Circulating serum microRNAs as novel diagnostic and prognostic biomarkers for multiple myeloma and monoclonal gammopathy of undetermined significance

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Supplementary Data for

Circulating serum microRNAs as novel diagnostic and prognostic biomarkers for multiple myeloma and monoclonal gammopathy of undetermined significance

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Supplementary methods

TaqMan Low Density Arrays (TLDA)

In the screening phase, we performed TaqMan Low Density Arrays (TLDA) analysis to identify differentially expressed miRNAs from serum samples (4 MM patients *vs* 4 healthy donors, 4 MM patients *vs* 5 MGUS patients). In brief, 60 ng of total miRNA/RNA was reverse-transcribed into cDNA using TaqMan MicroRNA Reverse Transcription Kit and 0.8 μl of each A or B Megaplex RT primers, version 3.0 (Life Technologies, CA, USA). To obtain sufficient amount of cDNA for TLDA analysis, pre-amplification step using TaqMan PreAmp MasterMix, TaqMan PreAmp Primers v3.0 and 2.5 μl of reverse transcription product was used. Then, 9μl of diluted pre-amplified product was loaded into TaqMan Array Human MicroRNA A+B Cards Set v3.0 (Life Technologies, CA, USA) enabling simultaneous quantification of 667 human miRNAs. All reactions and analyses were performed on the ABI 7900HT Instrument (Life Technologies, CA, USA) according to the standard manufacturers' protocols.

Normalization of TLDA data

The relative expression levels of target miRNAs were determined by the equation $2^{-\Delta CT}$, in which ΔC_T were calculated as follows: $\Delta C_T = C_{T\ miR\text{-}of\text{-}interest} - C_{T\ miR\text{-}16.}$ MiR-16 was chosen by GeNorm tool as a reference for normalization of miRNAs expression levels as it was stable across all samples (M value according to GeNorm tool = 0.20245). Relative miRNA levels were then calculated with the RQ Manager 1.2.

Reverse transcription and qPCR

Complementary DNA (cDNA) was synthesized in one run from all miRNA/RNA samples using miRNA-specific stem-loop primers according to the TaqMan MicroRNA Assay protocol (Applied Biosystems). For each RT reaction, 10 ng of miRNA/RNA sample, 50 nM of specific stem-loop RT primer, 1 × RT buffer, 0.25 mM each of dNTPs, 3.33 $U{\cdot}\mu l^{-1}$ MultiScribe reverse

transcriptase and 0.25 $U \cdot \mu l^{-1}$ RNase inhibitor (all from TaqMan MicroRNA Reverse Transcription kit, Applied Biosystems) were used. Reaction mixtures (15 µl) were incubated for 30 min at 16 °C, 30 min at 42 °C, 5 min at 85 °C and then held at 4°C. QPCR was performed using the Applied Biosystems 7500 instrument. The 20-µl PCR reaction mixture included 1.33 µl of RT product, 1 × TaqMan (AmpErase UNG) Universal PCR Master Mix and 1 µl of primer and probe mix of the TaqMan MicroRNA Assay kit (Applied Biosystems). Reactions were incubated in a 96-well optical plate at 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles at 95 °C for 15 s and 60 °C for 10 min. All reactions were run in duplicate and average threshold cycle was calculated. All reactions were run in duplicates using 7500 Real-Time PCR System. The resulting Cq values were determined using fixed threshold settings 0.2.

Absolute quantification of qPCR data

As there is still no reliable reference control for circulating miRNAs and U6RNA and 5S rRNA are degraded in serum, we performed absolute quantification to determine the copy number of each miRNA per 1ng of total miRNA/RNA isolated. Absolute quantification requires standard samples that are used to plot a curve of cycle threshold (Ct) *vs* copy number. For each assay, calibration curve was prepared by ten-fold serial dilution of synthetic single-strand miRNA (hsa-miR-222, hsa-miR-744, hsa-miR-130a, hsa-miR-34a, hsa-let-7d and hsa-let-7e, all IDT, Coralville, Iowa, USA) from 0,01 fM/L to 10^4 fM/L, and the levels of the synthetic miRNAs were assessed by qRT-PCR assay as described (Supplementary Information (SI), Figure S1).[1]

Assay precision of hydrolysis probe-based qPCR assay

Repeatability of miRNA/RNA extraction and analytical repeatability of the hydrolysis probe-based qPCR assay was performed on pooled serum from 20 HD using 10 TaqMan miRNA specific assays (hsa-miR-29a-002112, hsa-miR-21-000397, hsa-miR-130a-000454, hsa-miR-19b-000396, hsa-miR-16-000391, hsa-miR-34a-000426, hsa-let-7d-002283, hsa-let-7e-002406, hsa-miR-222-002276 and hsa-miR-744-002324) as described elsewhere (Supplementary Data, Figure S2).[1]

Interphase fluorescence in situ hybridization analysis (I-FISH)

The following aberrations were studied: gain 1q21, deletion 13q14, deletion 17p13 and translocation t(4;14). Hyperdiploidy status was determined by commercial probes mapping to chromosome 5 (LSI D5S23/D5S721), 9 (CEP9) and 15 (CEP15) (Abbott Molecular, Des Plaines, IL, USA).

