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

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ABSTRACT

Multiple myeloma still remains incurable in the majority of cases prompting a further search for new and better prognostic markers. Emerging evidence has suggested that circulating microRNAs can serve as minimally invasive biomarkers for multiple myeloma and monoclonal gammopathy of undetermined significance. In this study, a global analysis of serum microRNAs by TaqMan Low Density Arrays was performed, followed by quantitative real-time PCR. The analyses revealed five deregulated microRNAs: miR-744, miR-130a, miR-34a, let-7d and let-7e in monoclonal gammopathy of undetermined significance, newly diagnosed and relapsed multiple myeloma when compared to healthy donors. Multivariate logistical regression analysis showed that a combination of miR-34a and let-7e can distinguish multiple myeloma from healthy donors with a sensitivity of 80.6% and a specificity of 86.7%, and monoclonal gammopathy of undetermined significance from healthy donors with a sensitivity of 91.1% and a specificity of 96.7%. Furthermore, lower levels of miR-744 and let-7e were associated with shorter overall survival and remission of myeloma patients. One-year mortality rates for miR-744 and let-7e were 41.9% and 34.6% for the 'low' expression and 3.3% and 3.9% for the 'high' expression groups, respectively. Median time of remission for both miR-744 and let-7e was approximately 11 months for the 'low' expression and approximately 47 months for the 'high' expression groups of myeloma patients These data demonstrate that expression patterns of circulating microRNAs are altered in multiple myeloma and monoclonal gammopathy of undetermined significance and miR-744 with let-7e are associated with survival of myeloma patients.

Introduction

Multiple myeloma (MM) accounts for more than 10% of hematologic cancers.[1] In MM, malignant bone marrow plasma cells (BMPCs) undergo massive clonal expansion resulting in high levels of monoclonal immunoglobulin (mIg, M-protein) in blood and/or urine. This is often accompanied by other clinical symptoms, such as osteolytic lesions, increased calcium level, renal insufficiency and anemia.[1,2] MM evolves from a pre-malignant condition called monoclonal gammopathy of undetermined significance (MGUS) which progresses to MM at a rate of 1% per year.[3] Although there are serum markers used for diagnosis of MGUS and MM, such as levels of FLC or mIg,[4-6] recently a lot of attention has been paid to circulating microRNAs that could serve as new diagnostic and/or prognostic tools.[7-9]

MicroRNAs (miRNAs) are a class of short, non-coding, single stranded RNAs with regulatory function.[10,11] MiRNAs play crucial roles in a variety of basic biological processes; they even contribute to tumor formation and development.[12] In tumors, different miRNAs expression profiles compared to healthy tissues were described and resulting miRNAs signatures correlated with patients' survival and prognosis. Such observations highlighted miRNAs as promising biomarkers for diagnosis and even possible targets for therapies.[13]

So far, a number of studies, using BMPCs as the source of miRNAs, found several deregulated miRNAs in MM and MGUS, and implicated miRNAs in signaling pathways deregulated in MM pathogenesis.[14-17] Some of these miRNAs have a therapeutic potential *in vitro* and *in vivo*, such as miR-34a.[18] Nevertheless, obtaining a marker from the bone marrow (BM) is an invasive procedure for patients; therefore, there is still a need for a minimally invasive test that can be easily repeated. There is now a greater possibility of using miRNAs as biomarkers after the discovery that they are present in various body fluids.[19]

Moreover, they are very stable, as they are protected from degradation by association either with secreted membrane vesicles (exosomes, apoptotic vesicles) or with RNA-binding proteins (nucleophosmin, Argonaut 2 (Ago2)).[19-21] It was shown that the miRNAs profile of body fluids reflects physiological or pathological conditions and can be used for patient

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classification.[22] In this study, a new serum miRNAs expression profile, potent enough to distinguish newly diagnosed MM and MGUS patients from healthy controls, was created based on TaqMan Low Density Arrays (TLDA). This profile was validated by quantitative real-time PCR (qPCR) on a larger cohort of newly diagnosed and relapsed MM as well as MGUS patients. Moreover, miRNAs levels were correlated with clinical, biochemical and cytogenetic characteristics and survival data.

Methods

Patients and healthy donors

Peripheral blood (PB) serum samples from 103 newly diagnosed MM patients, 18 MM patients in relapse, 57 MGUS and 30 healthy donors (HD) from the Faculty Hospital Brno, Czech Republic, were obtained for this study. PB serum samples were collected as follows: centrifugation 3500 rpm/15 min/20°C. Samples were frozen as 0.5 mL aliquots, stored at -80°C and thawed only once. For 70 MM and 36 MGUS samples, BMPCs were obtained for routine interphase fluorescence *in situ* hybridization analysis (I-FISH), as described previously.[23] Patients' and donors' characteristics are described in Table 1 and in the *Online Supplementary Table S1*. For 6 newly diagnosed MM patients, BMPCs and exosomal and non-exosomal fraction from PB serum were collected. All patients signed an informed consent form approved by the hospital ethical committee before enrollment into this study.

