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Aberrant Levels of miRNAs in Bone Marrow Microenvironment and Peripheral Blood of Myeloma Patients and Disease Progression

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The bone marrow (BM) microenvironment of multiple myeloma (MM) is reported to play a role in the biology of disease. In this study, we found that the extracellular BM microenvironment in MM contains a unique miRNA signature detectable by miRNA microarray and quantitative real-time PCR, which is partially represented in the peripheral blood. Eleven miRNAs were significantly decreased in both BM and serum of MM patients in comparison with controls. Evaluation of these miRNAs in plasma of a separate cohort of MM patients and controls confirmed significantly aberrant levels of let-7a, let-7b, let-7i, miR-15b, miR-16, and miR-20a in both serum and plasma. We then studied the myeloma precursor diseases and found that a subset of the MM miRNAs exhibited aberrant expression in monoclonal gammopathy of undetermined significance and smoldering myeloma. miRNA analysis of enriched $CD138^{+}$ plasma cells from MM and monoclonal gammopathy of undetermined significance found that most of the validated MM BM signature miRNAs were significantly decreased in MM plasma cells. Gene expression profiling indicated that multiple targets of the decreased miRNAs found increased expression in MM plasma cells, including ATF2, HRAS, HDAC4, TGFB1, TGFBR1, and mitogen-activated protein kinases. The findings suggest that these miRNAs are detectable in aberrant levels in the peripheral blood of patients with plasma cell proliferation and may play a role in aberrant plasma cell proliferation and disease progression. *(J Mol Diagn 2015, 17: 669—678; http://dx.doi.org/10.1016/j.jmoldx.2015.06.006)*

Multiple myeloma (MM) is a malignant plasma cell (PC) neoplasm that evolves from an underlying asymptomatic precursor clonal PC proliferation designated monoclonal gammopathy of undetermined significance (MGUS). MGUS is present in >3% of the population aged >50 years and progresses to myeloma at a rate of nearly 1% per year.[1] Smoldering myeloma (SMM) represents an intermediate entity with increased bone marrow (BM) clonal PCs without symptomatic disease and carries an increased rate of progression to myeloma of nearly 10% per year.[2] Currently, no single factor can predict patients with MGUS that are likely to progress to MM. A biomarker of disease progression in the peripheral blood (PB) could assist in the early identification of patients evolving to MM. Recent data suggest that serum miRNAs are altered in MM and MGUS and may serve as diagnostic and prognostic biomarkers.[3,4]

miRNAs use a post-transcriptional gene regulation mechanism that was shown to play a role in development, differentiation, and tumorigenesis.[5–7] miRNAs are evolutionarily conserved small non-coding RNAs, which regulate gene

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expression by binding to the 3′ untranslated region of target mRNAs, leading to mRNA degradation or translational repression.[5–7] Individual miRNAs have the capacity to target many mRNAs. Previous studies have reported the ability of miRNA expression profiles to discriminate between specific types of cancer and the normal corresponding host tissue, to discriminate subclassifications of tumors,[8,9] and to serve as markers for disease prognosis and progression.[10]

miRNA is stable in PB. The profile of extracellular miRNAs may vary between serum and plasma, possibly related to platelet- or white blood cell-derived miRNAs, which may be released during clot formation in serum samples. Serum samples were shown to have higher levels of extracellular miRNAs than plasma.[11] Ideally, a robust miRNA biomarker shed into the PB should be significantly altered in both serum and plasma.

The BM microenvironment plays an important role in the regulation of abnormal PCs in MM.[12,13] Proposed mechanisms include effects of soluble mediators shed into the extracellular space, including cytokines, growth factors, chemokines, and/or miRNAs[13–15] or transfer of miRNAs exogenously from cell to cell via exosomes with functional capacity in the recipient cells.[16] Previous studies have reported aberrant miRNA expression in MM PCs associated with genetic subtypes of MM.[17–24] However, data on miRNA levels in the extracellular BM microenvironment or plasma of MM are virtually nonexistent, and studies of miRNAs in serum of MM are rare.[4,25]

Materials and Methods

BM, Serum, and Plasma Samples

The extracellular supernatant fluid from BM aspirates was obtained from 20 patients diagnosed with relapsed or refractory MM and eight healthy controls (HCs) from Dana-Farber Cancer Institute, Boston, MA, and from National Cancer Center and National Heart, Lung, and Blood Institute, Bethesda, MD. Of the 20 patients with MM, 6 were women and 14 were men; the median age was 64 years (range, 47 to 80 years). Available laboratory information indicated that serum monoclonal proteins were composed of 11 IgGκ, five IgGλ, two κ light chain only, and one λ light chain only.

Plasma samples were obtained from a separate cohort of 17 pretreatment MM patients (median age, 56 years; range, 40 to 73 years) and 20 HCs (median age, 52 years; range, 23 to 72 years). Plasma MM samples were composed of 11 IgGκ, three IgGλ, one IgAλ, and two light chain only. The median M-spike was 2.8 g/dL (range, 1.1 to 7.1 g/dL). Of the κ-restricted MM cases, the median κ:λ ratio was 62.7 (range, 2.89 to 10,235). Of the λ-restricted cases, the median κ:λ ratio was 0.03 (range, <0.01 to 0.12).