Statistical analyses

Standard descriptive statistics were applied in the analysis; median supplemented by min-max or interquartile range for continuous variables and absolute and relative frequencies for categorical variables. Statistical significance of differences in continuous variables among groups of patients was analyzed using nonparametric Kruskal-Wallis or Mann-Whitney U test; Wilcoxon paired test was used for the analysis of paired data. For discrete variables, chi-square test or Fisher's exact test was used. For the robust analysis of continuous parameters relationship, the Spearman correlation coefficient was adopted, Pearson correlation was adopted for the linearly dependent and normally distributed variables. Survival and progression rates were estimated using the Kaplan-Meier method. The overall survival (OS) and time-to-progression (TTP) were determined according to International Myeloma Working Group guidelines.[2] Differences in survival among subgroups of patients were compared using the log-rank test. Time-dependent receiver operating characteristic (ROC) was used for the identification of sensitivity, specificity and cut-off value of continuous variables for survival of patients in given time points. Cox proportional hazards models were used to assess the association of prognostic factors with OS and TTP. P-values below 0.05 were considered as statistically significant in all analyses. Data were statistically analyzed with IBM SPSS Statistics, v.20 and R v. 2.15.3 with survival ROC package.

Supplementary tables

Supplementary Table S1: Patients´ and healthy donors´ baseline characteristics used for TLDA.

	HD	MM	MGUS
No. of patients/donors	**4**	**4**	**5**
Gender: males-females	25%-75%	50%-50%	40%-60%
Age median (min-max) [years]	54.5 (54-56)	68 (60-89)	68 (58-75)
ISS stage: I-II-III	ND	25%-50%-25%	ND
Durie-Salmon stage: I-II-III	ND	0%-25%-75%	ND
Durie-Salmon substage: A-B	ND	50%-50%	ND
Ig isotype: IgG-IgA-LC only	ND	50%-25%-25%	ND
Light chains: kappa-lambda	ND	100%-0%	ND
No. of previous treatment lines			
None (First line treatment)	ND	4 (100%)	5 (100%)
Biochemical parameters:			
median (min-max)			
Hemoglobin (g/l)	ND	*78.8 (75-131)*	*135 (109-146)*
Thrombocytes (count x109)	ND	*139.5 (42.5-206)*	*253 (197-322)*
Calcium (mmol/l)	ND	*2.64 (2.49-3.11)*	*2.27 (2.26-2.54)*
Albumin (g/l)	ND	*28.6 (24-37.9)*	*42.4 (38.0-47.4)*
Creatinine (umol/l)	ND	*317.5 (69.0-595.0)*	*74.0 (66.0-156.0)*
β2-microglobulin (mg/l)	ND	*10.8 (2.44-19.3)*	*2.24 (1.81-4.26)*
Lactate dehydrogenase (ukat/l)	ND	*5.39 (4.0-18.69)*	*3.21 (1.92-3.49)*
C-reactive protein (mg/l)	ND	*7.3 (1.0-56.4)*	*2.3 (1.0-69.3)*
Monoclonal Ig (g/l)	ND	*63.1 (0-88.5)*	*8.4 (0-26.4)*
Plasma cell infiltration of bone marrow (%)	ND	*33.8 (15-57.2)*	*1.6 (0.4-5.0)*

Supplementary Table S2: MiRNAs used in validation phase: location of their gene on chromosome, putative targets (according to miRWalk database) and previous association with hematological malignancy are described.

miRNA	Location	Experimentally validated targets	Association with hematological malignancies
miR-222	Xp.11.3	APAF1, BCL2, CDKN1B, FOS	ALL
miR-744	17p12	TUBB4, APC2, JUNB	
miR-130a	11q12.1	IGF1, CCND2,TGFβ	CML
miR-34a	1p36.23	MYCN, E2F3, BCL2, CDK6	Myeloproliferative neoplasms
let-7d	9q22.32	APC2, TGFβRI, CDC25A, TP53	AML
let-7e	19q13.33	MAPK6, IGF1, MYCN, CDK6, APC2, TP53	ALL