MiRNA extraction

Total RNA enriched for miRNAs was extracted from all serum samples using miRNeasy Kit (Qiagen) modified for circulating miRNAs according to the manufacturer's instructions. MiRNA/RNA quantity was assessed on a NanoDrop ND-1000 Spectrophotometer (Thermo Scientific) as measurement of each sample 2 times with mean SD=0.292 ng/μL. All samples fit into the Nanodrop ND-1000 validated measuring range.

Exosomes precipitation

Exosomes were collected using ExoQuick Exosome Precipitation Solution (System Biosciences). Serum samples were centrifuged for 3500 rpm/10 min/4°C, 250 μL of serum was combined with 63 μL of ExoQuick, incubated for 30 min/4°C and centrifuged for 2 min/13000 rpm. Exosomal and non-exosomal fraction was used for miRNA/RNA extraction, as described above.

TaqMan Low Density Arrays

Megaplex profiling using human TaqMan Low Density miRNA Arrays A+B, v3.0 (TLDA) (Life Technologies) was performed to evaluate the expression of 667 miRNA (see *Online Supplementary Methods*). QPCR was performed on the ABI7900HT system; raw data were analyzed using SDS software v.2.3, RQ Manager v1.2.1 (Life Technologies).

Candidate miRNA confirmation by qPCR and quantification of miRNA

Individual TaqMan miRNA assays for 6 miRNA (hsa-miR-222-002276, hsa-miR-744-002324, hsa-miR-130a-000454, hsa-miR-34a-000426, hsa-let-7e-002406, hsa-let-7d-002283, Life Technologies) were used for qPCR on a 7500 Real-Time PCR System. QPCR and reverse transcription was performed following the manufacturer´s recommendations (see *Online Supplementary Methods*). Absolute quantification to determine the copy number of each miRNA per 1 ng of total miRNA/RNA was performed, as described previously[24] (*Online Supplementary Appendix* and *Online Supplementary Figure S1*). For determination of assay precision see the *Online Supplementary Methods*.

Interphase fluorescence in situ hybridization analysis

Interphase fluorescence *in situ* hybridization analysis (I-FISH) was performed as a part of routine diagnostic procedure on $CD138^+$ BMPCs, as previously described[25] (*Online Supplementary Methods*).

Statistical analysis

TaqMan Low Density Arrays data were analyzed according to the manufacturer's recommendations; multiple testing correction was applied using Benjamini-Hochberg correction for assessment of adjusted *P* values. For determination of the relative expression levels of target miRNAs see the *Online Supplementary Appendix*. Normalized expression data from the screening phase of the study were statistically evaluated using the R statistical computing language using the Bioconductor package and LIMMA model combined with hierarchical clustering (HCL).[26-28] Other statistical methods used are described in the *Online Supplementary Methods*. $P<0.05$ was considered statistically significant. Data were statistically analyzed with IBM SPSS Statistics, v.20 and R v.2.15.3 with survival ROC package.

Results

Low density arrays study

Screening of 667 miRNAs using TLDA was performed on 4 newly diagnosed MM patients, 4 HD and 5 MGUS samples to identify differentially expressed circulating miRNAs that could serve as putative biomarkers. Fourteen miRNAs were significantly deregulated (all $P<0.003$, adjusted $P<0.05$) between MM patients and HD: 7 miRNAs were up-regulated (miR-222, miR-218, miR-34a, miR-1274A, miR-138, miR-10b*, miR-1243), 7 miRNAs were down-regulated (miR-191, miR-130a, let-7d, miR-103, let-7e, miR-744, miR-151-5p) in MM patients (Figure 1). Out of these, miR-222, miR-744, miR-34a, miR-130a, let-7d and let-7e were further validated, as their position at the top of the list, fold change and favorable expression levels (Ct<30) were taken into account (*Online Supplementary Appendix* and *Online Supplementary Table S2*). However, no significant change in miRNAs expression was observed between MM and MGUS samples (*data not shown*). Therefore, we used the same 6 miRNAs to look for the difference between MGUS samples and healthy donors.

Validation of candidate miRNAs using qPCR

Since qPCR is more sensitive and more quantitative over a wider dynamic range than TLDA, we employed miRNA specific assays (miR-222, miR-744, miR-130a, miR-34a, let-7d and let-7e) on a larger cohort of 103 newly diagnosed MM patients and 30 HD to confirm the pattern of candidate miRNAs expression between MM/HD samples and also on 57 MGUS and 18 relapsed MM samples.

