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Original Article

Circulating serum microRNAs as diagnostic biomarkers for multiple myeloma

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Abstract: Objective: The aim of the present study is to investigate a new and better blood-based biomarker for the detection and observation of MM. Methods: Real-time quantitative RT-PCR assay was performed to evaluate the expression levels of the miRNAs (miR-29a, miR-155, miR-16, and miR-92a) circulating in the serum of different status MM patients. Results: Level of serum miR-29a significantly increased in newly diagnosed patients (MM1) as compared to HD ($P < 0.01$). Serum miR-155 levels were significantly lower in MM1 as compared to HD ($P < 0.001$); a combination of miR-29a and miR-155 was demonstrated to be an effective biomarker for distinguishing MM from HD, with an AUC (area under of ROC curve) of 0.8739, sensitivity of 80.77%, and specificity of 83.33%. Conclusion: Our results indicated that circulating serum microRNAs may serve as potential diagnostic biomarkers for multiple myeloma.

Keywords: MicroRNA, multiple myeloma, biomarkers

Introduction

Multiple myeloma (MM), a malignant disorder characterized by neoplastic growth of bone marrow plasma cells (PCs), accounts for approximately 13% of all hematological cancers and 1% of all cancers [1-3]. Although the high dose of chemotherapy with autologous stem cell support, and novel biologically targeted treatment, such as bortezomib or immunomodulatory drugs (lenalidomide and thalidomide), have improved the overall survival of patients, MM is still considered incurable [4]. Currently, the diagnosis of MM depends on the traditional bone marrow puncture, which is an invasive manipulation and cannot be repeated as often as needed. The presence of a specific marker in the peripheral blood will be non-invasive and make it convenient to obtain blood at various time points of treatment, even at the time of remission for frequent monitoring [5].

MicroRNAs (miRNAs) are a large family of post-transcriptional regulators of target gene expression that are short non-coding RNAs of approximately 18-22 nt in length [6]. They play an important role in a variety of physiological and pathological processes, such as development, differentiation, cell proliferation, apoptosis, and stress responses [7]. As of June 2014, 2581 mature human miRNAs have been recognized and listed in the recent version 21.0 of miRbase (mirbase.org). miRNAs have been reported to be involved in the initiation and progression of solid tumors as well as hematological malignancies [8]. Mansour et al reported that TAL1-mediated upregulation of miR-223 promotes the malignant phenotype in T-cell acute lymphoblastic leukemia (T-ALL) through repression of the FBXW7 tumor suppressors [9]. Rokah et al showed that miR-31, miR-155, and miR-564 are downregulated in chronic myeloid leukemia CML cell lines and patients in comparison to non-CML cell lines and healthy blood [10]. Moreover, miRNAs have been found to circulate in a stable form in body fluids, such as serum, plasma, saliva, and urine. Several studies have demonstrated the usefulness of serum miRNAs as potential biomarkers for detecting and monitoring cancer progression. The circulating serum miRNAs differ between healthy donors and patients in colorectal and lung cancer as well as diabetes [11]. A recent study described circulating miRNAs as useful, noninvasive diagnostic tools for breast cancer [12].

Table 1. Relative expression and characteristics of ROC curve

miRNAs	Relative expression			*p*-value HD-MM1	*p*-value MM1-MM2	AUC	*p*-value (AUC)	Sensitivity-specificity (%)
	HD	MM1	MM2					
miR29a	0.839	2.982	0.645	0.003** (p < 0.01)	0.028*	0.763	0.003**	81.48-72.22 (> 0.5544)
miR155	0.894	0.338	0.334	< 0.0001***	0.597	0.862	< 0.0001***	80.00-72.22 (< 0.6106)
miR16	1.487	5.613	3.467	0.024* (p < 0.05)	0.492	0.704	0.024*	72.00-66.67 (> 1.129)
miR92a	1.880	4.105	3.192	0.195	0.973	0.623	0.190	56.00-68.75 (> 2.292)
miR29a/miR155				< 0.0001***		0.874	< 0.0001***	80.77-83.33 (> 1.597)
miR16/miR155				< 0.001***		0.827	< 0.001***	76.00-72.22 (> 1.75)

*P < 0.05. ** P < 0.01. *** P < 0.001. HD, healthy donors; MM1, newly diagnosed multiple myeloma before chemotherapy; MM2, multiple myeloma after chemotherapy; AUC, Area under the ROC curve.