Supplementary Table S3: Validated miRNAs. Amount of copy numbers of validated miRNAs expressed as median value and interquartile range. Mann-Whitney U test was used to compare the values. Fold change between HD/MM and HD/MGUS and p values are presented. Significant values $p<0.05$ are marked with bold and italics.

miRNA	HD	MM	MGUS	MM		MGUS	
	median (25-75)	median (25-75)	median (25-75)	FC	P	FC	P
miR-222	5891 (4322-6939)	5824 (4032-9252)	ND ND	0.989	0.3022	ND	ND
miR-744	756 (501-909)	473 (219-725)	371 (293-568)	0.626	***0.0004***	0.491	***<0.0001***
miR-130a	9431 (7699-12134)	5618 (3586-9778)	6232 (3957-8652)	0.596	***0.0002***	0.661	***0.0001***
miR-34a	84 (65-125)	176 (110-275)	192 (116-319)	2.095	***<0.0001***	2.313	***<0.0001***
let-7d	3944 (2734-5135)	1944 (1030-3095)	1863 (1506-2864)	0.493	***<0.0001***	0.472	***<0.0001***
let-7e	8397 (6122-11233)	4222 (2728-6512)	3521 (2246-4700)	0.503	***<0.0001***	0.419	***<0.0001***

FC, fold change; ND, not done

Supplementary Table S4: Sensitivity, specificity, area under curve and cut-off values of serum miRNAs in MM and MGUS obtained by ROC analysis are displayed.

Multiple myeloma				
miRNA	**Sensitivity**	**Specificity**	**AUC**	**cut-off**
miR-744	72.8	66.7	0.715	650
miR-130a	57.5	90.0	0.722	6361
miR-34a	77.7	70.0	0.790	104
let-7d	64.1	86.7	0.804	2419
let-7e	88.8	63.3	0.829	8028
miR-34a+let-7e	80.6	86.7	0.898	
MGUS				
miRNA	**Sensitivity**	**Specificity**	**AUC**	**cut-off**
miR-744	85.7	66.7	0.795	653
miR-130a	76.8	70.0	0.753	8662
miR-34a	91.1	66.7	0.858	101
let-7d	68.4	86.7	0.823	2468
let-7e	78.6	86.7	0.912	4822
miR-34a+let-7e	91.1	96.7	0.976	

AUC, area under curve

Supplementary Table S5: Amount of copies of significantly deregulated miRNA (or miRNAs with a strong trend) in different ISS, DS stage and DS substage groups expressed as median value and interquartile range. Also, p values obtained by Mann-Whitney U test or Kruskal-Wallis test for differences between ISS groups are presented. Significant values $p<0.05$ are marked with bold and italics.

miRNA	ISS I	ISS II	ISS III	P value			P value
	median (25-75)	median (25-75)	median (25-75)	I vs II	II vs III	I vs III	total
miR-744	778 (364-947)	535 (241-784)	225 (148-501)	***0.046***	***0.001***	***<0.0001***	***<0.0001***
let-7d	2904 (1633-3813)	2128 (1433-3066)	1084 (761-2173)	0.264	***0.008***	***<0.0001***	***<0.0001***
let-7e	6359 (4112-8748)	4553 (2978-6870)	2834 (1832-4044)	0.053	***0.002***	***<0.0001***	***<0.0001***
miR-130a	7379 (4933-10205)	6010 (3745-11360)	4454 (3259-8712)	0.506	0.171	***0.015***	0.056
miRNA	**DS Stage I**	**DS Stage II**	**DS Stage III**	**P value**			**P value**
	median (25-75)	**median (25-75)**	**median (25-75)**	**I vs II**	**II vs III**	**I vs III**	**total**
let-7e	6512 (5231-7044)	3771 (2686-4965)	4071 2635-6135)	***0.008***	0.661	***0.011***	***0.024***
miRNA	**DS substage A**	**DS substage B**	**P value**				
	median (25-75)	**median (25-75)**	**total**				
miR-744	553 (254-828)	213 (116-404)	***<0.0001***				
let-7d	2065 (1367-3311)	935 (614-2032)	***0.021***				
miR-130a	6218 (4059-10129)	3573 (2913-8225)	***0.001***				
miR-34a	178 (117-286)	135 (85-261)	***0.001***				

Supplementary Table S6: Amount of copies of deregulated miRNAs in different patients group split according to presence of del(13q14) expressed as median value and interquartile range. Also, p values for differences between del(13q14) positive and del(13q14) negative group obtained by Mann-Whitney U test are presented. Significant values $p<0.05$ are marked with bold and italics.

miRNA	del(13q14) negative	del(13q14) positive	P value
	median (25-75)	**median (25-75)**	
let-7e	4705 (3552-6259)	3417 (1845-4892)	***0.019***
miR-744	535 (259-698)	302 (138-560)	0.056

