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The role of circulating miRNAs in multiple myeloma

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Multiple myeloma (MM) is a common malignant hematological disease. Dysregulation of microRNAs (miRNAs) in MM cells and bone marrow microenviroment has important impacts on the initiation and progression of MM and drug resistance in MM cells. Recently, it was reported that MM patient serum and plasma contained sufficiently stable miRNA signatures, and circulating miRNAs could be identified and measured accurately from body fluid. Compared to conventional diagnostic parameters, the circulating miRNA profile is appropriate for the diagnosis of MM and estimates patient progression and therapeutic outcome with higher specificity and sensitivity. In this review, we mainly focus on the potential of circulating miRNAs as diagnostic, prognostic, and predictive biomarkers for MM and summarize the general strategies and methodologies for identification and measurement of circulating miRNAs in various cancers. Furthermore, we discuss the correlation between circulating miRNAs and the cytogenetic abnormalities and biochemical parameters assessed in multiple myeloma.

miRNA, multiple myeloma, biomarker, diagnosis

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Multiple myeloma (MM) accounts for approximately 13% of all hematologic malignancies and 1% of all malignancies [1,2]. MM is a clonal B-cell malignancy characterized by the expansion of clonal plasma cells in the bone marrow, accompanied by abnormal accumulation of monoclonal antibodies [3,4]. Typically, MM evolves from a premalignant condition known as monoclonal gammopathy of undetermined significance (MGUS). The incidence of MGUS is approximately 3% in the general population 50 years of age or older, and approximately 1% of MGUS patients develop into MM each year [1]. Compared to MM, MGUS is characterized by a lower concentration of serum monoclonal antibody, less bone marrow plasmacytosis and a lack of organ damage, including hypercalcemia, renal impairment, anemia and bone lesions. Currently, there are no specific tests for distinguishing between these two conditions and estimating the risk of progression from MGUS to MM. Additionally, no current strategies exist to prevent the progression from MGUS to MM; therefore, novel biomarker which may predict progression is urgently needed in MGUS patients.

miRNAs are an abundant class of regulatory noncoding single-stranded RNA molecules approximately 20–23 nucleotides long. In general, miRNAs bind with imperfect complementarity to the 3′-untranslated region (3′-UTR (untranslated regions)) of a specific target mRNA to promote its degradation and/or inhibit its translation [5]. Many miRNAs display regulatory roles in all major biological processes, including cell motility, differentiation, proliferation and apoptosis [6,7]. Aberrant miRNA expression profiles are frequently discovered during the initiation and progression of cancers, including early or advanced disease stages, response, remission and relapse. Recently, it has been demonstrated that miRNAs are secreted into body fluids, including serum and plasma, and can maintain stability while freely circulating in the bloodstream.

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1 miRNAs and MM

As growing research has indicated that miRNAs played important roles in the occurrence, development, recurrence and drug resistance of MM, miRNA signatures may serve as potential biomarkers for myeloma diagnosis, prognosis, and response to treatment. For example, hemizygous and/or homozygous chromosomal deletion at the 13q14 locus in MM patients can result in the loss of or reduction in expression of miR-15 and miR-16, which is correlated with tumorigenesis and the development of MM [8,9]. Pichiorri et al. [10] first described comprehensive global miRNA expression profiling of MM, MGUS and normal plasma cells, which indicated that MM miRNA signatures modulated the expression of proteins critical to myeloma pathogenesis. In 2010, Gutierrez et al. [11] investigated the association between miRNA expression profiles and their corresponding target genes and found that the downregulation of several miRNAs resulted in overexpression of cyclin D2 (CCND2) in MM. These authors demonstrated for the first time that miRNA expression patterns in MM were associated with genetic abnormalities. Yyusnita et al. [12] revealed that several miRNAs, including downregulated let-7c and miR-16 and upregulated miR-449, miR-181a and miR-181b, exhibited similar expression patterns in peripheral blood compared to data obtained from bone marrow aspirates of MM patients. However, certain miRNAs, including miR-21, the miR-106b-25 cluster and miR-181a/b, promoted oncogenic activity during the malignant transformation of plasma cells in MM and MGUS [10]. Furthermore, patients with high expression levels of the miR-17-92 cluster had shorter progression-free survival (PFS) compared to those with low level expression, suggesting that this cluster of miRNAs was associated with tumorigenesis and poor prognosis in MM [13]. Additionally, miR-21 may display an oncogenic role in MM; therefore, antagonism of miR-21 by oligonucleotide inhibitors may result in anti-tumor activity against MM [14]. More recently, circulating miRNAs in body fluids (serum and plasma) have been shown to be significantly associated with MM pathogenesis.