Aberrant expression of circulating miRNAs has been reported in hematological malignancies, including MM. For instance, the first investigation on circulating miRNAs in MM revealed downregulated plasma miR-92a levels in patients with MM, but not in patients with smoldering MM or MGUS; additionally, the downregulation of miR-92a in plasma correlated with the response to treatment [13]. In another study, three serum miRNAs (miR-720, miR-1308, and miR-1246) were used as biomarkers for distinguishing MM/MGUS from healthy individuals [14]. Furthermore, a recent study on serum miRNAs showed that a combination of miR-34a and let-7e can distinguish MM/MGUS from healthy donors (HD), and lower levels of miR-744 and let-7e were associated with shorter overall survival and remission of MM patients [15]. Further research on the aberrant expression of miRNAs may possibly lead to the discovery of novel miRNA biomarkers for multiple myeloma. Therefore, in the present study, we chose four miRNAs (miR-29a, miR-155, miR-16, and miR-92a) based on their possible relationship with MM pathogenesis, and demonstrated their aberrant expression in MM serum. Through this study, we aim to establish a new blood-based biomarker for MM detection.

Materials and methods

Patients and processing of blood samples

The patients were diagnosed according to the NCCN (National Comprehensive Cancer Network) clinical practice guidelines for MM (16). Written informed consent was obtained from all the participants after explanation of the nature of the study, and the study was approved by the research ethics board of Xiamen University Zhongshan Hospital (Xiamen, China). We collected blood samples from 30 patients of MM1 who are newly diagnosed and 10 patients of MM2 who received chemotherapy and the status of them are at least reaching to very good partial response. The details of the patients are presented in **Table 1**. For healthy donor (HD) (n = 20) and these MM patients were diagnosed between July, 2013 and June, 2014 in the department of Hematology of Xiamen University Zhongshan Hospital. The peripheral blood samples were centrifuged at 3500 g for 10 min, and the serum was transferred into RNase/DNase-free tubes and stored at -80°C. All blood samples were processed within 2 h of obtaining them.

miRNA isolation from human serum

Total small RNA, including miRNAs, was isolated using the miRNeasy serum/plasma kit (Qiagen, Hilden, Germany), following the manufacturer's protocol for liquid samples. The total small RNA was stored at -80°C until further use.

qRT-PCR with serum samples

Total small RNA from the serum was reverse-transcribed to cDNA using RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Lithuania, EU). The miRNA-specific

