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The effects of MicroRNA deregulation on pre-RNA processing network in multiple myeloma

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Abstract

Over the last few years, a detailed map of genetic and epigenetic lesions that underlie multiple myeloma (MM) has been created. Regulation of microRNA (miR)-dependent gene expression and mRNA splicing play significant roles in MM pathogenesis; however, to date an interplay between these processes is not yet delineated. Here we investigated miR-mediated regulation of splicing networks at the transcriptome level. Our studies show that a significant number (78%) of miRs which are either up- or down-regulated in patient CD138+ MM cells, but not in healthy donors (HD) CD138+ plasma cells (PC), target genes involved in early stages of pre-mRNA splicing. We also identified deregulated miRs that target core splicing factors (SF) and modifiers (SM, enhancers/silencers) which cause altered splicing in MM. Our studies suggest that Let-7f, in combination other miRs which are frequently and significantly deregulated in patients with overt MM, targets genes that regulate intron excision. Importantly, deregulated expression of certain miRs in MM promote increased intron retention, a novel characteristic of the MM genome, by inducing deregulated expression of the genes that regulate the splicing network. Our studies, therefore, provide the rationale for therapeutically targeting deregulated miRs to reverse aberrant splicing and improve patient outcome in MM.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Introduction

Recent genomic studies have revealed new molecular characteristics of MM including recurrent mutations, copy number variations, as well as deregulated expression of epigenetic modifiers such as non-coding RNAs (miRs) and mRNA processing [1–6]. Targeting these alterations, alone and combination, may represent novel therapeutic approaches. miRs are non-coding RNAs which belong to a family of small RNA molecules that block mRNA translation, affect mRNA stability, regulate gene expression at the posttranscriptional level, and are an important component of the epigenetic network [7]. MiRs play a significant role in the regulation of various cellular processes [8, 9]. Importantly, miRs play a significant regulatory role in malignant transformation [8, 10].

Studies in MM have demonstrated the role of miRNAs in disease pathophysiology. For example, genome-wide miR and mRNA expression studies in newly diagnosed MM demonstrated the role of expressed miRNAs in MM progression [11]. Two independent studies reported miR-15a upregulation in newly diagnosed MM [11, 12], while a study in relapsed/refractory MM reported downregulation of this oncomir [13]. These studies suggest distinct roles of miR-15a in MM at different stages of disease development, highlighting the need to evaluate the functions of single miRs in the context of their target genes and/or gene pools. Multiple other miR-mRNA regulatory circuits have been reported in MM [14–21]. Interplay between *MYC* and the miR-17–92 cluster on chromosome 13 has been documented in MM [22]. Specifically, *MYC* upregulates miR-17–92 cluster, leading to *CDKN1A* and *MDM2* deregulation in mutated TP53 MM cells. Conversely, regulation of *MYC* through Let-7b mimic with an LNA-GapmeR has been proposed as a novel therapeutic strategy [23].

RNA-splicing is another circuit of epigenetic regulation in MM. Even though alternative splicing (AS) is a normal process, mistakes are inevitable. Previous studies of single genes and ongoing genome-wide transcriptome evaluation have detected different patterns of splicing alterations in MM patients [3, 24–29]. MM cells require *XBPI* expression and splicing into active *XBPIs* to grow and survive, and increased expression of spliced *XBPI* is associated with poor survival [24, 30]. MMSET is the result of a common t(4;14) translocation in MM and is associated with poor prognosis [31]; overexpression of MMSET isoforms is due to complex splicing in t(4;14) MM cases [26, 31–33]. Another example is *HAS1* which produces the extracellular matrix molecule Hyaluronan (HA), a ligand of CD44 and RHAMM. *HAS1* is spliced in MM patients, and *HAS1 Vb* splice variant overexpression is correlated with a poor outcome [27]. *RHAMM* is also spliced in MM, and RHAMM-splice variant/RHAMM-FL ratio both increases with disease burden and predicts for poor survival [34]. Importantly, ongoing whole-transcriptome RNA sequencing analysis of samples from newly diagnosed MM patient samples showed that splice variant expression may predict survival better than gene expression alone [35]. Most recently, NSG MM studies have identified recurrent splicing alterations in >30% patients, with the predominant (32%) recurrent alterations being intron retentions [36].