To accurately determine expression differences between groups, miRNAs were normalized as amount of miRNA copy numbers per 1 ng of total RNA/miRNA using absolute quantification approach. Standard curves for all 6 validated miRNAs were obtained (*Online Supplementary Appendix* and *Online Supplementary Figure S1*), and individual assays imprecision was also assessed (*Online Supplementary Appendix* and *Online Supplementary Figure*

Table 1. Patients' and healthy donors' base-line characteristics used for RT-PCR.

	MM	MGUS	HD
N. of patients/donors	103	57	30
Gender: males-females	49.5%-50.5%	66.7%-33.3%	46.7%-53.3%
Age median (min-max) [years]	66 (47-83)	67 (54-80)	55 (45-64)
ISS stage: I-II-III	34%-28%-38%	ND	ND
Durie-Salmon stage: I-II-III	10.9%-17.8%-71.3%	ND	ND
Durie-Salmon substage: A-B	79.6% - 20.4%	ND	ND
Ig isotype: IgG-IgA-IgM-IgD-LC only-	52.4%-27.2%- 1.9%-2.9%-10.7%	81.8%-3.6%-12.7%-0%-1.8%-	ND
NonSecr.-Biclonal	-3.9%-1.0%	0%-0%	
Light chains: kappa-lambda	59.2%-36.9%	53.7%-46.3%	ND
N. of previous treatment lines			
None (first-line treatment)	103 (100%)	57 (100%)	ND
First-line based treatment: thalidomide-Bortezomib -lenalidomide	76%-18%-8%	ND	ND
Biochemical parameters median (min-max)			
Hemoglobin (g/L)	108 (62.7-157)	138 (104-166)	ND
Thrombocytes (count x10^9)	215 (37.6-561)	233 (112-483)	ND
Calcium (mmol/L)	2.41 (1.85-4.94)	2.34 (2.04-2.67)	ND
Albumin (g/L)	39.0 (22.1-50.4)	43.8 (30.6-53.3)	ND
Creatinine (umol/L)	92.0 (48.0-884.0)	86.0 (50.0-779.0)	ND
β2-microglobulin (mg/L)	3.82 (1.10-42.6)	2.11 (1.21-35.0)	ND
Lactate dehydrogenase (ukat/L)	3.16 (1.15-18.69)	3.43 (1.92-7.88)	ND
C-reactive protein (mg/L)	4.0 (0-174.3)	3.1 (0-280.6)	ND
Monoclonal Ig (g/L)	26.65 (0-88.5)	8.7 (0-26.6)	ND
Plasma cell infiltration of bone marrow (%)	27.0 (10.0-94.0)	2.0 (0-8.4)	ND
Chromosomal abnormality			
13q14 deletion	30 (44.1%)	9 (23.7%)	ND
17p13 deletion	9 (13.2%)	1 (2.6%)	ND
Translocation t(4;14)	9 (18.4%)	3 (12.5%)	ND
1q21 gain	24 (37.5%)	3 (9.4%)	ND
Hyperdiploidy	29 (45.3%)	5 (15.2%)	ND

ND: not determined.

S2). As the difference in miR-222 expression between MM and HD was not significant (P=0.3022) it was excluded from further studies.

A significant decrease was observed in expression of miR-744, miR-130a, let-7d and let-7e (all P<0.001) in the MM group. However, miR-34a was significantly increased (P<0.0001) when compared to the HD group (*Online Supplementary Appendix* and *Online Supplementary Table S3*). These data confirm the results of the screening phase (for the correlation between TLDA and qPCR data, see *Online Supplementary Appendix* and *Online Supplementary Figure S1*). Similarly, expression of miR-744, miR-130a, let-7d and let-7e was decreased in MGUS samples (all P<0.0001) and the expression of miR-34a was increased in MGUS when compared to HD (P<0.0001) (*Online Supplementary Appendix* and *Online Supplementary Table S3*).

Receiver operating characteristics (ROC) curve analysis revealed that serum levels of all validated miRNAs can be used to distinguish MM and MGUS patients from HD (*Online Supplementary Appendix* and *Online Supplementary Table S4*). Moreover, multivariate logistical regression analysis showed that the combination of miR-34a and let-7e could improve the stratification power characterized with area under the curve (AUC) of 0.898, sensitivity of 80.6% and specificity of 86.7% for MM, and with AUC 0.976, sensitivity of 91.1% and specificity of 96.7% for MGUS (Figure 2).

MiRNA expression pattern correlates with biochemical parameters but not with PCs infiltration

To determine the correlation of miRNAs expression levels with clinical parameters, stage (ISS, Durie-Salmon (DS)) and percentage of BMPC infiltration, Spearman bivariate correlation was performed. All studied miRNAs significantly correlated with higher levels of hemoglobin: miR-744, miR-130a, let-7d and let-7e positively; miR-34a negatively. Moreover, levels of miR-744, miR-130a, let-7d and let-7e showed a significant positive correlation with thrombocyte count and a significant negative correlation with levels of creatinine and beta(β)2-microglobulin. Expressions of miR-744, let-7d, let-7e showed a significant positive correlation and miR-34a significantly negatively correlated with levels of albumin, and miR-744 and let-7e a significant negative correlation with C-reactive protein (CRP) level. Only let-7e expression showed a significant negative correlation with level of monoclonal immunoglobulin (Ig).

