCR study, we used Trizol™ for RNA isolation from CD138 positive MM cell lines (L363, KMS11, JLN3, RPMI-8226, U266, NCI-H929 and MM.1S), purified CD138 positive plasma cells from newly diagnosed MM patients (10 patients) and purified CD138 positive plasma cells (> 90%) from two healthy donors. L363, KMS11 and JLN3 cells were kindly provided by Dr. Leslie Brents (NIH, Bethesda, MD). MM.1S cells were kindly provided by Drs. Steven T. Rosen and Nancy Krett (Northwestern University, Chicago, IL). U266, NCI-H929 and RPMI-8226 cells were purchased from ATCC. All MM cell lines were maintained in RPMI-1640 containing 10% FBS, and 100 Units/ml penicillin and 100 µg/ml streptomycin (GIBCO). 2.5 µg/ml fungizone (Invitrogen) and 5 µg/ml plasmocin (InvivoGen) were added for MM.1S cells as additional antibiotics. The patient samples were obtained after informed consent and approval from the University of Chicago Institutional Review Board. Plasma cells from patient samples were isolated by CD138 selection using EasySep® Kit (Stem Cell Technologies, BC, Canada). Total RNAs of purified CD138 positive cells from healthy donors were purchased from All Cells, CA, USA.

**MiRNA microarray experiments and Data analysis**—MiRNA expression was determined using the miRCURY™ LNA (Locked Nucleic Acid) microarray (Exiqon, Vedbaek, Denmark). All miRNAs were isolated by Trizol™ and miRNeasy Mini Kit (Qiagen) was used to eliminate organic solvents. The quality of the RNA was verified by a 2100 Bioanalyzer (Agilent Technologies, CA, USA). The miRCURY™ LNA array used in this study contained 757 annotated human miRNA capture probes spotted in four subgrids, listed in the miRBase 11.0 version of the Exiqon array. After the hybridization the slides were scanned by the Agilent G2565BA Microarray Scanner System (Agilent Technologies) and image analysis was carried out using the ImaGene 8.0 software (BioDiscovery, CA, USA). The slides were pre-processed in ImaGene to ensure uniformity of hybridization before being subject to data analysis. Background correction was performed to remove nonbiological contributions to the measured signal [7]. The quantified signals were normalized using the global Lowess (LOcally WEighted Scatterplot Smoothing) regression algorithm [8] excluding spots that were flagged due to poor spot morphology. Hy3 and Hy5 labeling dyes were used to label the samples and the common reference pool respectively. The common reference pool represents a pool of all samples used in the miRNA study which contained two hundred nano grams of each sample labeled with Hy5. The common reference pool is specifically used in two-color cDNA microarray experiments for adjustment of experimental variation between slides. This approach named common reference design enabled us to compare directly between samples (Hy3) since a common factor is made (Hy5) [9,10]. The normalized Hy3- and Hy5-signals were presented as four (Hy3 (sample)/Hy5 (common reference pool)) ratios per probe ID. The median of the four ratios per probe ID was calculated and log<sub>2</sub>-transformed. Prior to the data analysis of the normalized signals two specific criteria were evaluated. The criteria for deciding that a miRNA

(probe ID) had failed on a particular array, is that 3 or more of the 4 replicated measures of this miRNA were flagged (ie. Poor spot morphology). Further, all capture probes with both Hy3- and Hy5-median signals lower than 1.5 X the median signal intensity of all probes on the given slide is excluded during further analysis.

After the extraction of normalized data, a candidate list of miRNAs was generated based on the filtering criteria on variation across all samples being a standard deviation (SD) >0.50 [11]. All genes SD>0.5 were selected as candidate genes. The cut-off SD > 0.5 is an arbitrary criteria corresponding to a regulation > 2 fold change when comparing the lowest and highest log<sub>2</sub>-transformed ratio per miRNA. This arbitrary cut-off approach is used when a group of samples do not fulfill at least 3 biological replicates used for statistical approaches. The clustering was performed on log<sub>2</sub> (Hy3 (sample)/Hy5 (common reference pool)) ratios which passed the filtering criteria on variation across samples; SD > 0.50 [11].