Supplementary Table S7: Details of multivariate Cox regression analysis for miR-744 and let-7e. Significant values $p<0.05$ are marked with bold and italics.

	miR-744				let-7e			
Parameter	**P value**	**Exp (B)**	**95.0% CI for Exp(B)**		**P value**	**Exp (B)**	**95.0% CI for Exp(B)**	
			Lower	**Upper**			**Lower**	**Upper**
Age	***0.041***	1.047	1.002	1.093	***0.024***	1.049	1.006	1.092
Hemoglobin	0.977	1.000	0.980	1.020	0.769	0.997	0.976	1.018
Thrombocytes	***0.033***	0.994	0.989	1.000	***0.019***	0.993	0.988	0.999
Albumin	***0.027***	0.941	0.891	0.993	0.025	0.939	0.889	0.992
Creatinin	0.829	1.000	0.997	1.004	0.956	1.000	0.997	1.004
β2-microglobulin	0.969	0.999	0.943	1.058	0.921	1.003	0.946	1.063
Lactate dehydrogenase	***0.021***	1.153	1.021	1.301	***0.024***	1.149	1.019	1.296
C-reactive protein	0.199	1.009	0.995	1.024	0.136	1.012	0.996	1.027
miRNA	0.902	1.020	0.743	1.400	0.472	1.193	0.738	1.928

Supplementary Table S8: Comparison of biochemical data between groups of MM patients with **A)** low/high levels of miR-744, **B)** low/high levels of let-7e using Mann-Whitney U test with defined cut-off for overall survival. Significant values $p<0.05$ are marked with bold and italics.

A

Median (range)	**miR-744 low expression**	**miR-744 high expression**	**P value**
Monoclonal Ig (g/l)	*32.0 (0-88.5)*	*24.1 (0-70.8)*	0.140
Hemoglobin (g/l)	*94.6 (62.7-136)*	*113.0 (86-157)*	***<0.0001***
Thrombocytes (count x10^9)	*169 (37.6-409)*	*242.5 (117-561)*	***<0.0001***
Calcium (mmol/l)	*2.40 (1.85-4.94)*	*2.41(2.04-4.48)*	0.738
Albumin (g/l)	*36.5 (22.1-46.8)*	*41.0 (28.0-50.4)*	***0.001***
Creatinine (umol/l)	*108 (48-541)*	*88.5 (52-884)*	***0.006***
β2-microglobulin (mg/l)	*5.92 (1.10-42.60)*	*3.12 (1.15-38.20)*	***<0.0001***
Lactate dehydrogenase (ukat/l)	*3.62 (1.15-18.69)*	*2.89 (1.57-8.09)*	***0.003***
C-reactive protein (mg/l)	*5.80 (0.99-174.3)*	*2.95 (0-62.00)*	0.094
BMPCs infiltration	*27.1 (10.0-71.2)*	*24.8 (10.6-94.0)*	0.650

B

Median (range)	**let-7e low expression**	**let-7e high expression**	**P value**
Monoclonal Ig (g/l)	*33.0 (0-88.5)*	*23.6 (0-70.8)*	0.266
Hemoglobin (g/l)	*96.15 (62.7-136)*	*114.0 (90.1-157)*	***<0.0001***
Thrombocytes (count x10^9)	*176.5 (37.6-409)*	*247 (86.9-561)*	***<0.0001***
Calcium (mmol/l)	*2.44 (1.95-4.94)*	*2.39 (1.85-3.62)*	0.359
Albumin (g/l)	*36.4 (22.1-46.8)*	*40.9 (25.1-50.4)*	***0.007***
Creatinine (umol/l)	*113 (48-541)*	*88.0 (49-884)*	***0.003***
β2-microglobulin (mg/l)	*5.78 (1.10-42.60)*	*2.94 (1.15-38.20)*	***<0.0001***
Lactate dehydrogenase (ukat/l)	*3.49 (1.15-18.69)*	*2.77 (1.57-11.50)*	***0.034***
C-reactive protein (mg/l)	*5.30 (0-174.3)*	*3.00 (0-62.00)*	0.178
BMPCs infiltration	*31.2 (10.0-94.0)*	*22.4 (10.6-86.2)*	0.100

Supplementary Table S9: ISS, DS and DS substage distribution between **A)** low/high miR-744 expression groups of MM patients, **B)** low/high let-7e expression groups of MM patients. The differences between distributions were tested using Chi-square or Fisher´s exact test, significant values $p<0.05$ are marked with bold and italics.