Similar data were obtained for MGUS patients, where levels of all studied miRNAs showed a significant positive correlation with hemoglobin level. In addition, levels of miR-744, miR-130a, let-7d and let-7e were significantly associated with levels of albumin and inversely correlated with levels of creatinine and β2-microglobulin. Also, levels of miR-744, miR-130a and let-7d showed a significant negative correlation with CRP levels. In contrast to MM patients, none of the studied miRNAs in MGUS correlated

with thrombocyte count (Table 2).

In MM, expression levels of miR-744, let-7d and let-7e were linked to advanced ISS stage; this trend was also observed for miR-130a, although it did not reach statistical significance. Also, only let-7e correlated with DS stage; levels of miR-744, miR-130a, let-7d and miR-34a were associated with DS sub-stage. However, none of the studied miRNAs in MM and MGUS correlated with percentage of PC infiltration in BM, which confirms previous observed findings[28] (*Online Supplementary Appendix* and *Online Supplementary Table S5*).

Level of circulating let-7e correlates with del(13q14) in PCs

Little is known about the origin of circulating miRNAs and their relationship with BMPCs. Therefore, the expression levels of five miRNAs were correlated with typical chromosomal MGUS and MM aberrations, such as gain of 1q21, 13q14 deletion, 17p13 deletion, translocation t(4;14) and hyperdiploidy (HY) status (HY of chromosomes 5, 9 and 15). We found that presence of del(13q14) in MM showed a significant correlation with lower levels of let-7e, and we also observed a trend for lower levels of miR-744 to be linked with this aberration (*Online Supplementary Appendix* and *Online Supplementary Table S6*).

Derivation of evaluated miRNAs

To further investigate potential derivation of all studied miRNAs, we measured their levels in exosomal and exosome-depleted supernatant of 6 newly diagnosed MM patients. Concentration of miR-744, miR-130a, let-7d and let-7e (all $P<0.05$) was found to be significantly higher in the exosome pellet compared to the exosome-depleted supernatant. However, there was no significant difference between these two fractions for miR-34a (*Online Supplementary Figure S4A*). For the same patients, we obtained miRNAs from BMPCs, and we observed levels of miR-744, miR-34a, let-7d and let-7e to be significantly

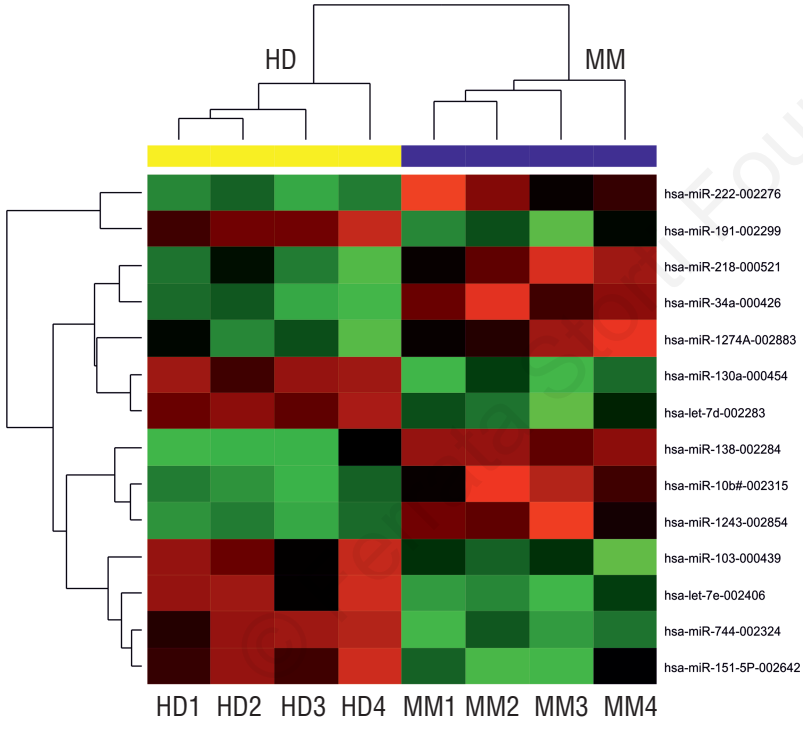

Figure 1. Hierarchical clustergram discriminating serum of MM patients and healthy donors according to differentially expressed miRNAs (yellow color indicates serum samples of HD, blue MM patients; adjusted $P<0.05$).

Table 2. Correlation of serum microRNAs in MM and MGUS with biochemical parameters. For correlation of the data, Spearman coefficient was adopted; significant coefficients of correlation ($P<0.05$) are marked with bold .