**Quantitative real-time PCR**—Reverse transcription (RT) and real-time PCR for miRNAs expression were performed using TaqMan MicroRNA Assays (Applied Biosystems), TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems), and TaqMan® Universal PCR Master Mix, No AmpErase® UNG (Applied Biosystems). TaqMan MicroRNA Assays, namely: *hsa-miR-193b* (Part No. 4395478), *hsa-miR-365* (Part No. 4373194) and *RNU6B* (Part No. 4373381) were used with amplification efficiencies of 100% for real-time PCR. RT and real-time PCR were carried out according to the manufacturer's instructions. Briefly, cDNAs were synthesized from total RNA using gene-specific primers. Reverse transcriptase reactions contained 5 ng of RNA samples, 7 µl of RT master mix including 0.15 µl of 100 mM dNTP mix, 1 µl of 50 U/µl MultiScribe™ Reverse Transcriptase, 1.5 µl of 10X Reverse Transcriptase Buffer, 0.19 µl of 20 U/µl RNase Inhibitor and 4.16 µl of Nuclease-free water, and 3 µl of stem-loop RT primer. The 15 µl of reactions were incubated for 30 min at 16°C, 30 min at 42°C, 5 min at 85°C, and then held at 4°C. Real-time PCR was performed using a Bio-Rad MiniOpticon Real-Time PCR Detection System (Bio-Rad, CA, USA). The 20 µl of PCR included 1 µl of TaqMan MicroRNA Assay, 1.33 µl of RT product, 10 µl of TaqMan 2X Universal PCR Master Mix, No AmpErase UNG and 7.67 µl of Nuclease-free water. Reactions were incubated in optical tubes at 95°C for 10 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 60 sec. All TaqMan MicroRNA Assays were designed with same conditions. All experiments were done in triplicate and data were calculated using the comparative Ct (2<sup>-ΔΔCt</sup>) method. U6 snRNA (*RNU6B*) was used as an internal control to normalize RNA input in the Q-PCR assay.

## RESULTS AND DISCUSSION

In order to identify commonly-perturbed miRNAs in myeloma cells we first carried out global miRNAs expression using 7 myeloma cell lines, purified plasma cells from 2 MM patients and 2 healthy donors, which lead to the identification of 22 commonly up-regulated and 6 commonly down-regulated miRNAs in myeloma cells (MM patient samples and MM cell lines) compared to the healthy donor samples (Tables I and II). The heat map and unsupervised hierarchical clustering of miRNAs shown in Figure 1A allowed us to identify 4 clusters, which were located in distinct chromosomes. Of the four miRNA clusters, *miR-17-92*, *miR-106b-25* and *miR-106a-92* were also identified in another study whereas *miR-193b-365* is a novel cluster that was not examined in that study or any other study in the context of myeloma [6]. The *miR-193b-365* cluster we have identified here has not been previously implicated in hematological malignancies. However, *miR-193b* and *miR-365* are highly perturbed in endometrioid adenocarcinoma and breast cancer respectively [12,13]. Therefore, our report provides the first evidence of up-regulation of *miR-193b* and *miR-365* in hematological malignancies. With respect to the other three clusters overexpression of *miR-17-92* cluster was reported in several hematological malignancies including Burkitt lymphoma,

mantle cell lymphoma, diffuse large B-cell lymphoma, acute lymphocytic leukemia, *MLL*-rearrangement leukemias and most recently in myeloma [6,14–16], in addition to various solid tumors including lung cancer [17,18]. On the other hand *miR-106b-25* cluster was up-regulated mostly in solid tumors such as in gastric cancers [19]. *miR-17-92* and *miR-106b-25* clusters have been suggested to function as oncogenic miRNAs over the MYC/E2F1/TGF $\beta$  network [20]. *miR-106a-92* cluster has been reported to be overexpressed in leukemia as well as in certain solid tumors such as colon and prostate [17,21,22].