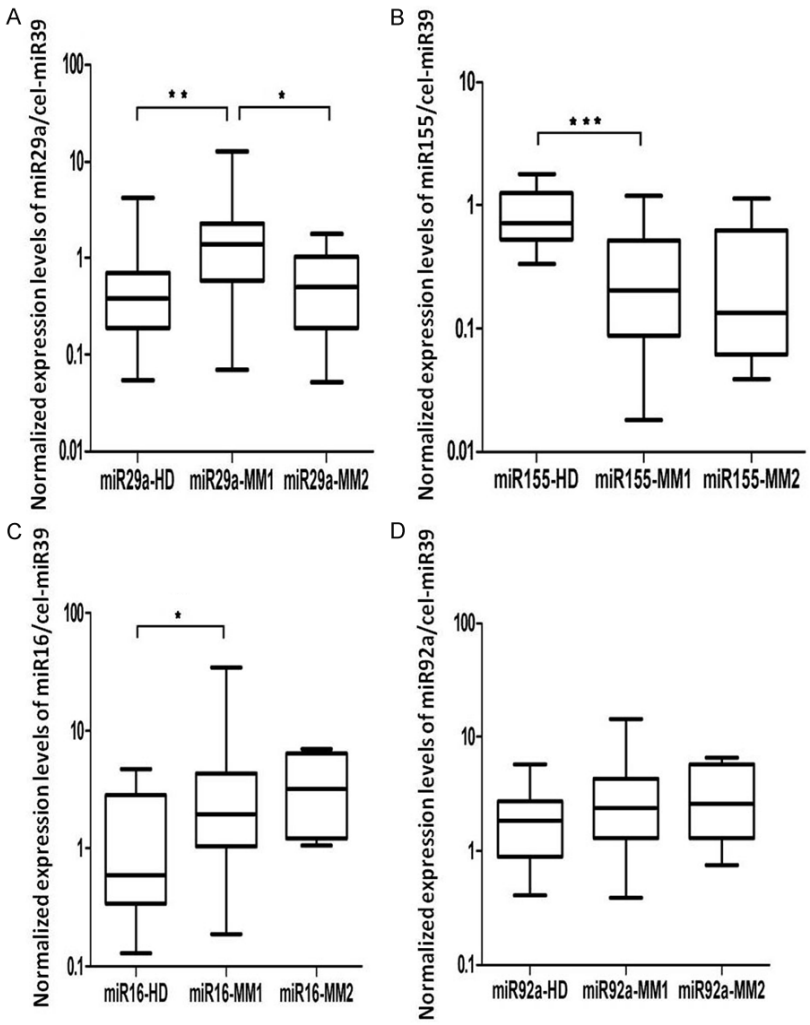

Figure 1. The level of serum miRNA expression in myeloma patients. Comparison of expression level of serum miR-29a (A), miR-155 (B), miR-16 (C), and miR-92a (D) (log10 scale on *y*-axis) in healthy donors (HD) (*n* = 20), in patients with newly diagnosed multiple myeloma (MM1) (*n* = 30), and in patients after treatment (MM2) (*n* = 10). Expression of each miRNAs is normalized to the expression of cel-miR-39. Lines represent mean value, 25-75% quartile and min-max values. Statistically significant differences were determined using Mann-Whitney U test.

primers used for the reaction were cel-miR-39, hsa-miR-29a, hsa-miR-155-5p, hsa-miR-16-5p, and hsa-miR-92a-3p (Sangon Biotech Co. Ltd., Shanghai, China). qRT-PCR was performed using the AccuPower 2×Greenstar qPCR Master Mix kit (Bioneer, Daejeon, Korea), according to the manufacturer's instructions and the levels of cel-miR-39, miR-29a, miR-155, miR-16, and miR-92a in serum were detected. qRT-PCR was performed on an ABI PRISM 7500 instrument (Applied Biosystems), using the manufacturer's recommended cycling conditions. Ct data were obtained using SDS v1.4.0 software (Applied Biosystems) with the default threshold settings.

qRT-PCR data was analyzed using the SDS v1.4.0 software (Applied Biosystems) (settings: automatic baseline, threshold 0.2). Expression data were normalized to the expression of cel-miR-39 (C. elegans miR-39) reference miRNA (miRNeasy Serum/Plasma Spike-In control; QIAGEN). The expression level of the cel-miR-39 is the most stable and gives no significant difference in the cel-miR-39 Ct values between control and MM samples. Comparative qRT-PCR was performed in duplicates, which included no-template controls. ΔCt was calculated by subtracting the Ct of cel-miR-39 from the Ct of miRs. The relative quantitative value of miRs was calculated by the $2^{-\Delta\Delta CT}$ equation (17). The $2^{-\Delta\Delta CT}$ method was used for analysis, where ΔΔCT = (average CT_{miRNA}-average $CT_{cel\text{-}miR\text{-}39}$)$_{MM}$-(average CT_{miRNA}-average $CT_{cel\text{-}miR\text{-}39}$)$_{HD}$.

Statistical analysis

GraphPad v5.0 software (Graphpad Software Inc., La Jolla, CA, USA) was used for statistical analysis. The Mann Whitney U test was used to determine statistical significance between two groups and one-way ANOVA for three or more groups, using the median value as the high/low cut-off point. Area under the curve (AUC), sensitivity, and specificity for serum miRNAs levels were determined using receiver operator characteristic (ROC) analysis. All calculations were performed using GraphPad Prism v.5.0. *P*-values of less than 0.05 were considered statistically significant. Results were processed using the non-parametric Mann Whitney U test.