	Multiple myeloma					MGUS				
rS	miR-744	miR-130a	miR-34a	let-7d	let-7e	miR-744	miR-130a	miR-34a	let-7d	let-7e
Monoclonal Ig (g/L)	**-0.175**	-0.011	0.135	-0.087	**-0.199**	0.059	0.062	0.116	0.000	0.051
Hemoglobin (g/L)	**0.543**	**0.283**	**-0.258**	**0.387**	**0.585**	**0.383**	**0.465**	**0.270**	**0.290**	**0.424**
Thrombocytes (count x10^9)	**0.555**	**0.390**	-0.190	**0.427**	**0.515**	-0.007	-0.092	-0.127	0.000	0.024
Albumin (g/L)	**0.355**	0.093	**-0.204**	**0.302**	**0.355**	**0.464**	**0.341**	0.221	**0.401**	**0.309**
Creatinine (µmol/L)	**-0.415**	-0.354	-0.007	**-0.310**	**-0.406**	**-0.369**	**-0.330**	-0.090	**-0.468**	**-0.367**
β2-microglobulin (mg/L)	**-0.575**	**-0.236**	0.170	**-0.439**	**-0.571**	**-0.451**	**-0.279**	**-0.211**	**-0.484**	**-0.277**
Lactate dehydrogenase (µkat/L)	**-0.207**	-0.095	0.141	**-0.202**	-0.176	-0.115	-0.115	-0.180	-0.061	-0.216
C-reactive protein (mg/L)	**-0.221**	-0.086	0.122	-0.178	**-0.253**	**-0.331**	**-0.349**	-0.019	**-0.299**	-0.224

higher in BMPCs than in exosomal fraction (all $P<0.05$) (*Online Supplementary Figure S4B*). Interestingly, levels of miR-130a were comparable in BMPCs and exosomes ($P=0.8438$). However, there was no correlation found between miRNAs from BMPCs and from exosomal fraction (*data not shown*).

Dynamics of miRNA levels during disease progression

As deregulated miRNAs expression in MGUS and MM patients was observed at the time of diagnosis, the next step was to check if this profile changes during disease progression. For 18 MM patients, who had not undergone PBMC transplantation, serum samples at the time of diagnosis and in relapse (after 2 lines of treatment) were collected. All of the miRNAs in MM samples differed significantly from HD at the $P<0.0001$ (miR-744: FC=0.270; miR-130a: FC=0.487; miR-34a: FC=10.083; let-7d: FC=0.243; let-7e: FC=0.300). Moreover, a significant increase of miR-34a (FC=3.560; $P<0.0001$) and decrease of let-7d (FC=0.460; $P=0.0182$) was found in relapsed samples compared to samples at the time of diagnosis. For miR-744 and let-7e, a trend toward lower expression was observed; however, no change in expression between diagnostic and relapsed sample was observed for miR-130a (*Online Supplementary Appendix* and *Online Supplementary Figure S5*).

Analyses of overall survival and time to progression

Furthermore, miRNAs expression was verified as a possible indicator of survival. Univariate Cox proportional hazards survival model with one explanatory variable showed prognostic impact for serum miR-744 (HR 0.670 [HR95%CI: 0.548; 0.819]; $P<0.0001$) and for let-7e (HR: 0.611 [HR95%CI: 0.450; 0.829]; $P=0.002$) for the MM cohort of patients. To characterize the prognostic significance of this miRNA, a multivariate Cox proportional hazards survival model was used. The variables in the multivariate model were the only variables which remained statistically significant when potential predictors were combined with miRNA expression and forced into the model. The results showed that neither miR-744 nor let-7e is independently associated with overall survival (OS) when combined with other factors (miR-744: $P=0.902$; let-7e: $P=0.472$) (*Online Supplementary Appendix* and *Online Supplementary Table S7*). Survival cut-off points were established based on time-dependent ROC analysis (*data not shown*), which showed suitable AUC for a 0.5-1.5 year time period for miR-744 and a 1.5 year time point for let-7e.