The fold increase in expression of this new cluster, *miR-193b* and *miR-365*, ranged from 2–6 fold depending on the myeloma sample compared to CD138 plasma cells isolated from the 2 healthy donors (Figure 1B). To further confirm that up-regulation of this cluster was common in myeloma, we carried out Q-PCR using RNA isolated from a total of 10 (CD138 purified) patient samples along with RNA isolated from 2 healthy donors. As shown in Figure 2 by Q-PCR we observed 2–12 and 2–7 fold increase in expression of *miR-193b* and *miR-365* respectively depending on the patient samples. In addition to this novel cluster we have identified several new miRNAs that are up-regulated and a smaller set of miRNAs that are down-regulated in myeloma cells using the same version 11.0 array in our studies (Tables I and II).

In order to obtain further insight into the organization and sequence conservation of *miR-193b-365* cluster we performed cluster and nucleotide sequence alignment of multiple species respectively. The organization of *miR-193b-365* cluster was highly conserved among vertebrates such as Human, Chimpanzee, Macaque, Mouse, Chicken and Zebrafish (Figure 3A). It was not conserved in Fly, Worm and Yeast suggesting that organization of this cluster evolved recently and is important in vertebrates. Furthermore, we investigated sequence alignment of mature *miR-193b* and *miR-365* and their homologues among vertebrates and flies which revealed that the *miR-193b* sequence was highly conserved with the exception of one nucleotide and *miR-365* sequence was perfectly conserved among vertebrates but not in *Drosophila* (Figure 3B). In order to obtain insight into whether *hsa-miR-193b* and *hsa-miR-365* are paralogs that may act in concert to target the same mRNA or mRNA family, we next searched similarity of mature *hsa-miR-193b* and *hsa-miR-365* sequences using ClustalW2 analysis program. This analysis showed a 57.7% similarity between the two sequences suggesting that *miR-193b* and *miR-365* potentially can cooperate to target some of mRNA families in order to synergize the phenotypic outcome (Figure 3C).

In the current study we were successful in identifying a common set of miRNAs that are perturbed in myeloma by considering microarray and Q-PCR data obtained from every sample examined without discarding any data. This approach allowed us to identify a cluster that has not been previously recognized in hematological malignancies. Furthermore we were able to identify multiple new miRNAs and confirm several other clusters that have been identified recently in myeloma. Although the new miRNAs identified by this study that is not part of the *miR-193b-365* cluster need to be further tested using a large number of primary myeloma samples, it provides us the basis for future studies to understand the role/s played by specific miRNAs in regulating gene transcription in MM.