Serum samples from 12 HCs (median age, 42 years; range, 21 to 68 years) and recently diagnosed and/or pretreatment patients at the NIH Clinical Center were as follows: 17 MGUS [median age, 61 years (range, 38 to 78 years); median M-spike, 0.65 g/dL (range, 0.3 to 1.3 g/dL); free light chain analysis for κ cases, median κ:λ = 1.4 (range, 1.2 to 5.0) and for λ cases, median κ:λ = 0.6 (range, 0.29 to 0.82)]; 17 SMM [median age, 62 years (range, 40 to 81 years); M-spike median, 2.6 g/dL (range, 1 to 4.5 g/dL); free light chain analysis for monoclonal κ cases κ:λ = 26.7 (range, 1.5 to 807) and for λ cases, 0.02 (range, 0.01 to 0.78)]; 13 MM (median age, 58 years [range, 42 to 75 years]; median M-spike, 2.2 g/dL [range, 1 to 8.6 g/dL]; free light chain analysis for monoclonal κ cases κ:λ = 59.3 [range, 4.98 to 468) and for λ cases 0.23 (range, <0.01 to 0.66)]. All patients were enrolled on institutional review board-approved protocols and provided written informed consent before samples were obtained.

CD138$^+$ Cell Isolation

CD138$^+$ cells were freshly isolated and enriched from 10 MGUS and eight MM patient BM aspirates with the use of CD138$^+$ selection kits (Miltenyi Biotec Inc., Auburn, CA).

Table 1 PCR and Reverse Transcription Primer and Probe Sequences

Primer name	Sequence
hsa-let-7a	5′-GGGCCTGAGGTAGTAGGTTGTATAGTT-3′
hsa-let-7b	5′-GTGCCTGAGGTAGTAGGTTGTGTGGTT-3′
hsa-let-7i	5′-CTGGCTGAGGTAGTAGTTTGTGCTGTT-3′
miR-106b	5′-GTGCCTAAAGTGCTGACAGTGCAGAT-3′
miR-155	5′-GGGCCTTAATGCTAATCGTGATAGGGGT-3′
miR-15a	5′-GTGCCTAGCAGCACATAATGGTTTGTG-3′
miR-15b	5′-GTGCCTACTGTAGCAGCACATCATGGTTTAC-3′
miR-16	5′-GTGCCTAGCAGCACGTAAATATTGGCG-3′
miR-192	5′-GTGCCCTGACCTATGAATTGACAGCC-3′
miR-19b	5′-GCCAGTTTTGCAGGTTTGCATCCAGC-3′
miR-206-3p	5′-GTGCCTGGAATGTAAGGAAGTGTGTGG-3′
miR-20a	5′-GGGCCTAAAGTGCTTATAGTGCAGGTAG-3′
miR-21	5′-GGGCCCTAGCTTATCAGACTGATGTTGA-3′
miR-223	5′-GTGCCCGTGTATTTGACAAGCTGAGTT-3′
miR-34a	5′-GGTGGCAGTGTCTTAGCTGGTTGT-3′
miR-361	5′-GTGCCTTATCAGAATCTCCAGGGGTAC-3′
miR-370	5′-GCCTGCTGGGGTGGAACCTGGT-3′
miR-595	5′-GAAGTGTGCCGTGGTGTGTCT-3′
miR-671	5′-AGGAAGCCCTGGAGGGGCTGGAG-3′
miR-939	5′-TGGGGAGCTGAGGCTCTGGGGGTG-3′
RNU6	5′-CACGCAAATTCGTGAAGCGTTCCAT-3′
RNU38B	5′-GGGCAGTAAGTGAAGATAAAGTGTGTCTGA-3′
RNU44	5′-GGCAAATGCTGACTGAACATGAAGGTC-3′
RNU48	5′-CTCTGAGTGTGTCGCTGATGCC-3′
RNU66	5′-GGCTGAGGTGGTTCTTTCTATCCTAGT-3′
PCR reverse primer	5′-GTCCGAGGTATTCGATCCTAAC-3′
TaqMan probe	5′-/56-FAM/TCTCCTCGG/ZEN/TATCGAGTC-GCACT/3IABkFQ/-3′
RT anchor primer	5′-CGACTCGATCCAGTCTCAGGGTCCGAGGTAT-TCGATCCTAACCCTCTCCTCGGTATCGAGTCG-CACTTTTTTTTTTTTV-3′

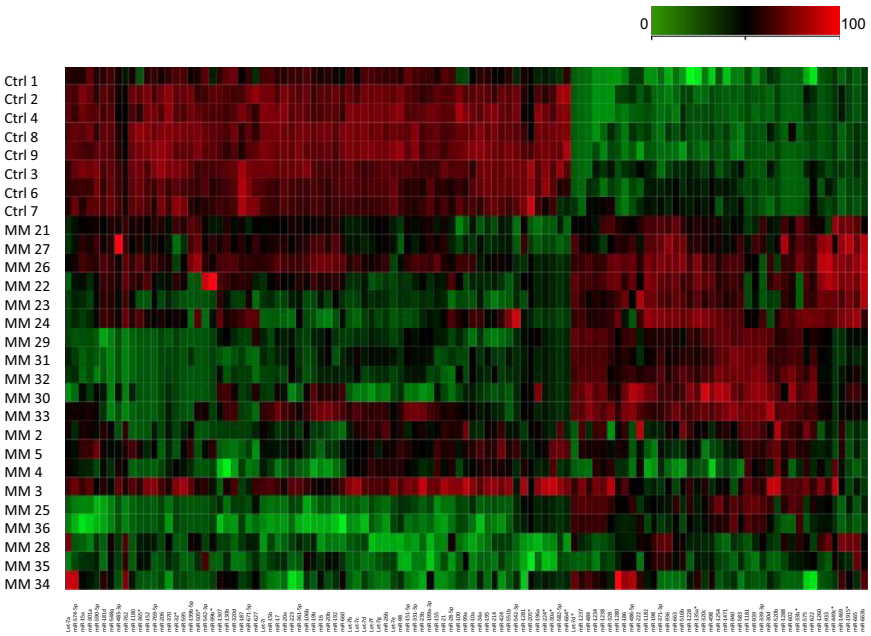

Figure 1 Myeloma miRNA signature in the bone marrow extracellular microenvironment. Heatmap on the basis of hierarchical clustering of 111 miRNAs differentially expressed in the bone marrow extracellular microenvironment of MM in comparison with healthy control marrow as measured by miRNA array ($P < 0.05$). Twenty MM and eight control samples are shown in the heatmap. Ctrl, control; MM, multiple myeloma.