To determine the prognostic impact of defined miR-744 and let-7e expression cut-off values, we compared OS between the 'low' and the 'high' expression subgroups (Figure 3A and B). For miR-744, worse 1-year OS was indicated in the 'low' expression subgroup of patients (43 of 103) in comparison with the 'high' expression group (60 of 103) ($P<0.0001$). One-year mortality rate for the 'low' miR-744 expression group was 41.9% (95%CI: 28.8%; 57.9%), and for the 'high' expression group it was 3.3% (95%CI: 0.8%; 12.7%), respectively. Similarly for let-7e, worse 1-year OS was indicated in the 'low' expression subgroup of patients (52 of 103) in comparison with the 'high' expression group (51 of 103) ($P=0.001$). One-year mortality rate for the 'low' let-7e expression group was 34.6% (95%CI: 23.4%; 49.2%) and for the 'high' expression group 3.9% (95%CI: 1.0%; 14.8%). In the same way, the Cox model showed prognostic impact for serum miR-744 (HR: 0.690 [HR95%CI: 0.584; 0.817]; $P<0.0001$) and let-7e (HR: 0.552 [HR95%CI: 0.424; 0.718]; $P<0.0001$) in time to progression (TTP) for the MM patient cohort. Only MM patients who had an event after first-line of therapy were taken into account (86 of 103). We compared TTP between miR-744 'low' and 'high' expression subgroups and between let-7e 'low' and 'high' expression subgroups using the cut-off value defined by time-dependent ROC analysis. The analysis showed suitable AUC for a 1-2 year time period for miR-744 and a 1-year time point for let-7e (Figure 3C and D). Shorter TTP was indicated in patients in the 'low' miR-744 expression subgroup (37 of 86) in comparison with the 'high' expression group of patients (49 of 86) ($P<0.0001$), and median time of remission was approximately 11.5 months (95%CI: 6.49; 16.50) for the 'low' expression and approximately 47.5 months (95%CI: 24.63; 70.37) for the 'high' expression groups, respectively. For let-7e, shorter TTP was indicated in the 'low' expression subgroup of patients (43 of 86) in comparison with the 'high' expression subgroup of patients (43 of 86) ($P<0.0001$), and median time of remission was approximately 11.5 months (95%CI: 7.17; 15.83) for the 'low' expression and approximately 47.5 months (95%CI: 31.61; 63.39) for the 'high' expression groups, respectively.

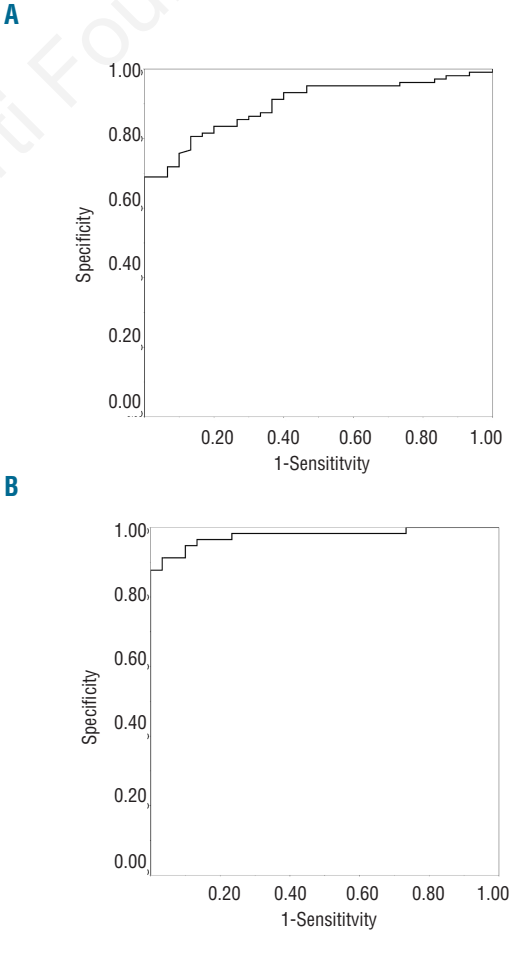

Figure 2. ROC curves curves for combination of serum miR-34a and let-7e yielded in (A) AUC of 0.898, sensitivity 80.6% and specificity 86.7% in discriminating MM from HD and in (B) AUC of 0.976, sensitivity 91.1% and specificity 96.7% in discriminating MGUS from HD.

Biochemical and stage characteristics (ISS, DS, DS sub-stage) of presented groups of MM patients and *P*-values are provided in the *Online Supplementary Appendix* for both the 'high' and the 'low' miR-744 and let-7e expression groups (*Online Supplementary Appendix* and *Online Supplementary Tables S8A* and *B, S9A* and *B*). The miR-744 and let-7e 'low/high' expression groups were significantly different in levels of hemoglobin, thrombocytes, albumin, creatinine, β2-microglobulin and lactate dehydrogenase ($P<0.05$). Significant differences between groups in ISS and DS sub-stage distribution ($P<0.05$) were also observed.

Interestingly, a significant association between group of patients with lower expression of let-7e and occurrence of del(13q14) ($P=0.031$) was found. There was no difference between the 'high' and the 'low' miR-744 and let-7e expression groups in terms of the occurrence of the other analyzed cytogenetic abnormalities (*data not shown*).