## Acknowledgments

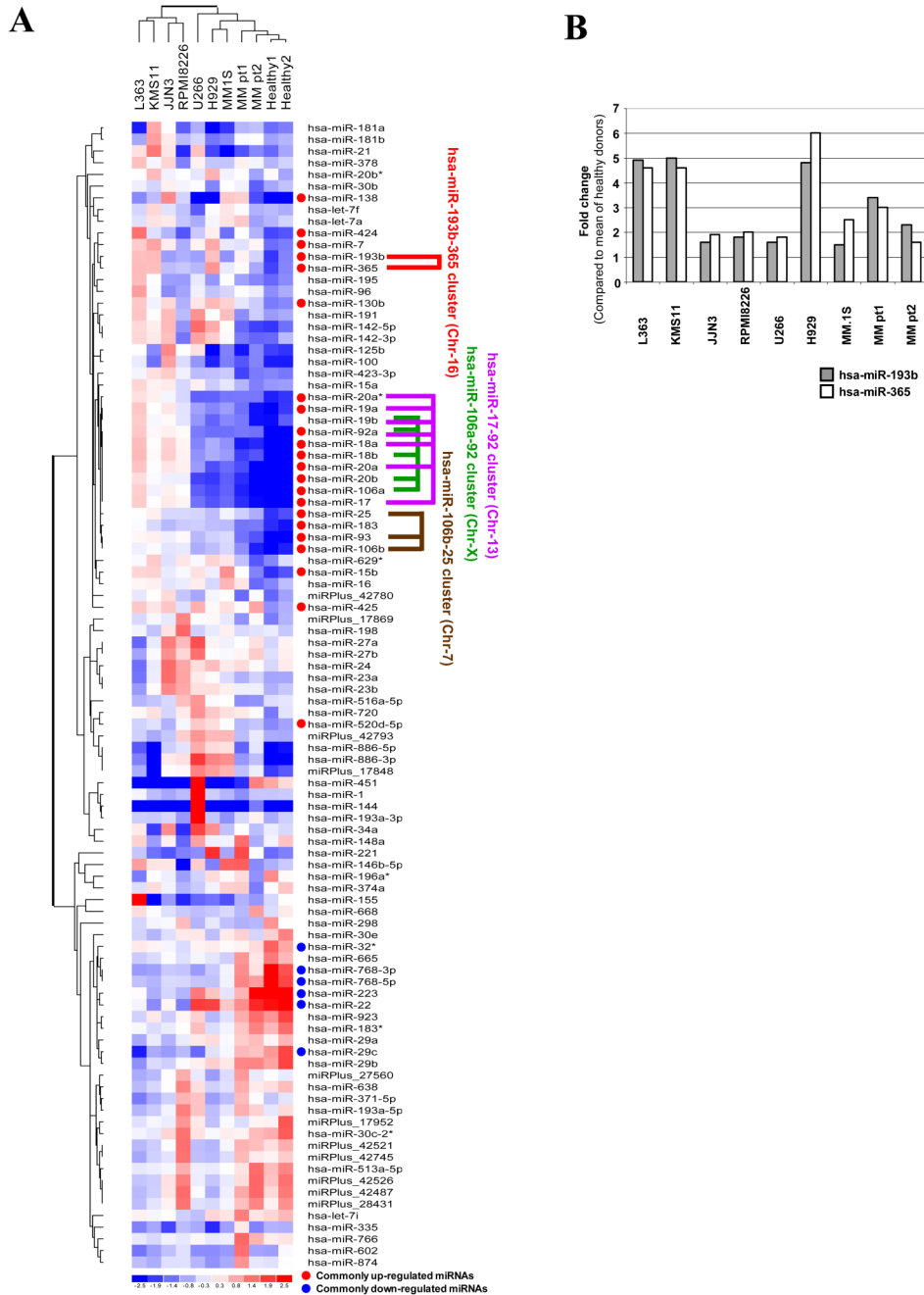
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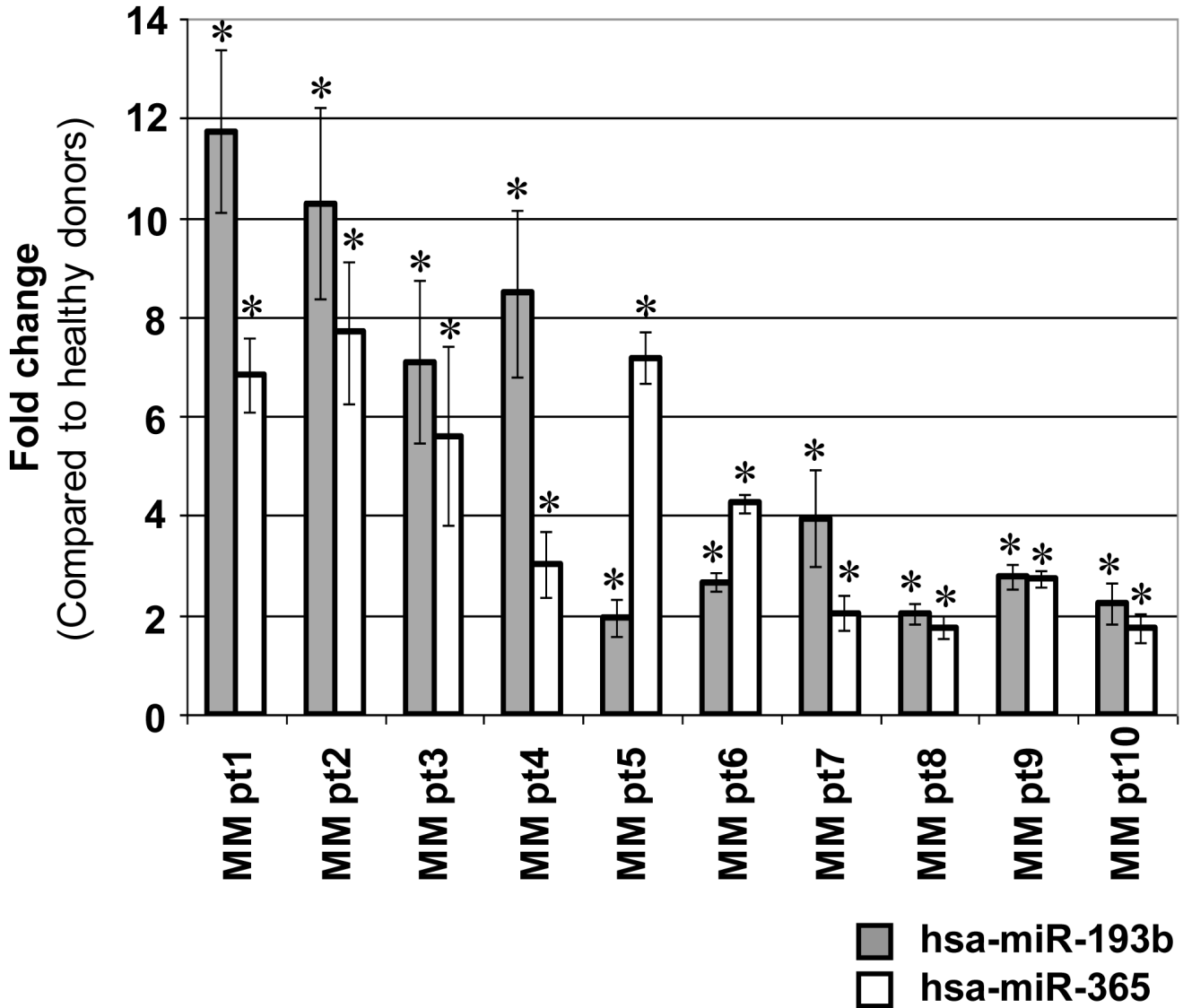
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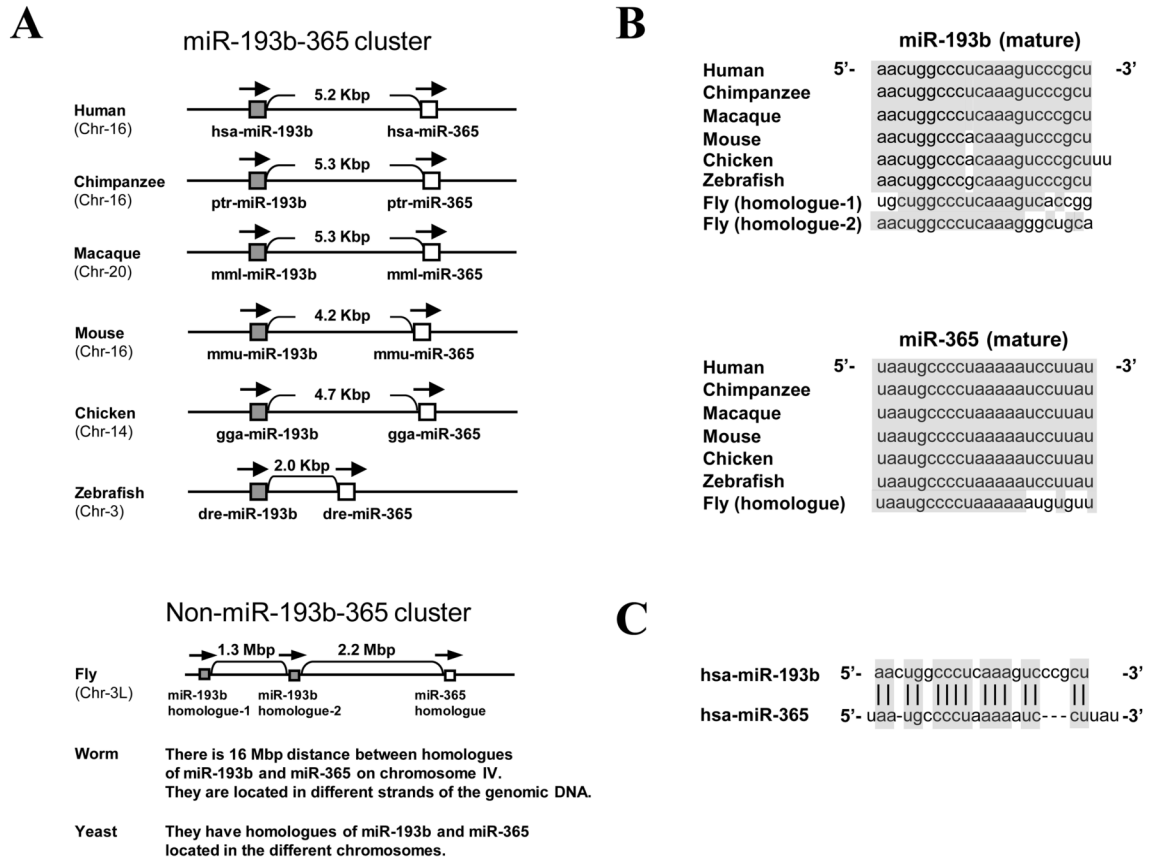
**Figure 1.** Differential expression of miRNAs in multiple myeloma. (A) Heat map and unsupervised hierarchical clustering. The clustering was performed on log<sub>2</sub> (Hy3 (sample)/Hy5 (common reference pool)) ratios which passed the filtering criteria on variation across samples; standard deviation >0.50. miRNAs that are commonly up-regulated and are commonly down-regulated in CD138 positive myeloma cells compared to CD138 positive cells from healthy donors are indicated in addition to identified miRNA clusters. (B) Expression of the *hsa-miR-193b-365* cluster in MM cells (CD138) compared to CD138 positive cells from healthy donor. The data were plotted after conversion of log ratios to fold changes. The mean value of two healthy samples was used as a control.