The average percentage of PCs in the enriched samples was 75.5% (range, 50% to 95%).

RNA Isolation

Total RNA was isolated from 400 μL of BM supernatant fluid, 100 to 200 μL of serum, 50 μL of plasma, and from CD138-enriched PCs when indicated with the use of miRNeasy Mini Kits (Qiagen, Germantown, MD). RNA concentration was determined with the NanoDrop 2000 instrument (Thermo Scientific, Wilmington, DE).

Agilent Human miRNA Array

Total RNA (100 ng) and spike-in controls were cyanine 3-cytidine biphosphate-labeled with the Agilent miRNA

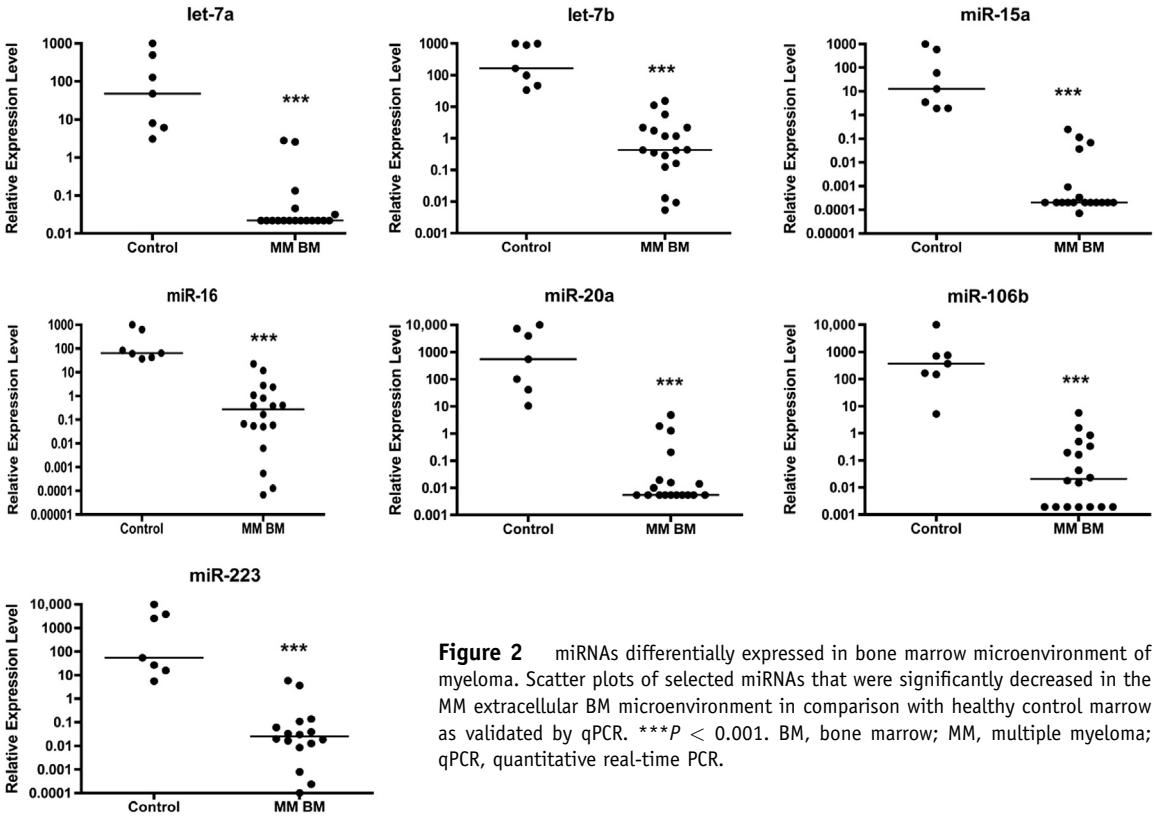

Figure 2 miRNAs differentially expressed in bone marrow microenvironment of myeloma. Scatter plots of selected miRNAs that were significantly decreased in the MM extracellular BM microenvironment in comparison with healthy control marrow as validated by qPCR. ***$P < 0.001$. BM, bone marrow; MM, multiple myeloma; qPCR, quantitative real-time PCR.

Table 2 miRNAs Decrease in BM and PB of MM, SMM, and MGUS as Validated by qPCR

miRNA	BM MM	PB MM	PB SMM	PB MGUS
Let-7a	X	X	X	
Let-7b	X	X	X	
Let-7i	X	X	X	X
miR-15a	X	X	X	X
miR-15b	X	X	X	
miR-16	X	X	X	X
miR-19b	X			
miR-20a	X	X	X	
miR-21	X	X		
miR-34a	X			
miR-106b	X	X	X	X
miR-155	X			
miR-192	X			
miR-206	X			
miR-223	X	X		
miR-361	X	X		
miR-370	X			
miR-595	X			

X indicates significant difference between healthy controls and disease groups, $P < 0.05$.