A

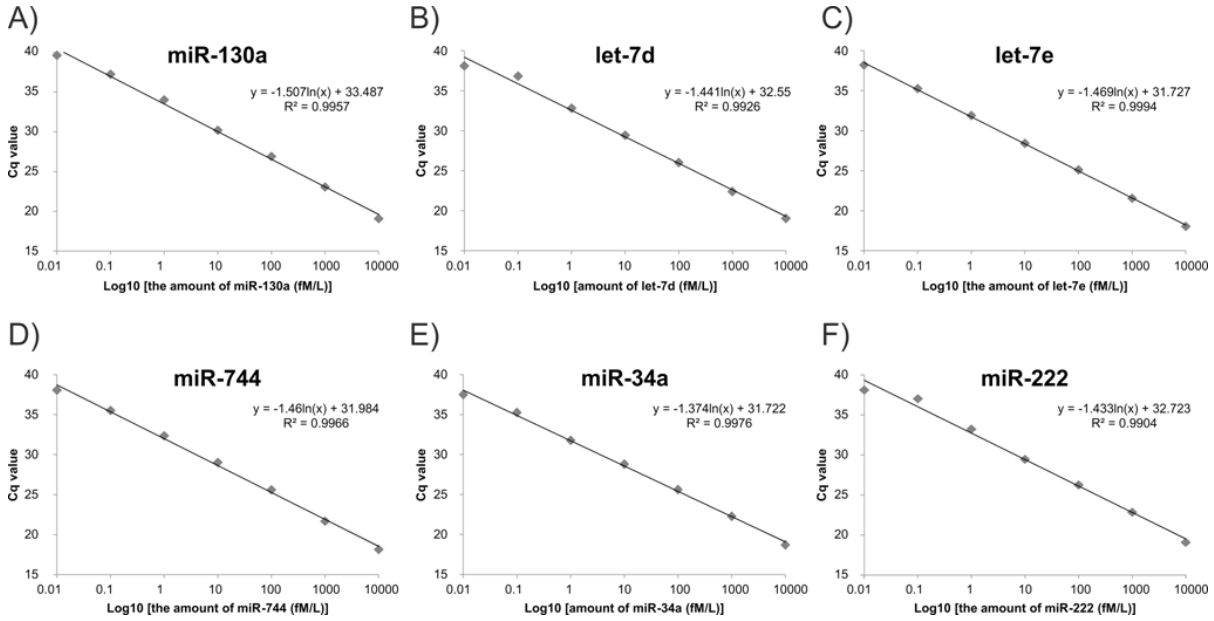
Group	ISS stage			
	I	II	III	
LOW miR-744	17.1% (7/41)	22.0% (9/41)	60.9% (25/41)	***p<0.0001***
HIGH miR-744	45.8% (27/59)	32.2% (19/59)	22.0% (13/59)	
	Durie-Salmon stage			
	I	**II**	**III**	
LOW miR-744	4.8% (2/43)	19% (8/43)	76.2% (33/43)	p=0.221
HIGH miR-744	15.5% (9/58)	17.2% (10/58)	67.3% (39/58)	
	Durie-Salmon substage			
	A	**B**		
LOW miR-744	67.4% (29/43)	32.6% (14/43)		***p=0.013***
HIGH miR-744	88.3% (53/60)	11.7% (7/60)		

B

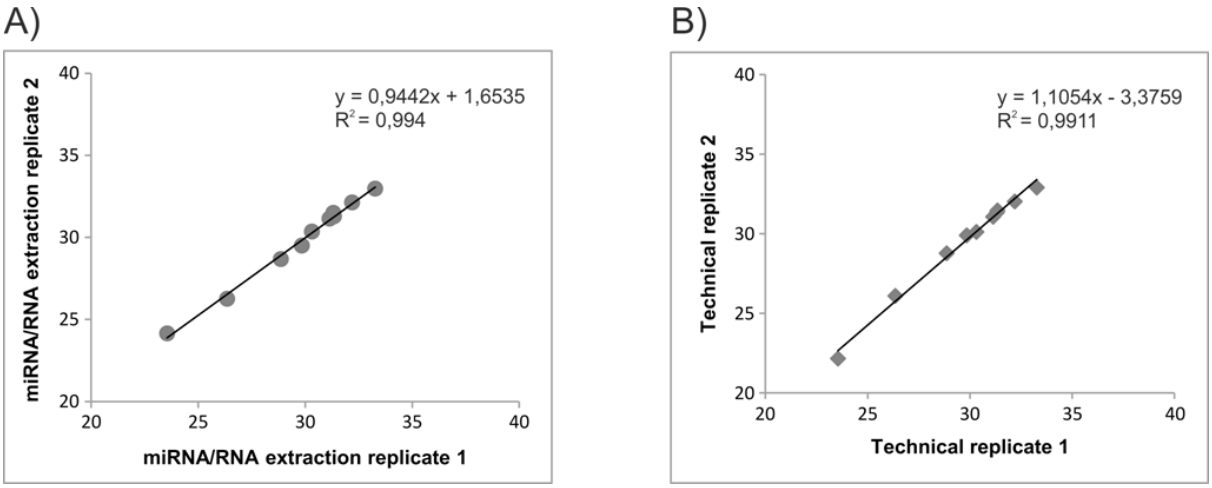
Group	ISS stage			
	I	II	III	
LOW let-7e	19.6% (10/51)	23.6% (12/51)	56.8% (29/51)	***p<0.0001***
HIGH let-7e	48.9% (24/49)	32.7% (16/49)	18.4% (9/49)	
	Durie-Salmon stage			
	I	**II**	**III**	
LOW let-7e	3.9% (2/51)	21.6% (11/51)	74.5% (38/51)	p=0.062
HIGH let-7e	18.0% (9/50)	14.0% (7/50)	68.0% (34/50)	
	Durie-Salmon substage			
	A	**B**		
LOW let-7e	67.3% (35/52)	32.7% (17/52)		***p=0.003***
HIGH let-7e	92.2% (47/51)	7.8% (4/51)		