Recently, a variety of evidences suggested interaction between MM cells and bone marrow microenvironment (BMME) components played an increasingly important role in MM pathogenesis and progression. Aberrant expression of some miRNAs in MM-BMSCs (bone marrow stromal cells) or exosome resulted in altered expression of miR-related targets including cytokines, adhesion molecules, DNA methyltransferase and signaling molecules (Table 1) [15–21]. It has been confirmed that interleukin-17 (IL-17) produced by the tumor microenvironment played a crucial role in promoting cell proliferation, inhibiting cellular and facilitating cell migration. Sun et al. demonstrated that negative correlation between miR-192 expression and IL-17 or IL-17RA expression was observed in bone marrow specimens derived from MM patients [15]. Furthermore, Shen et al. [16] indicated that miR-202 served as a negative modulator that can regulate BAFF by inhibiting MM cell survival, growth, and adhesion in BMME. Up-expression of miR-202 in BMSCs resulted in MM cells more sensitive to bortezomib. More interestingly, a recent study suggested that exosomal miR-135b endosome-derived from hypoxia-resistant MM (HR-MM) cells can control MM angiogenesis through directly inhibiting the expression of factor-inhibiting hypoxia-inducible factor 1 (FIH-1) in endothelial cells [17].

2 Biogenesis and strategies for the identification of circulating miRNA in cancer

It remains unclear whether cell-free tumor-related miRNAs

Table 1 Overview of specific miRNAs and their role in the MM microenviroment

miRNA	Up-regulation/ Down-regulation	Gene targets	Description of miRNAs functions	Reference
miR-192 miR-215	Down-regulation	IL-17Rs(IL-17RA and IL-17RE)	IL-17/miR-192/IL-17Rs regulatory feedback loop regulated MM cell proliferation, apoptosis and migration	[15]
miR-202	Down-regulation	B cell-activating factor (BAFF)	Regulating MM cell survival, growth, and adhesion in BMME and induction of BMSC mediated drug resistance	[16]
miR-135b	Up-regulation	Facter-inhibiting hypoxia-inducible factor 1 (FIH-1)	Exosomal miR-135b suppressed FIH-1 in endothelial cells and regulated tumor angiogenesis	[17]
miR-126-3p miR-140	Down-regulation	DNA methyltransferase-1 (DNMT-1)	miR-126-3p and miR-140 suppressed DNMT1 expression and re-expressed RANKL in MM cells	[18]
miR-29b	Down-regulation	DNMT-3A and DNMT-3B	miR-29b inhibited DNMT-3A and DNMT-3B expression and reduced global DNA methylation	[19]
miR-21	Up-regulation	RhoB	Induction of BMSC-mediated drug resistance in MM cells partially through NFκB pathway and by repressing RhoB	[20]
miR-15a/16	Down-regulation	Not mentioned	IL-6 secreted by BMSCs inhibited miR-15a/16 expression and promoted drug-resistance in myeloma cells	[21]

in the circulation are secreted by tumor cells or immunocytes in the tumor microenvironment or are the by-product of tumor cell death and lysis. One hypothesis is that passive release occurs during cell injury [22]. A second opinion is that the high rate of proliferation and cell lysis in tumors might contribute to the abundance of miRNAs in the circulation because a pattern of nonspecific miRNA release has been observed in various tumors [23]. Furthermore, the third possibility is that massive circulating miRNAs derive from immunocytes in the tumor microenvironment or the immune response regulated by the appropriate organ system [24]. Cell-free miRNAs in the blood stream are very stable and can be protected from degradation by encapsulation in lipid vesicles including microvesicles, exosomes, and apoptotic bodies. These vesicles are capable of binding with argonaute 2 (AGO2), nucleophosmin 1 (NPM1) and high- density lipoprotein (HDL). Additionally, understanding the origins of circulating miRNAs requires insight into the association between expression levels of circulating miRNAs and the corresponding tumor cells that they affect. Most studies found consistent trends between changes in tumor tissue miRNAs and those observed in circulating miRNAs [25,26]. However, some of the cellular miRNAs may be selectively released into the serum [27]. Therefore, it is feasible to distinguish pathogenesis from normal physiology by the detection of certain circulating miRNAs originating from different tissue cells.