BM, bone marrow; MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; PB, peripheral blood; qPCR, quantitative real-time PCR; SMM, smoldering myeloma.

labeling kit (Agilent Technologies, Palo Alto, CA) according to the manufacturer's protocol. Labeled RNA was purified with Micro Bio-Spin 6 columns (Bio-Rad, Hercules, CA), dried, and re-dissolved in hybridization buffer and hybridized on Agilent high-density human miRNA arrays (version 3, release 12, with 851 human miRNAs on the array) at 56°C and 20 rpm for 20 hours. After washing, array images were scanned on Agilent's G2505C scanner and processed with Agilent's Feature Extraction software version 10.7.3.1.

qPCR

Quantitative real-time PCR (qPCR) was used for validation of miRNA array and miRNA quantitation. For RNA from BM samples, reverse transcription was performed from 50 ng of total RNA with pooled TaqMan reverse transcription primers with the use of High-Capacity cDNA Reverse Transcription Kit on a GeneAmp PCR system 9700 instrument (Applied Biosystems, Foster City, CA). TaqMan real-time PCR was performed in triplicate on a StepOne Plus instrument (Applied Biosystems). For RNA from PB samples and from $CD138^+$ cells, qPCR was performed with a polyadenylation step before reverse transcription as described previously[26] with some modifications. Briefly, 70 ng of RNA was poly-adenylated with the use of the poly-(A) polymerase (MCLAB, South San Francisco, CA) according to the manufacturer's recommendations. Reverse transcription was conducted with an anchor primer (Table 1). Real-time PCR was performed on the StepOnePlus instrument by using a forward primer (mature miRNA sequence) and a universal reverse primer and a universal FAM-ZEN-IABKFQ-labeled TaqMan probe (Integrated DNA Technologies, Coralville, IA) (Table 1). A Ct value of 37 was assigned to miRNAs with undetermined Ct values. The relative miRNA expression level was calculated as 2^{-Ct}. For miRNA quantitation of RNA isolated from $CD138^+$ cells, small nuclear or nucleolar RNAs (RNU6, RNU38B, RNU44, RNU48, RNU66) were amplified and used as internal controls. The relative miRNA expression level was calculated as $2^{-\Delta Ct}$. Primer sequences are included in Table 1.

NanoString nCounter Assay

Gene expression profiling was conducted on purified PCs of eight MM and five MGUS samples with the use of nanoString nCounter assay (nanoString Technologies, Seattle, WA)[27] with the Human Inflammation panel that contained 183 genes related to signaling pathways important in cell proliferation, apoptosis, and inflammation. The total RNA (4 μL) from enriched PCs was hybridized with the capture and reporter probes and incubated overnight at 65°C according to the manufacturer's recommended protocol. mRNA was quantified in the nCounter Digital Analyzer. The data were normalized to the spike-in controls and to the geometrical mean of six housekeeping genes in each hybridization reaction.

Statistical Analysis

Data from the Agilent miRNA array were analyzed with Agilent's GeneSpring GX software version 11.5.1. The array data were normalized to the data point of 75th percentile signal strength and to a set of spike-in and control probes on the array, respectively. The differences between the means of experimental groups were analyzed by two-tailed Mann-Whitney rank sum test. The miRNAs with significant P value ($P \leq 0.05$) and fold change (at least twofold) in both normalization methods (75th percentile and control probes) were selected for further analysis. The signal intensity values and fold changes presented in figures and supplemental tables are from data normalized by control gene probes. Raw data and data normalized by control gene probes were deposited in the Gene Expression Omnibus database (*http://www.ncbi.nlm.nih.gov/geo*; accession number GSE49261). The differences between the means of experimental groups of normalized qPCR assay data were also analyzed by two-tailed Mann-Whitney rank sum test. $P < 0.05$ was considered significant. Hierarchical clustering analysis and heatmap generation were performed with JMP software version 11 (SAS Institute, Cary, NC). Scatter plots were generated with GraphPad Prism software version 6 (GraphPad Software, La Jolla, CA).

Results

Unique miRNA Signature in the Extracellular BM Microenvironment of Myeloma

To identify miRNAs related to MM that may be shed into the extracellular BM microenvironment and subsequently