Supplementary Figures

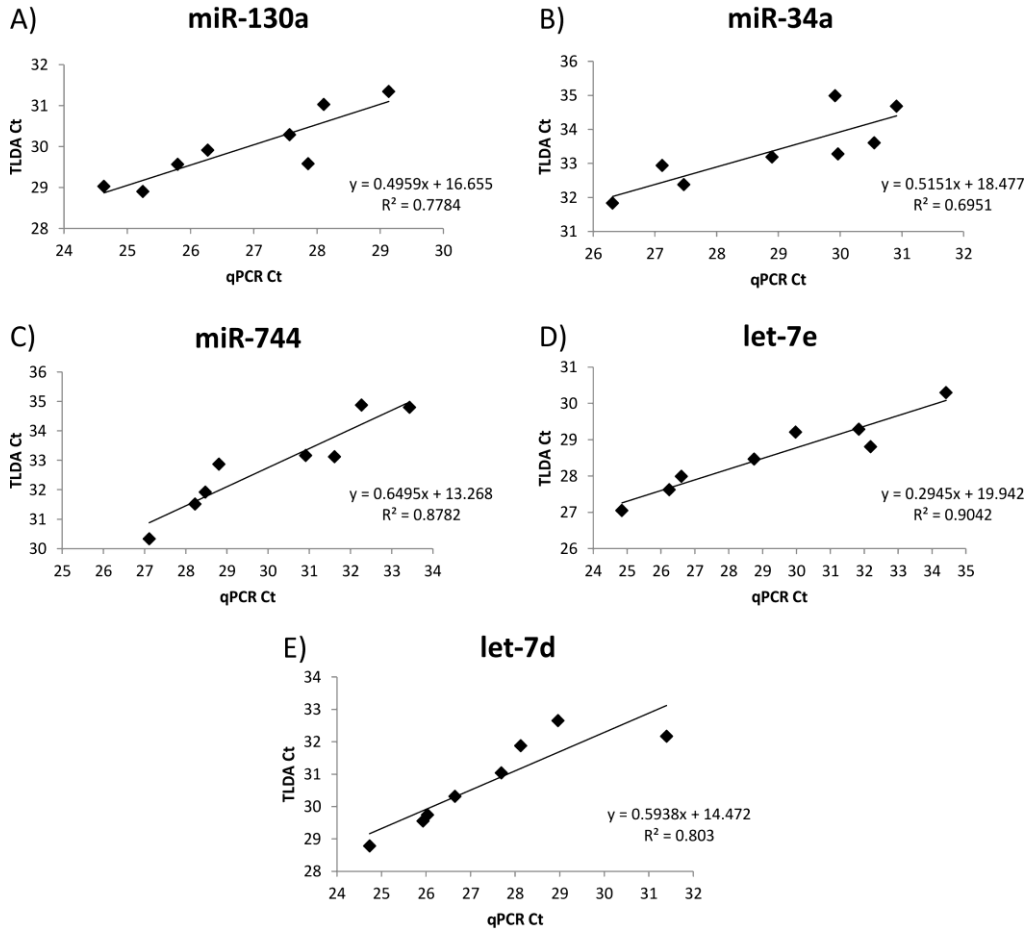
Supplementary Figure S1: Using synthetic, single-stranded miRNAs, standard curves were generated for each miRNA of interest: miR-130a, let-7e, let-7d, miR-744, miR-34a and miR-222 (**A-F**). Each of synthetic miRNA was ten-fold serially diluted from 0.01 fM/L to 10^4 fM/L to generate standard curves using specific miRNA assays. The obtained Cq values were plotted versus the $\log_{10}$ of the amount of synthetic miRNAs. Each point represents the mean of triplicate.