In general, strategies for identifying tumor-related circulating miRNAs employ similar methodologies among various cancers (Figure 1). The first step is efficient extraction and purification of miRNAs from serum or plasma. It is important to avoid miRNA degradation and contamination by additional substances in body fluids. Contaminations may result from the presence of intact blood cells in the serum or plasma or lysis of these cells (hemolysis) during sampling and processing [28–30].

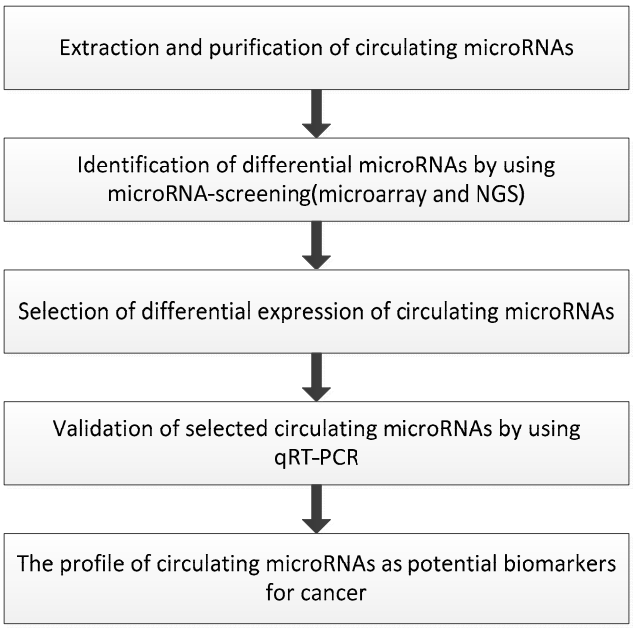

Figure 1 Flow chart of experimental design for the identification of circulating miRNA biomarkers in cancer.

After purification, it is vital to obtain global profiles of miRNA signatures and compare the differential expression of miRNAs between tumor patients and healthy controls using the following three methodologies: qRT-PCR, miRNA microarray, and next-generation sequencing (NGS). Diagnostics based on circulating miRNAs rely on accurate measurements of miRNAs from the serum or plasma with high sensitivity and precision. Real time RT-PCR has been widely used as a sensitive and precise tool to detect low-abundance circulating miRNAs; however, there are crucial methodological challenges involved in the normalization during detection of circulating miRNAs. Recent studies have indicated that a combination of several miRNAs can serve as a stable reference for the normalization of circulating miRNAs, and this method is statistically superior to the single reference gene approach previously used [31,32]. Moreover, to accurately determine the differential expression of miRNAs between groups, absolute quantification of circulating miRNAs appears to be the more reliable way. NGS and microarray are also effective techniques for identification of circulating miRNAs, but are less frequently used due to increased starting material requirements. Furthermore, the validation of NGS and microarray data need subsequently perform at the individual patient level by qRT-PCR. In particular, the application of NGS and microarray techniques is challenging for most laboratories as these approaches are expensive to validate and NGS itself remains costly and labor intensive. These issues may explain why qRT-PCR-based profiling strategies are more frequently used [33]. However, NGS has the notable advantage of distinguishing among unknown RNA species that would not be amplified by qRT-PCR. This may correlate with the emerging notion that miRNA 5′- and 3′-end structural variants, termed isomiRs, are frequently expressed and have been linked to tumorigenesis [34].

3 Diagnostic, prognostic and predictive biomarkers of circulating miRNA in MM

Distinctive circulating miRNAs profiles may be observed in MM patients compared to healthy donors and MGUS patients, and this provides an important auxiliary tool for distinguishing MM patients from healthy controls and MGUS patients (Table 2). Kubiczkova et al. [35] obtained serum miRNA expression profiles by taqman low density arrays (TLDA), and validated these results by qRT-PCR in a cohort of newly diagnosed, relapsed MM and MGUS patients. A combination of miR-34a and let-7e could distinguish MM patients from healthy individuals with a sensitivity of 80.6% and a specificity of 86.7%; MGUS patients could be distin-