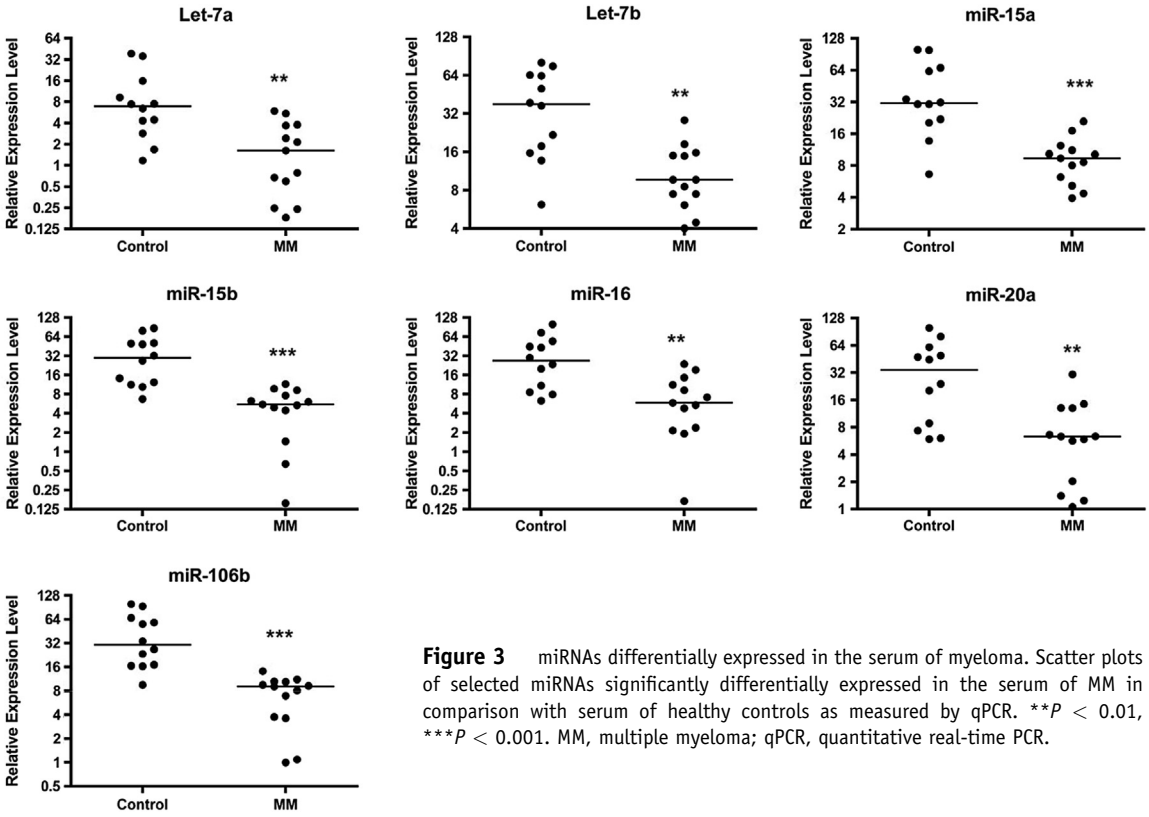

Figure 3 miRNAs differentially expressed in the serum of myeloma. Scatter plots of selected miRNAs significantly differentially expressed in the serum of MM in comparison with serum of healthy controls as measured by qPCR. $^{**}P < 0.01$, $^{***}P < 0.001$. MM, multiple myeloma; qPCR, quantitative real-time PCR.

released into the PB, miRNA profiling of the extracellular BM of MM was performed. RNA was isolated from the extracellular supernatant fluid of BM aspirates obtained from 20 patients diagnosed with MM and eight HCs who underwent BM aspiration. RNA was analyzed on a hybridization-based high-density miRNA array platform that contained probes for 851 human miRNAs. On the basis of twofold or greater difference in mean values between controls and MM ($P < 0.05$), we identified 111 miRNAs that were significantly differentially expressed in MM (Supplemental Table S1). Among these, 69 miRNAs were down-regulated and 42 miRNAs were up-regulated in MM BM. Hierarchical clustering analysis revealed a unique miRNA signature in MM, which was distinct from the control marrows (Figure 1). This signature included eight members of the let-7 family of miRNAs (let-7a, -7b, -7c, -7d, -7e, -7g, and -7i), which were sixfold to 17-fold decreased ($P < 0.03$) in MM BM. Among the MM signature miRNAs, we chose 26 miRNAs for validation and further analysis, taking into consideration those miRNAs with most significant P values, fold changes, and potential targets by TargetScan analysis. These miRNAs were assayed by qPCR in RNA samples from BM microenvironment of 18 MM and seven controls. Eighteen miRNAs were validated by qPCR and were significantly decreased in the MM BM microenvironment ($P < 0.05$). The validated miRNAs included let-7a, let-7b, let-7i, miR-15a, miR-15b, miR-16, miR-19b, miR-20a, miR-21, miR-34a, miR-106b, miR-155, miR-192, miR-206, miR-223, miR-361, miR-370, and miR-595 (Figure 2 and Table 2).

BM MM miRNA Signature Is Represented in the Serum of MM Patients

To investigate whether aberrant miRNA profiles detected in the MM BM microenvironment were also detectable in the PB serum of MM patients, qPCR with the use of a poly-A–based stem-loop method was performed with RNA isolated from serum samples of 13 MM patients and 12 HCs. The 18 miRNAs, validated and found down-regulated in BM microenvironment, were assayed in the serum. Greater than 60% (11 of 18) of the miRNAs demonstrated significantly decreased serum levels in MM patients in comparison with controls. These miRNAs included let-7a, let-7b, let-7i, miR-15a, miR-15b, miR-16, miR-20a, miR-21, miR-106b, miR-223, and miR-361 (Figure 3 and Table 2). Hierarchical clustering analysis found that eight miRNAs (let-7a, let-7b, let-7i, miR-15a, miR-15b, miR-20a, miR-106b, and miR-361) were capable of distinguishing MM from controls, forming a miRNA signature of MM in serum (Supplemental Figure S1).