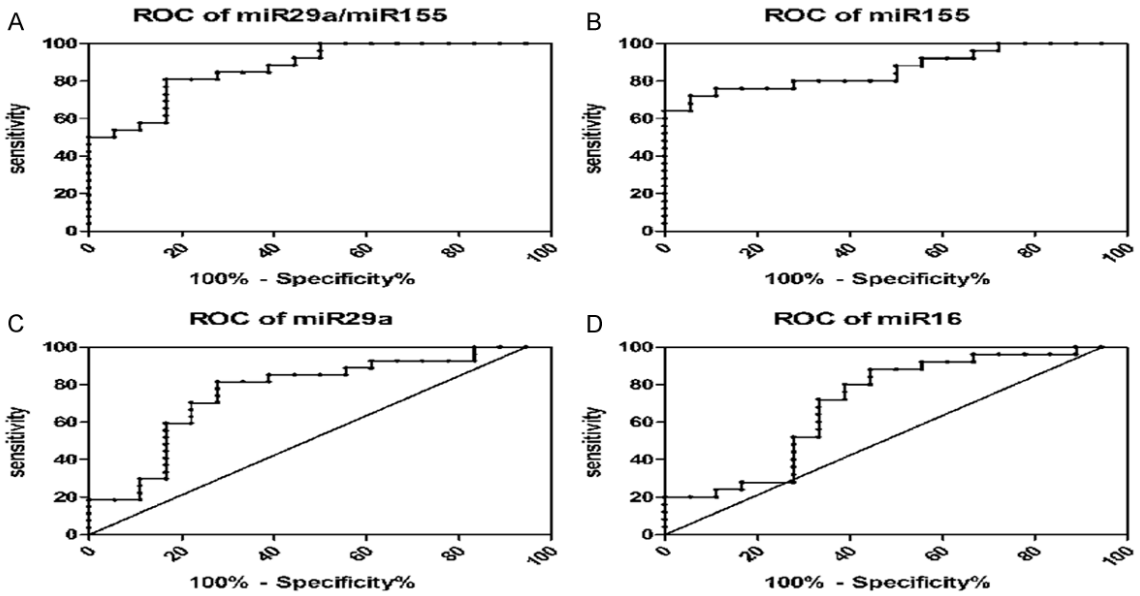

Figure 2. The comparison of miRNA expression as diagnostic biomarkers for myeloma parents. (A) A combination of serum miR-29a/miR-155, noted as the most promising in distinguishing between MM1 and HD with an AUC of 0.8739 ($P < 0.0001$). Other miRNAs that distinguished between MM1 and HD are (B) miR-155 ($P < 0.001$); (C) miR-29a ($P < 0.01$); and (D) miR-16 ($P < 0.05$).

Table 2. miR29a expression level in different clinical phases

	A (mean)	B (mean)	C (mean)
miR-29a relative level	2.56072	1.27651	0.820959
Bone marrow plasma cells	65%	31%	5%
M-protein (IgG)	109	54	31.8
Globulin (GLO)	74	44	31
B2-Microglobulin (B2 m)	39.5	15.6	5.97

A: Diagnostic phase, B: PR phase after treatment, C: CR phase.

Results

Expression of miRNAs in serum of MM patients

Our results indicated that the level of miRNA in serum was due to aberrant expression in MM patients. In newly diagnosed MM patients (MM1), serum miR-29a level was higher as compared to healthy donors ($P < 0.01$); and following treatment with chemo-drugs, the level of miR-29a was found to descend in MM2 ($P < 0.05$) (**Figure 1A**). Compared with the HD samples, serum miR-155 level was significantly lower in MM1, while it showed no significant difference between MM1 and MM2 ($P = 0.597$) (**Figure 1B**). In addition, the level of miR-16 in serum was upregulated in MM1 when compared to HD ($P < 0.05$), while there was no significant difference observed between MM1 and MM2 ($P = 0.492$) (**Figure 1C**). Serum miR-92a expression level did not reach statistical significance ($P = 0.1949$) (**Figure 1D**), and there were no significant difference of serum miR-92a level between MM1 and MM2 ($P > 0.5$). The level of serum miR-29a, miR-155, miR-16, and miR-92a in HD, MM1, and MM2 patients are listed in **Table 1**.

miRNAs as biomarkers for distinguishing MM patients from HD

The receiver operating characteristic curve (ROC) was generated by comparing the $2^{-\Delta\Delta CT}$ values of patients with those of HD. AUC (area under the curve of ROC) refers to the diagnostic value of the research target, which shows that larger the AUC value, higher the accuracy of the classification effect. The analysis showed that the combination of miR-29a/miR-155 might serve as the most useful biomarker for differentiating MM1 from HD. The AUC obtained for this combination of markers was 0.8739 (95% confidence interval [CI]: 0.7725-0.9754), with 80.77% sensitivity (95% CI: 60.65%-93.45%) and 83.33% specificity (95% CI: 58.58%-96.42%), at the cutoff level of 1.597 (**Figure**