**Figure 2.**

Expression of *hsa-miR-193b* and *hsa-miR-365* in purified CD138 positive cells from 10 MM patients determined by Q-PCR. The data are shown as fold change in expression of *hsa-miR-193b* and *hsa-miR-365* compared to expression levels observed in purified CD138 positive cells of two pooled healthy donors. Q-PCR was done in triplicate and data were calculated using the comparative Ct ( $2^{-\Delta\Delta Ct}$ ) method. U6 snRNA was used as an internal control. All Q-PCR data were statistically significant (\* $P < 0.05$ ). The P values of *hsa-miR-193b* and *hsa-miR-365* for each myeloma patient sample were 0.008 and 0.005 (MM pt1), 0.014 and 0.015 (MM pt2), 0.023 and 0.047 (MM pt3), 0.016 and 0.032 (MM pt4), 0.046 and 0.002 (MM pt5), 0.004 and 0.001 (MM pt6), 0.034 and 0.036 (MM pt7), 0.014 and 0.029 (MM pt8), 0.007 and 0.003 (MM pt9), 0.038 and 0.044 (MM pt10) respectively. The mean signal intensities for *hsa-miR-193b* in myeloma patient samples and healthy donors were 429 and 157 respectively. The mean signal intensities for *hsa-miR-365* in myeloma patient samples and healthy donors were 351 and 135 respectively.