Table 2 Synopsis of the potential of circulating miRNAs as biomarkers of MM

Body fluid	Sample size	Methodology	Upregulated-miR	Downregulated-miR	References
Serum	Newly diagnosed MM patients (*n*=103) vs MM patients in relapse (*n*=18) vs MGUS (*n*=57) vs healthy donors (*n*=30)	Taqman low density miRNA arrays (TLDA) and qRT-PCR	miR-34a	let-7d let-7e miR-130a miR-744	[35]
Serum	MGUS patients (*n*=15) vs MM patients (*n*=24) vs normal hospitalized controls (*n*=20) vs normal healthy controls (*n*=13)	Microarray and qRT-PCR	miR-1246 miR-720	miR-1308	[36]
Plasma	Symptomatic MM (*n*=138) vs smoldering MM (*n*=8) vs MGUS (*n*=22) vs healthy volunteers (*n*=21)	Taqman low density miRNA arrays (TLDA) and qRT-PCR	miR-146a miR-16 miR-186 miR-454 miR-483-5p	miR-17 miR-19a miR-19b miR-20a miR-92a	[37]
Serum	MM patients (*n*=91) vs healthy controls (*n*=30)	qRT-PCR	miR-142-5p miR-29a miR-660	–	[38]
Serum	MM patients (*n*=40) vs healthy controls (*n*=30)	qRT-PCR	miR-202	–	[39]
Plasma	Newly diagnosed MM patients (*n*=40) vs healthy individuals (*n*=20)	Microarray and qRT-PCR	miR-483-5p	miR-20a	[40]
Serum	Newly diagnosed symptomatic MM patients (*n*=108) vs healthy donors (*n*=56)	Microarray and qRT-PC	miR-135b-5p miR-214-3p miR-33b miR-3658 miR-4254	miR-19a miR-92a	[41]
Plasma	MM patients (*n*=40) vs healthy controls (*n*=28)	Taqman low density miRNA arrays (TLDA) and qRT-PCR	miR-148 miR-181a miR-20a miR-221 miR-99b	–	[42]
Serum	MM patients (*n*=38) vs MGUS (*n*=8) vs healthy controls vs paired MM patients (*n*=17) at CR and relapse after autologous stem-cell transplantation (ASCT)	TaqMan human microRNA arrays and qRT-PCR	–	miR-16 miR-25 miR-20a miR-25 miR-660	[43]

guished from healthy donors with a sensitivity of 91.1% and a specificity of 96.7%. Jones et al. [36] revealed that circulating miR-720 and miR-1246 were upregulated, and miR-1308 downregulated in MM patient sera. Further study found that the combination of miR-720 and miR-1308 was useful in distinguishing healthy controls, as well as patients with unrelated diseases from pre-cancerous myeloma and MM patients. Additionally, miR-1246 and miR-1308 expression could distinguish MGUS from MM patients [36]. Furthermore, low expression of plasma miR-92a in symptomatic MM patients was found compared with healthy donors [37]. As previously reported, miR-92a was identified as the most important potential diagnostic marker in several malignancies, including colorectal cancer [44], breast cancer [27], non-Hodgkin's lymphoma [45], and gastric cancer [46]. The upregulation of miR-29a was helpful in discrimination between MM and healthy donors with a sensitivity of 88% and a specificity of 70% [38]. The miR-202 expression was significantly higher in MM patients than in healthy controls, and the sensitivity and specificity for miR-202 in this diagnosis of MM patients were 80.0% and 60.0%, respectively [39]. Recently, increased miR-483-5p and decreased miR-20a expression display a diagnostic role in discriminating between MM patients and healthy subjects [40]. Furthermore, Hao et al. [41] found that a combination of miR-19a and miR-4254 could distinguish MM patients from healthy controls with a sensitivity of 91.7% and a specificity of 90.5%.

More recently, some studies have supported the fact that circulating miRNAs could function as non-invasive biomarkers to estimate the prognosis of MM patients. miR-744 and let-7e were verified as possible indicators of survival in MM, and low miR-744 and let-7e levels were associated with shorter overall survival (OS) and remission, and shorter time to progression compared to patients with high expression of these molecules [35]. These poor clinical outcomes can be partially explained by the fact that the gene for miR-744 is located in the 17q12 region where various tumor-related genes are closely situated, including TP53, BRCA1, and FBXO47. Deletion of chromosome 17q12 has been previously associated with poor survival in cancers [47,48]. Furthermore, the high levels of circulating miR-20a was positively correlated with miR-148a and shorter relapse-free time (RFT) in MM patients [42]. Additionally, expression of some miRNAs showed dynamic changes in MM plasma or serum during disease progression, and thus, continual detection of miRNA levels was beneficial for monitoring disease status and assessing the