miR-dependent gene regulation and mRNA splicing both are epigenetic processes that modulate the protein repertoire at the transcriptional level. Although the independent effects

of these processes in MM cells have been shown [37], the interaction between them has not yet been investigated. Our study is the first attempt to define an association between deregulation of miR expression in MM and its effects on pre-mRNA-splicing. Here, we show that a significant number (78%) of the miRs that are deregulated in MM patients modulate expression of splicing factors (SF) and splicing modifiers (SM, enhancers/silencers) which mediate early stages of AS. Moreover, our results also identified new miRs and core SF interactions in MM, which coordinately regulate splicing at the gene expression level. Our analysis further demonstrates that miR Let-7f modulates SF/SM (*PTBP3*, *KHSPR*, *EFTUD2*, *SUPT6H*) transcripts that are regulators of intron retention in MM cells, associated with development of aggressive disease. These studies, therefore, provide the framework for novel therapeutics targeting miRs to modulate splicing and improve patient outcome in MM.

Material and methods

Sample preparation and miR expression profiling

After obtaining written informed consent, we evaluated miR expressions in tumor cells from 78 patients with newly diagnosed MM, as well as from 9 healthy donors (HD) and 12 MM cell lines. Details of these analyses are described in Supplementary Method S1–S2.

In silico target genes prediction and annotation analyses

The web server DIANA-miRPath-V.3 [38] and the Database for Annotation, Visualization, and Integrated Discovery (DAVID) [39] was used to assess merged effects of deregulated miRs in MM on RNA processing. Details of these analyses are described in Supplementary Method S3.

Target gene expression profiling

Target gene expression profiles were evaluated in 41-MM, 33 monoclonal gammopathy of undetermined significance (MGUS), smoldering MM (sMM), and 5-HD. Dataset GDS4968 was downloaded from the GEO. Data were analyzed using the Partek Genomics Suite, according to the standard pipeline.

Cell culture and transient transfection of the antagomir/pre-miR

Our studies were carried out on OPM2 MM cell line. We used DMRIE-C and Oligofectamine (2:1) reagent cocktails. These cells were transfected with LNA antagomir anti-let-7f and/or pre-miR-155 according to the manufacturer's instructions (Exiqon/Ambion). As a control, OPM2 cells were transfected with a scrambled antagomir. Probes were custom conjugated with fluorophores to monitor their proper delivery by flow cytometry or fluorescent microscopy (Supplementary Fig. 1).

Results

miR expression profiling in MM patients

In order to define unique miR signatures in MM, we performed genome-wide mature miR expression profiling of purified CD138⁺MM cells from patients, HD plasma cell (HD-PC)

samples, and MM cell lines. We first compared expression profiles of 141 filtered miRs in all MM patients to the miR expression levels detected in HD-PC. This analysis identified one upregulated intronic miR (miR-193b) in 40% of MM samples ($p = 0.0001$); as well as 16 downregulated miRs that were >20 fold downregulated in MM patients relative to HD-PC. The frequency of these downregulated miRs in MM patients ranged from 45 to 85% (Supplementary Table 1A).

An unsupervised, hierarchical clustering analysis of 141 filtered miRs identified two major groups amongst MM patients (Fig. 1, Groups-A and -B). All MM cell lines were grouped together and formed a subnode with Group A (32 patients), while all HD samples were grouped together with Group-B (34 patients). Based on our unsupervised clustering analyses, Group-A patients have similar proliferation index as cell lines, since they cluster together and share similar miR profiles.

We next performed miRs expression profiling in Group-A and -B patient samples compared to HD-PCs. In Group-A, miR expression profiling identified significant upregulation of intergenic/intronic, intronic/UTR, and intronic miRs; as well as downregulation of exonic, intergenic, UTR, and intronic miRs, compared to HD-PCs (Supplementary Table 1B). miR expression profile in Group-B identified only one upregulated intergenic miR; as well as exonic, UTR, intergenic, and intronic downregulated miRs, compared to HD-PCs (Supplementary Table 1C). Importantly, our analysis demonstrates that the intergenic miR Let-7f (70% patients) and intronic miR-585 (80% patients) are most significantly ($P = 0.001$) and most frequently upregulated in MM patients. In Group-A samples, expression of miR-585 was 9 ± 0.3 fold higher and Let-7f 6.4 ± 0.29 fold higher than in HD-PC; in contrast, in Group-B samples their expression is downregulated compared to HD-PC (Supplementary Fig. 2). The miR expression profiling did not identify differentially expressed miRs in Group C patient samples as compared to miRNA expression in HD PC. Thus, we focus our studies on Group A and Group B patients.