**Figure 3.** Organization and sequences of *miR-193b-365* cluster is highly conserved among vertebrates. (A) Conservation of the *miR-193b-365* cluster among vertebrates. Using miRBase (version 12.0) (<http://microrna.sanger.ac.uk/sequences/index.shtml>), FlyBase (<http://flybase.org/>), WormBase (<http://www.wormbase.org/>) and *Saccharomyces* Genome Database (<http://www.yeastgenome.org/>), conservation of the *miR-193b-365* cluster was investigated among organisms. (B) Sequence alignment of mature *miR-193b* and *miR-365* and their homologues among vertebrates and fly. Using miRBase (version 12.0) and FlyBase, mature *miR-193b* and *miR-365* sequences and their homologues were confirmed among vertebrates and fly. (C) *miR-193b-365* cluster composed of two paralogs; *miR-193b* and *miR-365*. Similarity of mature *miR-193b* and *miR-365* sequences was searched using ClustalW2 (<http://www.ebi.ac.uk/Tools/clustalw2/index.html>) program.

Table I

Fold increase in expression of commonly up-regulated miRNAs in MM compared to samples from healthy donors.

microRNAs	L363	KMS11	JJN3	RPM18226	U266	H929	MM.LS	MM pt1	MM pt2	SD
<b>hsa-miR-138</b>	<b>4.8</b>	<b>6.8</b>	<b>23.7</b>	<b>7.9</b>	<b>1.7</b>	<b>1.8</b>	<b>15.5</b>	<b>14.9</b>	<b>3.2</b>	<b>1.63</b>
hsa-miR-106a	17.0	11.1	13.6	12.4	4.0	3.5	4.2	2.7	1.7	1.49
hsa-miR-20a	15.8	10.4	12.5	11.7	5.3	3.2	3.9	3.1	1.6	1.45
<b>hsa-miR-17</b>	<b>16.1</b>	<b>11.3</b>	<b>13.2</b>	<b>12.2</b>	<b>4.4</b>	<b>3.5</b>	<b>4.1</b>	<b>2.7</b>	<b>2.1</b>	<b>1.44</b>
<b>hsa-miR-18a</b>	<b>13.7</b>	<b>8.9</b>	<b>11.8</b>	<b>9.4</b>	<b>3.8</b>	<b>5.0</b>	<b>4.1</b>	<b>1.8</b>	<b>2.0</b>	<b>1.37</b>
<b>hsa-miR-20b</b>	<b>13.2</b>	<b>10.2</b>	<b>9.3</b>	<b>9.3</b>	<b>2.9</b>	<b>2.7</b>	<b>3.5</b>	<b>2.5</b>	<b>1.8</b>	<b>1.34</b>
<b>hsa-miR-18b</b>	<b>11.7</b>	<b>7.5</b>	<b>10.0</b>	<b>7.9</b>	<b>3.4</b>	<b>4.1</b>	<b>3.6</b>	<b>1.9</b>	<b>2.3</b>	<b>1.25</b>
hsa-miR-93	7.7	8.1	6.5	7.6	4.5	5.3	5.7	2.8	2.3	1.13
hsa-miR-92a	9.3	6.8	7.1	6.6	2.6	2.4	2.4	2.6	2.4	1.11
<b>hsa-miR-19a</b>	<b>8.0</b>	<b>5.4</b>	<b>6.4</b>	<b>4.5</b>	<b>2.8</b>	<b>2.4</b>	<b>2.8</b>	<b>2.0</b>	<b>1.1</b>	<b>1.08</b>
<b>hsa-miR-424</b>	<b>8.9</b>	<b>2.7</b>	<b>2.0</b>	<b>1.1</b>	<b>2.3</b>	<b>3.8</b>	<b>1.3</b>	<b>3.3</b>	<b>2.0</b>	<b>0.97</b>
hsa-miR-15b	4.9	5.0	3.2	3.5	4.5	3.3	7.8	3.7	1.6	0.97
hsa-miR-106b	5.0	5.5	4.7	4.9	3.2	3.6	4.3	2.6	1.2	0.96
<b>hsa-miR-193b</b>	<b>4.9</b>	<b>5.0</b>	<b>1.6</b>	<b>1.8</b>	<b>1.6</b>	<b>4.8</b>	<b>1.5</b>	<b>3.4</b>	<b>2.3</b>	<b>0.88</b>
<b>hsa-miR-365</b>	<b>4.6</b>	<b>4.6</b>	<b>1.9</b>	<b>2.0</b>	<b>1.8</b>	<b>6.0</b>	<b>2.5</b>	<b>3.0</b>	<b>1.6</b>	<b>0.87</b>
<b>hsa-miR-20a*</b>	<b>5.3</b>	<b>4.8</b>	<b>4.0</b>	<b>3.2</b>	<b>1.4</b>	<b>1.5</b>	<b>1.7</b>	<b>1.7</b>	<b>1.7</b>	<b>0.87</b>
<b>hsa-miR-7</b>	<b>3.6</b>	<b>4.8</b>	<b>2.9</b>	<b>1.5</b>	<b>2.8</b>	<b>4.6</b>	<b>2.2</b>	<b>2.1</b>	<b>2.8</b>	<b>0.78</b>
<b>hsa-miR-183</b>	<b>4.5</b>	<b>3.5</b>	<b>2.8</b>	<b>3.2</b>	<b>3.3</b>	<b>3.0</b>	<b>2.9</b>	<b>1.8</b>	<b>1.9</b>	<b>0.72</b>
<b>hsa-miR-130b</b>	<b>3.4</b>	<b>2.1</b>	<b>3.1</b>	<b>1.4</b>	<b>2.8</b>	<b>3.8</b>	<b>3.2</b>	<b>2.0</b>	<b>1.7</b>	<b>0.70</b>
hsa-miR-25	3.6	4.1	2.8	2.8	2.7	2.3	3.4	2.6	2.1	0.68
<b>hsa-miR-425</b>	<b>2.8</b>	<b>2.3</b>	<b>2.9</b>	<b>1.6</b>	<b>3.3</b>	<b>2.0</b>	<b>2.4</b>	<b>2.0</b>	<b>3.6</b>	<b>0.63</b>
<b>hsa-miR-520d-5p</b>	<b>1.5</b>	<b>1.7</b>	<b>1.2</b>	<b>1.9</b>	<b>3.0</b>	<b>2.6</b>	<b>2.0</b>	<b>1.3</b>	<b>1.4</b>	<b>0.52</b>