prognoses of MM patients. Yoshizawa et al. [37] found that differential expression of miR-92a in different stages of MM. miR-92a plasma levels were lower in partial MM patients and did not normalize normal in subjects with a partial response (PR) or very good PR to treatment, however, miR-92a levels did reach baseline in subjects who achieved complete remission. A recent study indicated that high miR-92a serum levels showed an independent correlation with poor survival in colorectal cancer [49]. The detection of miR-34a and let-7d was helpful in assessing the prognosis of MM, and high miR-34a expression and low let-7d expression were observed in relapsed MM patients compared to levels in patients at the time of diagnosis [38]. Qu et al. [40] revealed that miR-483-5p showed association with PFS, and the median PFS was 15 mon in patients with high miR-483-5p levels compared to 21 mon in patients with low miR-483-5p levels.

MGUS is usually referred as an asymptomatic precancerous stage of malignant expansion of a clonal plasma cell. A good biomarker is urgently required and would be of great value for predicting MM tumorigenesis in MGUS patients and discriminating between these two conditions. Recently, Jones et al. [36] revealed that the combination of miRNA-1246 and -1308 expression can distinguish MM from MGUS with a sensitivity of 79.2% and a specificity of 66.7%. Unfortunately, no single serum miRNA was able to discriminate between these two conditions. Moreover, plasma miR-92a concentration may serve as a biomarker for monitoring the therapeutic response and assessing disease progression from MGUS to MM. The miR-92a plasma level in MUGS patients was significantly higher compared to that in MM patients, but there was no significant difference in plasma miR-92a levels between MM patients with smoldering disease and MGUS patients. Furthermore, the expression of plasma miR-92a in MGUS patients maybe act as a promising parameter not only for determining disease status but also whether further treatment was required [37]. Reportedly, combination with miR-19b and miR-331 was a marker of shorter PFS, because longer PFS after ASCT was associated with high expression of miR-19b or miR-331 in MM patients [43].

4 Circulating miRNA expression patterns are associated with biochemical parameters in MM

Spearman bivariate correlation was performed to understand the relevance of circulating miRNA expression levels to clinical parameters in MM patients (Table 3). The results indicated that expression levels of four miRNAs (miR-744, miR-130a, let-7d and let-7e) showed positive correlations with hemoglobin and thrombocyte counts; however, expression of these miRNAs was also negatively correlated with levels of creatinine and β2-microglobulin. Furthermore, miRNA-34a was negatively correlated with hemoglobin. Three circulating miRNAs including miR-744, let-7d and let-7e were positively correlated with albumin levels, whereas miRNA-34a showed a significant negative correlation with albumin. In particular, let-7e levels showed significant negative correlations with the level of monoclonal immunoglobulin [35]. Jones et al. [36] demonstrated that miR-1246 showed a slight significant inverse correlation with paraprotein (M-protein), which indicated that miRNA-1246 expression may be suppressed by increased paraprotein production or secretion. Yu et al. [39] found that the relative expression of serum miR-202 was higher in MM patients compared to healthy subjects, and significantly positively correlated with serum β2-microglobulin and κ light chain concentrations but was not correlated with the lactic acid dehydrogenase (LDH) and λ light chain concentrations.

5 Circulating miRNA expression is associated with cytogenetic abnormalities in MM

Detailed genomic analysis has revealed that MM was characterized by complex cytogenetic abnormalities, such as chromosomal translocations involving mutations of t(4;14), t(6;14) and t(14;16); deletion of 13q14 and 17p13; rearrangement of immunoglobulin heavy-chain genes (IGH); and amplification of 1q21 [50–53]. The tumor suppressor p53 which is located in 17p13.1 is rarely inactivated by

Table 3 Correlation between circulating miRNAs and biochemical parameters in MM

miRNA	Biochemical parameters	Negative/Positive	References
miR-130a, miR-744, let-7d, let-7e	Hemoglobin	Positive	[35]
	Thrombocytes count	Positive	
	Creatinine and β2-microglobulin	Negative	
let-7d, let-7e, miR-744	Albumin	Positive	
let-7e, miR-744	C-reactive protein (CRP)	Negative	
let-7e	Monoclonal immunoglobulin (Ig)	Negative	
miR-34a	Hemoglobin	Negative	
	Albumin	Negative	
miR-1246	Paraprotein (M-protein)	Negative	[36]
miR-202	β2-microglobulin and κ light chain concentrations	Positive	[39]