MM PB miRNA Signature Validation in Plasma of a Separate Cohort of MM

The spectrum of miRNAs normally present in serum and plasma is inherently somewhat different because serum contains higher levels of platelet-derived miRNAs released during the process of coagulation and clot formation.[11] Because MM patients often have thrombocytopenia, it is possible that some

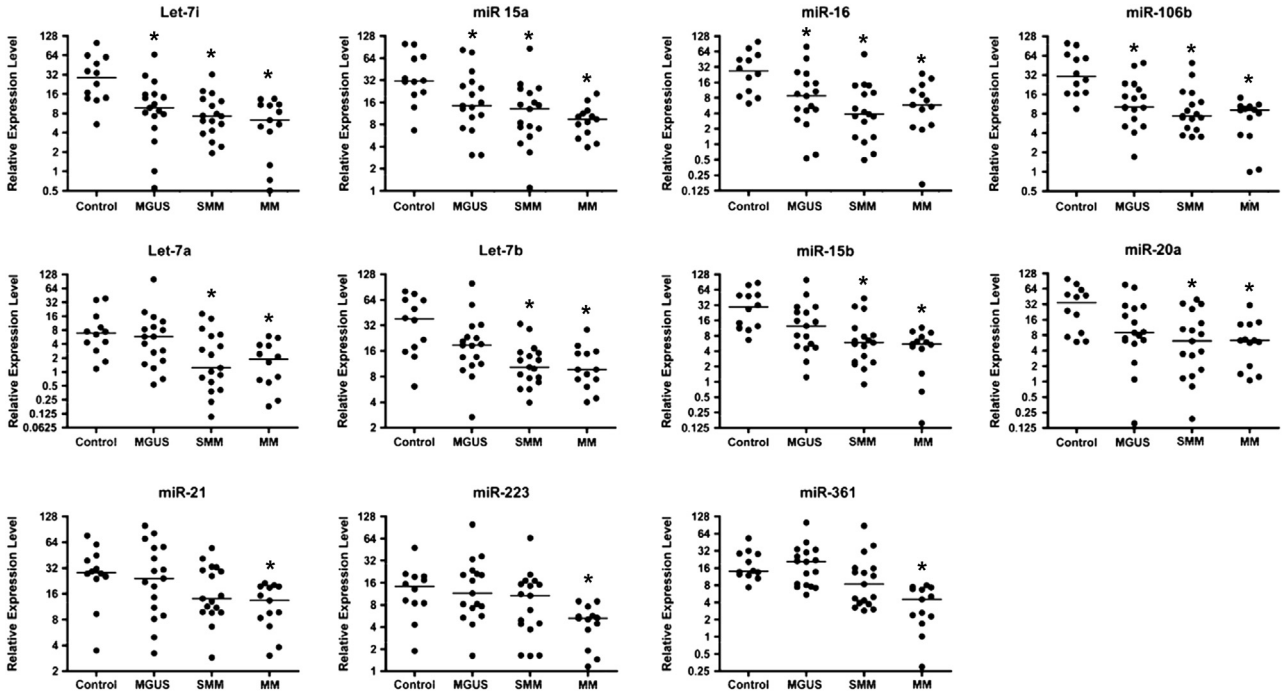

Figure 4 miRNAs differentially expressed in serum of MGUS, SMM, and myeloma. Scatter plots of selected miRNAs differentially detected in peripheral blood of MGUS, SMM, and (MM) by qPCR. In comparison with the serum of healthy controls, Let-7i, miR-15a, miR-16, and miR-106b are significantly decreased in serum of MGUS, SMM, and MM; Let-7a, let-7b, miR-15b, and miR-20a are significantly decreased in serum of SMM and MM; and miR-21, miR-223, and miR-361 are significantly decreased in the serum of MM. Values for MGUS and SMM were not statistically significant. *$P < 0.05$ compared to control. MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; qPCR, quantitative real-time PCR; SMM, smoldering myeloma.

differences in miRNA profiles between MM and controls in serum could be related to platelet levels. We sought to evaluate miRNA signature (let-7a, let-7b, let-7i, miR-15a, miR-15b, miR-20a, miR-106b, and miR-361) detected in the MM serum samples in a separate cohort and assay the miRNAs in plasma. miRNAs were assayed by ABI TaqMan qPCR in the plasma of a separate cohort of 17 MM patients and 20 HCs. Among eight signature miRNAs found significantly decreased in MM serum samples, six of them (let-7a, let-7b, let-7i, miR-15a, miR-15b, and miR-20a) were found significantly decreased in plasma samples of MM patients (Supplemental Figure S2).

Down-Regulated Expression of a Subset of MM-Associated miRNAs Is Present in MGUS and SMM Serum

To determine whether miRNAs in our MM signature may play a role in MM precursor disease and disease progression, serum miRNA analysis was performed in 17 MGUS, 17 SMM, and 13 MM patients and 12 HCs in parallel. The 11 miRNAs, initially found decreased in MM PB as shown in Figure 3 and Table 2 (let-7a, let-7b, let-7i, miR-15a, miR-15b, miR-16, miR-20a, miR-21, miR-106b, miR-223, and miR-361), were evaluated in MGUS, SMM, MM, and control serum samples. As expected, all of the 11 miRNAs were decreased in the MM PB samples on repeat analysis. Interestingly, only 36% (4 of 11) of the miRNAs (let-7i, miR-15a, miR-16, and miR-106b) were significantly decreased in MGUS PB, suggesting that aberrant expression of these miRNAs may be associated with early events in PC neoplasia (Figure 4 and Table 3). Seventy-three percent of the miRNAs (8 of 11) were decreased in SMM (let-7a, let-7b, let-7i, miR-15a, miR-15b, miR-16, miR-106b, and miR-20a) (Figure 4 and Table 3). Twenty-seven percent (3 of 11) of the miRNAs (miR-21, miR-223, and miR-361) were significantly decreased in MM but not in MGUS/SMM, suggesting that down-regulation of this group of

Table 3 Aberrant miRNA Levels in Serum Associated with MGUS, SMM, MM, and Disease Progression

miRNA	PB MGUS	PB SMM	PB MM
Let-7i	X	X	X
miR-15a	X	X	X
miR-16	X	X	X
miR-106b	X	X	X
Let-7a		X	X
Let-7b		X	X
miR-15b		X	X
miR-20a		X	X
miR-21			X
miR-223			X
miR-361			X

X indicates significant difference between healthy controls and the stage of plasma cell dyscrasia, $P < 0.05$.

MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma; SMM, smoldering myeloma.

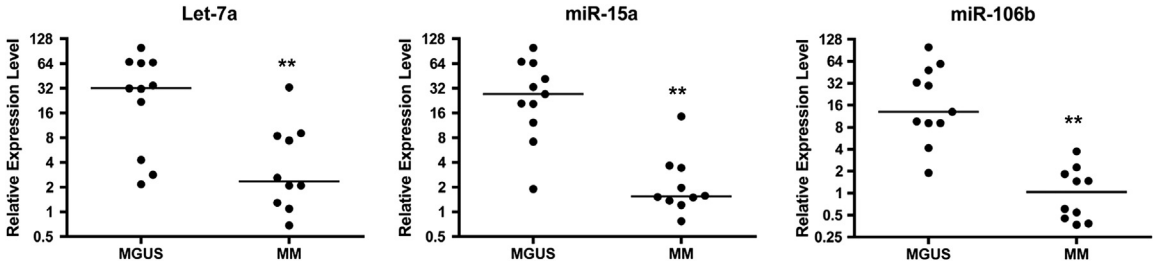

Figure 5 miRNAs differentially expressed in plasma cells of MGUS and myeloma. Scatter plots of selected miRNAs differentially expressed in CD138$^+$ plasma cells of MGUS and MM. $^{**}P < 0.01$. MGUS, monoclonal gammopathy of undetermined significance; MM, multiple myeloma.

miRNAs may be related to later events in disease progression (Figure 4 and Table 3).

CD138$^+$ Plasma Cells from MM Patients Show Similar miRNA Profile to PB and BM Supernatant Fluid

BM of MM patients typically contains increased CD138$^+$ monoclonal PCs, which comprise a significant component of the cellular marrow (10% to 100%).[27,28] In contrast, healthy BM typically contains <4% of CD138$^+$ polyclonal PCs.[29] Hence, it is extremely difficult to isolate adequate quantities of CD138$^+$ PCs from healthy donors as controls for analysis because of low numbers of PCs in normal control marrow. MGUS BM contains 5% to 9% PCs,[29,30] which are relatively easier to isolate in comparison with HC PCs. Although a subset of the miRNAs aberrantly expressed in MM BM and PB were altered in MGUS, there were also a number of miRNAs in MGUS that were not aberrantly expressed and were found at levels similar to those seen in HCs.

To determine whether the aberrant miRNA profiles detected in the BM and PB of MM and not in MGUS are attributable to malignant MM PCs, CD138$^+$ PCs were enriched from BM aspirates of 10 MM and 11 MGUS patients. The levels of 18 miRNAs, found decreased in MM BM (Table 2), were assessed in CD138$^+$ PCs of MM and MGUS by qPCR. The results indicated that 14 miRNAs (let-7a, miR-15a, miR-19b, miR-20a, miR-21, miR-106b, miR-34a, miR-155, miR-192, miR-206, miR-223, miR-361, miR-370, and miR-595) were significantly down-regulated in CD138$^+$ PCs of MM compared with that of MGUS (Figure 5 and Supplemental Table S2). Among these 14 miRNAs, only 2 miRNAs were significantly differentially expressed in the serum of MGUS (miR-15a and miR-106b), which is in contrast to the serum of MM whereby 7 of the 14 miRNAs (let-7a, miR-15a, miR-20a, miR-21, miR-106b, miR-223, and miR-361) found differential expression, suggesting that the differential miRNA expression in the serum of MM and MGUS mirrors the expression patterns in MM and MGUS PCs for many of the miRNAs assayed.

To further examine the expression of potential targets that might be affected by altered miRNA levels in CD138$^+$ cells, the expression of a panel of 184 genes (Supplemental Table S3), including genes involved in the regulation of proliferation, apoptosis, and NF-κB cell signaling pathways, was examined with the nanoString nCounter assay platform.[31] Thirty-seven of the 184 genes in the assay exhibited significantly increased expression in MM PCs ($P < 0.05$; >twofold) relative to MGUS PCs (Supplemental Table S4). Many of these genes encode proteins that regulate cell proliferation, such as *ATF2*, *HRAS*, *HDAC4*, *TGFB1*, *TGFBR1*, and mitogen-activated protein (MAP) kinases (*MAP2K1*, *MAP2K4*, *MAP2K6*, *MAP3K1*, *MAP3K5*, *MAP3K7*, *MAP3K9*, *MAPK1*, *MAPK8*, *MAPKAPK2*, *MAPKAPK5*), and transcripts of these genes are previously validated targets of several miRNAs found decreased in BM, serum, and malignant PCs of MM. The validated targets of miR-15b include (mRNA) MAP2K4 and (mRNA) MAPKAPK5,[32] which were increased 14-fold and twofold, respectively, in MM PCs. Let-7 family miRNAs target multiple gene transcripts,[33,34] which were significantly increased in MM PCs, including (mRNA) HRAS (9.6-fold increase), (mRNA) HDAC4 (17-fold increase), (mRNA) TGFB1 (3.4-fold increase), and (mRNA) TGFBR1 (4.6-fold increase). miR-20a and miR-21 also target (mRNA) TGFB1 and (mRNA) TGFBR1,[35–37] underscoring the significant increase in expression of gene targets of the miRNAs that were aberrantly decreased in MM.