Supplementary Figure S2: Repeatability of miRNA/RNA extraction and RT-qPCR assay. **A)** The repeatability of miRNA/RNA extraction was assessed using pooled serum from 20 healthy donors that was divided into two equal portions (each 400µl). Total miRNA/RNA was extracted from this two pooled serum samples, respectively. Further, amounts of ten miRNAs (including miR-29a, miR-21, miR-130a, miR-19b, miR-16, miR-34a, let-7d, let-7e, miR-222 and miR-744) were determined in triplicates using TaqMan stem-loop primers and TaqMan primer-probe qPCR assays. MiRNAs levels, presented as Cq values from the replicate assays were plotted against each other. Coefficient of correlation between the two replicates is included. **B)** The repeatability of RT-qPCR assay was assessed using detection of the same ten miRNAs as above in the two same miRNA/RNA samples extracted from pooled serum from 20 healthy donors. Each reaction was performed in triplicate. Similarly, the Cq values of the miRNAs from the replicate assays were plotted against each other and coefficient of correlation between the two replicates is included as well.

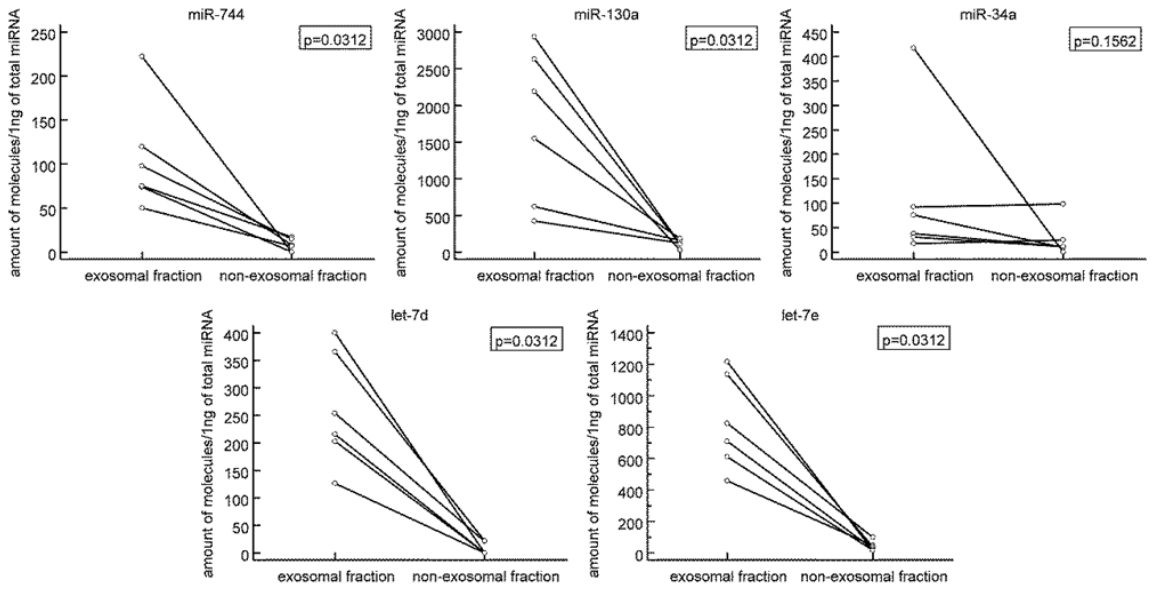


Supplementary Figure S3: Correlation plot between Ct values obtained by TLDA and qPCR. Ct values for 5 miRNAs: miR-130a, miR-34a, miR-744, let-7d and let-7e (A-E) obtained by TLDA for 4 MM patients and 4 HD are plotted vs Ct values obtained by qPCR.