Table 4 Correlation between circulating miRNAs and cytogenetic abnormalities in MM

miRNA	Cytogenetic abnormalities	Negative/Positive	References
let-7e, miR-744	del(13q)	Negative	[35]
miR-19a	del(13q14) and 1q21 amplification	Positive	[41]
miR-99b	t(4;14)	Positive	[42]
miR-211	del(13q)	Negative	[42]

mutations or deletions, the ectopic expression of p53 can downregulate miR-192, miR-194, and miR-215 in a subset of newly diagnosed MM patients. These miRNAs serve as enhancers in the pharmacological activation of the p53 pathway, in addition, they target the insulin-like growth factor (IGF) pathway, preventing enhanced migration of plasma cells into bone marrow [54]. Huang et al. [42] investigated the cytogenetic aberrations of 12 MM patients using micro-FISH, and detected serum miRNA expression by qRT-PCR. Interestingly, a high level of miR-99b expression was associated with a t(4;14) translocation (IGH; FGFR3) (Table 4), which suggested that miRNA-99b maybe promote oncogenesis in MM. Furthermore, the presence of a t(4;14) chromosomal abnormality consistently indicated poor prognosis in MM patients [55]. Deletion of chromosome 13 was a common cytogenetic abnormality in MM and was associated with the clonal expansion of MM cells [8,9,56]. miR-221, an important oncogenic miRNA, was upregulated in numerous MM patients [57,58]. Expression of plasma miR-221 was specifically upregulated in the sera of MM patients compared to healthy controls, however, the miR-221 expression level was lower in MM samples with del(13q) than in those without del(q13) [42]. In addition, the presence of del(13q14) in MM patients has a significant correlation with lower levels of let-7e, as well as miR-744, in the serum, which indicated worse OS and time to progression (TTP) [35]. Several studies have reported that lower levels of miR-744 were associated with 1q12 amplification or t(4;14) in MM cells, both of which have been previously described as poor prognostic indicators for MM [59,60].

6 Circulating miRNA expression is associated with drug resistance in MM

miRNA expression profiles are useful for prognosticating clinical resistance in MM patients. Aberrant expression of numerous miRNAs has also been observed in the drug-resistant myeloma cell lines, suggesting that dysregulated miRNA might be involved in the generation of drug resistant MM cells [61,62]. Hao et al. [21] identified that miRNA-15a/-16 expressions significantly correlated with drug resistance of MM cells. Expression of miRNA-15a/-16 was suppressed by interleukin-6 (IL-6) produced by BMSCs, which resulted in significantly increasing growth and survival of MM cells after treatment with cytotoxic agents. Serum miR-19a can function as a valuable predictor of PFS and OS in MM patients. MM patients with low miR-19a levels had improved responses to bortezomib compared to subjects with high miR-19a expression, experienced a significantly extended survival upon bortezomib-based therapy [41]. Qu et al. [40] indicated that the median PFS of patients with high levels of plasma miR-483-5p was 15 mon, in comparison with 21 mon for patients with low levels of plasma miR-483-5p after application of several chemotherapy regimens including TAD, VD, TCD, PAD, TD and VTD.

7 Conclusions

Exploration of circulating miRNAs as novel biomarkers for MM is still a “booming” field. Growing studies indicate that the expression profiles of circulating miRNAs are valuable tools to diagnose patients and estimate prognosis as well as predicting response to treatment, clinical therapeutic outcomes, and disease recurrence. However, some critical problems must be resolved to establish circulating miRNAs as biomarkers for MM. First, it is helpful to understand whether circulating miRNAs originate from tumor cell death and lysis, or instead from secretion by tumor cells or immunocytes in blood circulation. Furthermore, identifying the relationship between tissue miRNAs and circulating miRNAs will be valuable to elucidate the origin and function of circulating miRNAs. Second, to improve the diagnostic accuracy of this approach, a well-characterized panel of miRNAs specific to MM, MGUS, and chemotherapeutic responses should be established. Finally, larger sample sets including long-term clinical data are urgently required for future studies because these recent results are from small studies that lack long-term outcome data. In the coming years, we believe that widespread application of miRNA biomarkers in clinical practice will become routine in the diagnosis, prognosis and prediction of MM.

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