Discussion

In this study we generated miRNA profiles of the extracellular BM microenvironment of MM, demonstrating a unique miRNA signature associated with MM that is partially represented in the serum. Moreover, analysis of MGUS and SMM suggests that a subset of circulating aberrant miRNAs (miR-21, miR-223, and miR-361) are associated with later stages of myelomagenesis and may be related to malignant transformation from precursor disease to myeloma. miRNA analysis of purified PCs of MM and MGUS suggested that differential miRNA expression in the BM microenvironment and serum of MM and MGUS is mirrored in PCs, providing further evidence that the aberrant levels of miRNAs detected in the serum are related to the disease state of the PC dyscrasia.

Decreased levels of let-7a, let-7b, and let-7i and miR-15a, miR-15b, miR-16, miR-20a, miR-21, miR-106b, and miR-361 were found in both the BM supernatant fluid and the PB of the MM patients. The let-7 family of miRNAs is expressed at significantly low levels in human cancer and stem cells on the basis of previous studies.[10,38] Let-7 miRNAs target many important transcripts of proteins that regulate oncogenesis, cell cycle, proliferation, and apoptosis, including multiple oncoproteins (eg, RAS, MYC, and HMGA2) and the pluripotency-promoting factor LIN28. Interestingly, feedback mechanisms exist between let-7 family members and their targets. For example, MYC also directly regulates let-7 transcription by binding to let-7 promoters. LIN28 inhibits the processing of primary let-7 miRNAs.[33,34] Hence, unregulated MYC expression in MM may in part be related to diminished levels of let-7 miRNAs.

Myelomagenesis is a multistep process.[39] This is underscored by the fact that MM is consistently preceded by a precursor disease state of MGUS and/or SMM.[40,41] With the use of conventional cytogenetic analysis, the chromosomal translocation between the immunoglobulin heavy chain gene (14q32) and an oncogene (often 11q13, 4p16.3, 6p21, 16q23, and 20q11) is observed in approximately 50% of MM cases.[42,43] Similar distributions are present already in MGUS and SMM.[43,44] These translocations result in overexpression of oncoproteins that are believed to be critical initiating events in the pathogenesis of MM.[45] *MYC* overexpression is associated with progression from MGUS to MM[46,47] and is associated with a poor prognosis.[39,48] In our study, let-7a and let-7b [both regulators of (mRNA) MYC and regulated by MYC] were decreased only at the SMM and MM stages in the blood and exhibited normal expression levels in MGUS. The findings suggest that the pattern of let-7a and let-7b expression in MM and MGUS may be related to increased *MYC* expression in MM and disease progression. Only let-7i was found decreased at the precursor disease stage of MGUS, in addition to SMM and MM, suggesting that some let-7 family miRNAs may play a role in myelomagenesis in the early stages of PC dyscrasia.

miR-15a/16 cluster targets multiple antiapoptotic proteins or oncoproteins, such as BCL2, MCL1, CCND1, and WNT3A.[49] Decreased expression or deletion of miR-15a and miR-16 was reported in chronic lymphocytic leukemia, myelodysplastic syndrome, pituitary adenomas, and prostate carcinoma.[10,49–53] We found miR-15a and miR-16 were significantly decreased in serum samples of MGUS, SMM, and MM. Consistent with these findings, Roccaro et al[18] observed decreased expression of miR-15a and miR-16 in MM PCs. They found that miR-15a and miR-16 regulate proliferation through inhibition of AKT kinase, ribosomal-protein-S6, MAP kinases, and NF-κ B activator MAP3KIP3, and that decreased expression of miR-15a and miR-16 promoted growth of MM cells *in vitro*. These findings suggest a mechanism for the effects of diminished miR-15a and miR-16 in PC neoplasia. Furthermore, recent data indicate that the expression of miRNAs are globally suppressed in MM cells because of hypermethylation of DNA in the miRNA regions.[54] Similarly, another study found that miR-155 was down-regulated in MM cell lines and patient samples because of DNA methylation.[55]

Previous studies indicated that miRNA processing enzymes, Dicer and AGO2, are up-regulated in MGUS and MM,[21,56] leading to increased levels of miRNAs in MGUS or MM. Huang et al[25] reported that the overall miRNA levels in MM blood was higher than in controls, and the levels of miR-148a, miR-181a, miR-20a, miR-221, and miR-99b were increased in the plasma of MM patients. They reported that high levels of miR-20a and miR-148a were related to shorter relapse-free survival. In our study, we did not observe a significant difference in total miRNA levels in blood samples between MM and controls. In addition, we found that miR-20a was decreased in both BM supernatant fluids and PB of MM patients.

Conclusion

In summary, we found that the levels of let-7a, miR-15a, miR-20a, miR-21, miR-106b, miR-223, and miR-361 were decreased in the BM microenvironment, PB, and $CD138^+$ PCs of MM. Our findings suggest that the antiproliferative and proapoptotic miRNAs, such as let-7 family members and miR-15a/16 clusters, are down-regulated in the microenvironment of MM. Down-regulation of these miRNAs is detectable in the PB and may parallel disease progression from the precursor lesion of MGUS to the fully malignant stage of myeloma. These findings suggest that measuring the expression of miRNAs associated with myeloma progression in the PB may hold promise for predicting disease progression in MGUS and SMM. Further investigations into the roles of aberrantly expressed miRNAs, found in MM BM and blood samples, in relation to tumor progression in MM and precursor diseases is warranted.

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Supplemental Data

Supplemental material for this article can be found at *http://dx.doi.org/10.1016/j.jmoldx.2015.06.006*.

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