2A). miR-29a, for MM1, yielded an AUC of 0.7634 (95% CI: 0.6141-0.9127), with a sensitivity of 81.48% (95% CI: 61.92%-93.70%) and a specificity of 72.22% (95% CI: 46.52%-90.31%) at the cutoff level of 0.5544 (**Figure 2B**). The results showed that miR-155, with an AUC of 0.8622 (95% CI: 0.7540-0.9705), revealed 80.00% sensitivity (95% CI: 59.30%-93.17%) and 72.22% specificity (95% CI: 46.52%-90.31%) at the cutoff level of 0.6106 (**Figure 2C**). At a cutoff value of 1.129, miR-16 discriminated between MM1 and HD with an AUC value of 0.7044 (95% CI: 0.5376-0.8713), a sensitivity of 72.00% (95% CI: 50.61%-87.93%) and a specificity of 66.67% (95% CI: 40.99%-86.66%) (**Figure 2D**). The AUC, sensitivity, and specificity of serum miR-29a, miR-155, miR-16, and miR-92a in HD and MM1 are listed in **Table 1**.

Discussion

Although the source and function of circulating miRNAs still remain unclear, circulating miRNAs may not always be directly associated with malignant cells [18, 19]. Since peripheral blood is easily accessible, serum miRNAs are seriously being investigated for use in cancer detection. Several studies have reported the potential use of circulating miRNAs as biomarkers for detecting and monitoring the progression of hematological malignancies, including MM [5, 13-15]. Unfortunately, there is limited information available on the circulating miRNAs and their clinical use in MM, and the results are neither similar nor conclusive [20]. Sevcikova et al. reported an upregulated serum miR-29a and its application as a diagnostic biomarker in newly diagnosed MM patients [5]. In the present study, we detected four circulating serum miRNAs, viz. miR-29a, miR-155, miR-16, and miR-92a, out of which, miR-29a, miR-155, and miR-16 displayed the ability to discriminate between MM and HD. However, the combination of serum miR-29a and miR-155 (AUC, 0.8739; sensitivity, 80.77%; and specificity, 83.33%) proved to be a more powerful diagnostic biomarker for differentiating MM from HD. Furthermore, we verified that the measurement of serum miR-29a level in MM patients is useful for initiation of chemotherapy and monitoring of the disease status.

Studies have suggested that serum miR-155 is upregulated and acts predominantly as an oncogene; it plays a key role in the pathogenesis of hematological malignancies, including malignant lymphoma, acute myeloid leukemia, and myelodysplastic syndrome [21-23]. However, certain reports have indicated that miR-155 does not act as an oncogene but as a tumor suppressor in chronic myeloid leukemia and Caski cells [24, 25]. Additionally, an investigation on the expression level of serum miR-155, miR-15a, miR-16-1, and miR-29c showed that these miRNAs are downregulated in diffuse large B cell lymphoma and they can be used as promising novel biomarkers [26]. Similar to these findings, our results showed that miR-155 is more downregulated in the serum of MM patients as compared to HD ($P < 0.001$), indicating that miR-155 may act as tumor suppressor in MM. ROC analysis further showed that serum miR-155 may be a useful marker for newly diagnosed MM (AUC, 0.8622; sensitivity, 80.00%; specificity, 72.22%).

In this study, we continuously monitored the level of miR29a in three myeloma patients, before and after treatment. Our data indicated that the expression of miR-29a changed with the evolution of MM condition: for newly diagnosed patients, the expression of miR-29a was significantly upregulated, and was downregulated to normal levels after treatment, which is consistent with the changes in bone marrow plasma cells and MM protein levels (**Table 2**). Our study presents preliminary results showing that the level of circulating serum miRNAs may form a useful indicator (biomarker) to evaluate the effect of treatment on MM. If our hypothesis is confirmed, these miRNAs can be used as a simple and quick tool for the diagnosis and chemotherapy evaluation of MM.

In summary, the present study revealed that circulating serum miR-29a, miR-155, and miR-16 show the potential to discriminate newly diagnosed multiple myeloma patients from healthy donors. Serum miR-29a and miR-16 expression levels were found to be upregulated, while miR-155 was downregulated in MM patients. Meanwhile, serum miR-29a level in MM patients could prove useful for the initiation of chemotherapy and for monitoring the disease status. Our data suggested that the expression of specific miRNAs may contribute to MM diagnosis and monitoring, and their roles in the pathogenesis of this disease should be examined further.

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Disclosure of conflict of interest

None.

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