Discussion

It has been shown that miRNAs are present as circulating molecules in human body fluids and thus may serve as a new class of powerful and minimally invasive biomarkers.[7,8,29-31] However, the studies differ regarding deregulated miRNAs, the array platforms used and the normalization methods adopted. The origin of circulating miRNAs and their function is still unclear as circulating miRNAs may not always be directly associated with malignant cells but may also reflect indirect effects, could be secreted by non-malignant cells, or actively taken up by malignant cells.[32,33]

In this study, TLDAs were used to identify circulating miRNAs that are differently expressed in MM serum samples and could reflect this pathological condition. Fourteen differently expressed miRNAs between MM and HD serum samples were identified. Out of these, five miRNAs (miR-744, miR-130a, let-7d, let-7e and miR-34a) were cho-

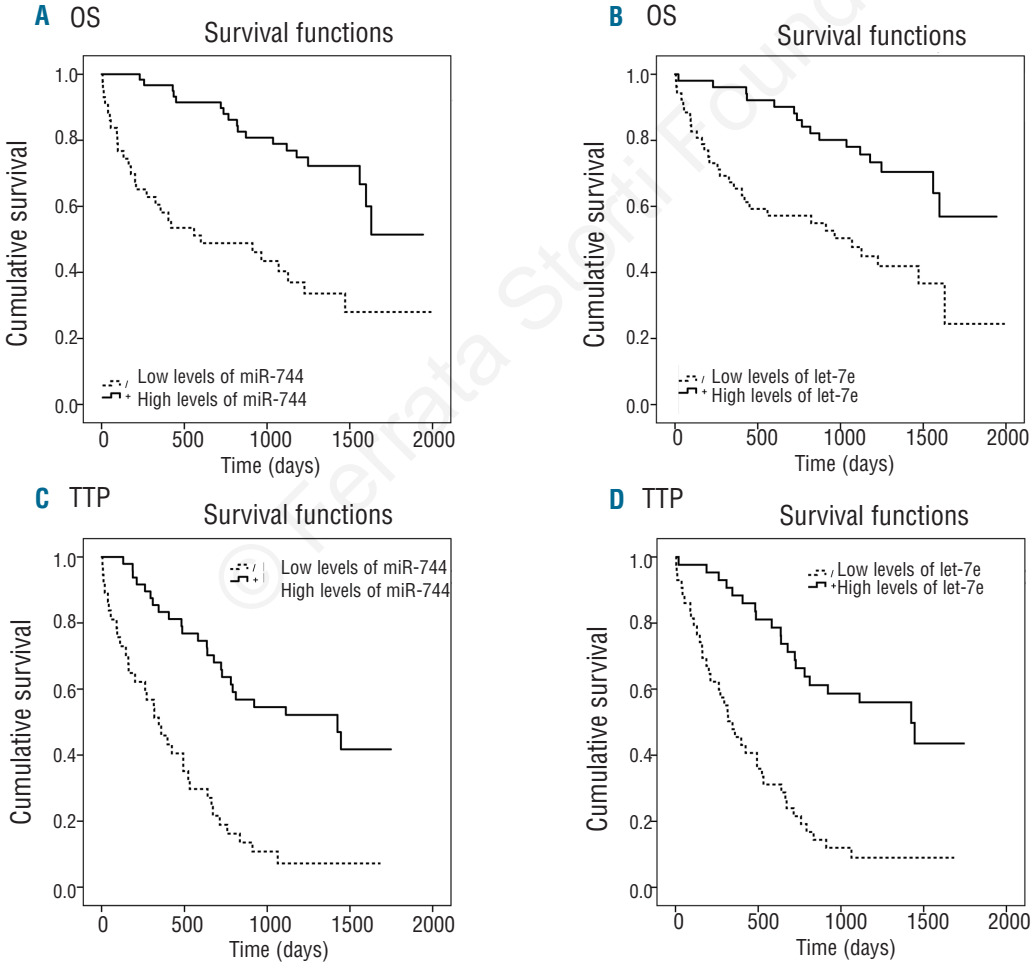

Figure 3. Kaplan-Meier curves of miR-744 and let-7e and their association to (A) (B) OS (C) (D) TTP. The thresholds of cut-off points were determined using a time-dependent ROC analysis. For miR-744 OS, the cut-off value was derived from 0.5-1.5 years survival, for let-7e it was derived from 1.5 years survival. Similarly for TTP, the cut-off values were derived for miR-744 from 1-2 years progression, for let-7e from 1 year progression. Log2 scale of amount of copies/1ng of miRNA/RNA was used for miRNA expression in this analysis. The Y axis represents survival probability and the X axis represents time of follow up in days.

sen and confirmed as significantly deregulated on a bigger cohort of MM and MGUS patients using an absolute quantification approach. At this point, since no miRNA is accepted as a standard for serum samples, this is probably the most accurate method of quantification of serum miRNAs in MM. Therefore, to accurately determine the expression differences between groups, miRNA levels were normalized to amount of miRNAs copy numbers per 1 ng of total RNA/miRNA.