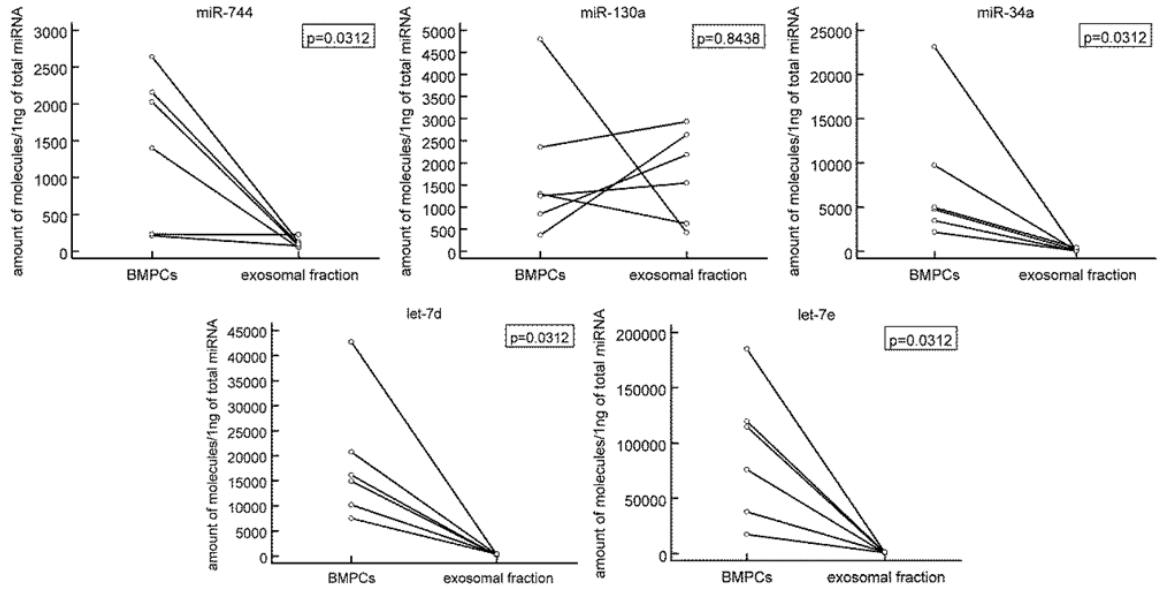


Supplementary Figure S4: Comparison of miRNA levels between **A)** exosomal and exosome-depleted samples and **B)** levels in BMPCs and exosomal samples. Wilcoxon test for paired samples was used to compare the data and $p<0.05$ was considered as statistically significant.

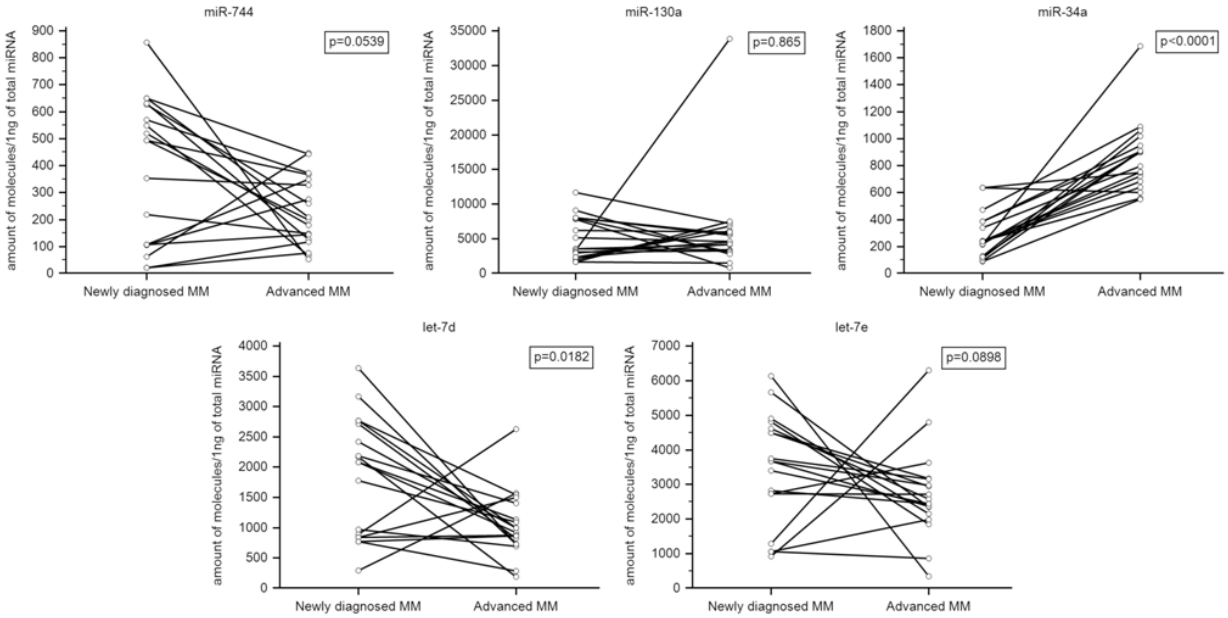
A



B



Supplementary Figure S5: Box plots of serum miRNAs in 18 patients' serum samples taken at the time of diagnosis and in relapse, after 2 lines of treatment. Wilcoxon test for paired samples was used and $p<0.05$ was considered as statistically significant.



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