Fold increase in expression of miRNA common to myeloma were derived by comparing to expression in CD138 positive plasma cells from two healthy donors. The miRNAs shown in bold face are ones that were newly identified by the current study whereas the ones that are underlined were identified by the current study as well as by a recent report [6]. SD values were calculated from four technical replicates. SD >0.50.

Table II

Fold decrease in expression of commonly down-regulated miRNAs in MM compared to samples from healthy donors.

microRNAs	L363	KMS11	JJN3	RPMI8226	U266	H929	MM1S	MM pt1	MM pt2	SD
hsa-miR-223	0.1	0.1	0.1	0.1	0.3	0.2	0.1	0.2	0.9	1.61
hsa-miR-22	0.2	0.1	0.2	0.1	0.7	0.6	0.3	0.4	0.8	1.40
hsa-miR-768-3p	0.1	0.1	0.2	0.1	0.1	0.2	0.2	0.5	0.3	1.28
hsa-miR-29c	0.1	0.2	0.2	0.2	0.1	0.3	0.4	0.6	0.6	1.19
hsa-miR-32*	0.5	0.5	0.4	0.3	0.5	0.5	0.5	0.5	0.6	1.19
hsa-miR-768-5p	0.1	0.1	0.2	0.2	0.2	0.1	0.2	0.5	0.4	1.16

Fold decrease in expression of miRNA common to myeloma were derived by comparing to expression in CD138 positive plasma cells from two healthy donors. The miRNAs shown are ones that were newly identified by the current study. Previously no miRNAs have been shown to be down-regulated. SD values were calculated from four technical replicates. SD >0.50.