Analytical characteristics of the five miRNAs (miR-744, miR-130a, miR-34a, let-7d and let-7e) showed that they can all discriminate MGUS and MM from HD. However, the combination of serum miR-34a and let-7e (the highest sensitivity of 91.1% and specificity of 96.7% for MGUS, and 80.6% sensitivity and 86.7% specificity for MM) proved to be an even more powerful discriminating tool.

In the group of MM patients, most of the five miRNAs were observed to be associated with some of the clinical parameters, ISS or DS sub-stage. Particularly in the cases of miR-744, let-7d and let-7e, lower levels were associated with advanced ISS stage. As lower levels of miR-744, miR-130a and let-7d are related to the advanced DS sub-stage, they might reflect the renal impairment that often develops in MM patients. This observation is further supported by the relation of lower miRNA levels to higher creatinine and β2-microglobulin levels. Lactate dehydrogenase (LDH) level helps to assess tumor burden, and the level of β2-microglobulin reflects the tumor mass.[6,34] Furthermore, anemia associated with MM is caused by inadequate erythropoietin levels consequent to renal impairment and the effect of inflammatory cytokines.[35] C-reactive protein (CRP) as well as albumin levels are known to be hallmarks of tumor activity.[36,37] Taking all these facts into consideration, we can anticipate that serum miRNA levels are associated with tumor mass and disease activity. Interestingly, such correlation pattern with biochemical parameters was observed also for MGUS.

However; as no correlation with infiltration of BMPCs in MM and MGUS was observed, which is in concordance with previously presented data from another group,[29] our observations further suggest that circulating miRNAs reflect other MM pathological effects as well. To further investigate potential derivation of all studied miRNAs, we estimated their levels in exosomal and exosome-depleted fractions and in BMPCs. Four miRNAs were observed to be present primarily in exosomes, which is consistent with previous observations that exosome fraction is highly enriched in miRNAs.[38] Moreover, all of the studied miRNAs were found to be abundantly present in BMPCs when compared to levels in exosomes. Interestingly, levels of miR-130a were comparable in exosomal fraction and in BMPCs, suggesting their involvement in intercellular communication. However, as we did not find any linear dependence between miRNA levels in exosomal fraction/exosome-depleted fraction and miRNA levels in BMPCs, it is not clear whether they originate from BMPCs.

Different miRNAs expression was confirmed also in 18 paired MM samples taken at diagnosis and at relapse with higher levels of miR-34a and lower levels of let-7d, suggesting that deregulated levels of miRNAs reflect patient condition and are associated with more advanced disease.

To the best of our knowledge, the possibility of a prognostic serum miRNAs marker in MM has not yet been investigated. In this study, lower levels of miR-744 and let-7e were found to be significantly associated with the worse OS and TTP of MM patients. It should be mentioned that this is related to a short-time period (1-2 years). For-miR-744, the observation could be partially explained by the fact that the gene for miR-744 lies in the 17p12 region, close to the *TP53* gene (17p13). Deletions at chromosome 17p13.1-17p12 were previously found to be associated with poor survival.[39] Also, low *TP53* gene expression, which is highly correlated with loss of heterozygosity of the TP53 locus, was associated with shorter event-free survival and OS.[40]

However, we were not able to prove the relationship between low levels of miR-744 and deletion of *TP53*, and thus we cannot say that absence of the 17p13.1-17p12 region can fully explain the lower levels of miR-744.

As patients were not equally distributed across ISS stage, we assume that miR-744 and let-7e impact on OS and TTP could be explained by ISS heterogeneity. However, no differences in DS stage between groups with low/high expression of miR-744 were observed, but they were observed between groups with 'low/high' expression of let-7e. Interestingly, the miR-744 'low' expression group of patients was associated with presence of 1q21 amplification or t(4;14), which have been previously described as unfavorable prognostic factors for MM.[41,42]

The 'low/high' miR-744 and let-7e groups of MM patients were also observed to be clinically heterogeneous, which was demonstrated by different levels of albumin, creatinine, β2-microglobulin, LDH, hemoglobin and thrombocyte count between groups. As mentioned above, all listed parameters are known to be markers of tumor mass and disease activity.[34,35,43] Although our initial findings concerning clinical data, such as OS and TTP, show that these miRNAs are not an independent factor, but rather a hallmark of a complex pathological process that accompanies MM, they both reflect disease status and thus can serve as new auxiliary peripheral blood prognostic markers for MM.

In conclusion, we have identified for the first time a profile of five serum miRNAs which are deregulated in MM and MGUS sera. Levels of miR-744, miR-130a, let-7d and let-7e were significantly decreased whereas miR-34a was increased in MM and MGUS. Deregulated levels of miRNAs were observed in advanced MM suggesting that they are stable markers of MM. Moreover, levels of miR-744 and let-7e might be useful as a marker of patients' survival. Even though additional larger-scale studies are needed to address other biological characteristics of these miRNAs, it is obvious that circulating serum miRNAs have diagnostic and prognostic implications for MGUS and MM patients.

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