We next compared altered expression levels of miRs between Group-A and Group-B patients. We identified 5 miRs, which were significantly downregulated in both Groups-A and -B (Supplementary Table 1B, C). Furthermore, We identified miRs that were significantly downregulated only in Group-A or Group-B patients (Supplementary Table 1B, C). No common miR was upregulated between the two groups. This significant downregulation of miRs, which is due to defective miR biogenesis, is a general characteristic of many types of malignancies including myeloma.

Identification of pathways regulated by differentially expressed miRs in MM patients

We next used the miR pathway analysis web-server DIANA-miRPath to identify pathways targeted by those miRs which are deregulated (up- or downregulated) in MM patient samples. These analyses showed that 78% (18 of 23) miRs deregulated in MM patients target genes involved in RNA processing, specifically RNA splicing (Supplementary Tables 2–3). Consistent results were obtained from analyses of miRs deregulated in Group-A and Group-B patients: 69% (11 of 16) and 93% (13 of 14) deregulated miRs were evident in Groups-A and -B, respectively, targeting genes involved in mRNA processing (Supplementary Table 3).

The above analyses showed that a significant number of the genes targeted by upregulated miRs in MM patient samples vs HD-PC mapped to chromosomes (Ch)-1, -2, -17, -19 vs Ch-1, respectively (Fig. 2a). In contrast, downregulated miRs in Groups-A vs Group-B MM patients target genes mapped to Ch-1, -6, and -17, vs Ch-1 and -17, respectively.

Next, we determined the biological features of genes targeted by miRs deregulated in MM patients. We performed GO annotation and KEGG enrichment analyses of target genes by querying DAVID-v6.8. database. GO CC annotation showed that a significant number of gene products targeted by deregulated miRs are enriched in the nucleus (>60%) and nucleoplasm (>75%), and are part of spliceosome complexes (~48%) (Fig. 2b, Supplementary Table 3A). To examine the function of miRs differentially expressed in MM, we next annotated miR target genes based on their molecular functions and determined their associations with biological processes. According to BP annotation, genes targeted by miRs upregulated in MM are enriched for gene products which are components of the spliceosome complex involved in mRNA 3' end processing, RNA capping, export from the nucleus, regulation of nuclear import, as well as trans-esterification reactions or regulation of transcription (Fig. 2c, Supplementary Table 3B).

Finally, we carried out deregulated miR target gene enrichment analyses, and identified a number of signaling pathways affected by deregulated miRs ($P < 0.005$). We specifically identified four signaling pathways affected by upregulated miRs, and four pathways affected by down-regulated miRs. Our studies suggest that pathways commonly modulated by deregulated miRs control spliceosome assembly, mRNA stability, and mRNA transport (Table 1). Furthermore, multiple genes within each pathway are targeted by deregulated miRs, suggesting coordinated control of affected pathways by deregulated miRs (Fig. 3a, b).

Validation of deregulated miR target genes in MM patients

We next validated the expression of those transcripts targeted by deregulated miRs in Dataset GDS4968. We performed unsupervised clustering analyses of deregulated miR target genes, and identified their expression levels compared to HD-PC. Our analyses showed that one cluster is enriched with MM samples and the other with sMM samples regardless of whether miRs are upregulated or downregulated. These clusters are not homogeneous, since our clustering analyses were done on a small number of genes selectively targeted by deregulated miRs (Fig. 4).

We evaluated expression profiles of transcripts targeted by Group-A and Group-B up- and down-regulated miRs as compared to HD-PC. Among the 85 genes targeted by Group-A upregulated miRs, we identified 36 transcripts that are differentially expressed (22-downregulated, 14-upregulated) in MM patients compared to HD-PC ($P < 0.05$, Fig. 4a). In Group-B, we found only 4 genes significantly altered by upregulated miRs. Analysis of genes targeted by downregulated miRs showed that 28 of 80 genes were significantly deregulated (11-upregulated, 17-downregulated) by Group-A miRs, while 35 of 91 genes were significantly differentially expressed (15-upregulated, 20-downregulated) by Group-B miRs (Fig. 4b). Gene enrichment and annotation analyses showed that most of the downregulated target genes are involved in the formation of exon junction complexes or regulate pre-mRNA 3' end processing; or are affecting U1, U2, U4/6, and U5 spliceosome

components. Some genes are part of Complex-A or a post-spliceosomal complex, while others are involved in the assembly of Complex-B during the splicing process (Fig. 3b).

Effects of Let-7f on splicing molecules in MM

To evaluate the functional consequences of deregulated miRs in MM patients, we focused our validation studies on Let-7f, since it is most frequently and significantly deregulated in MM patient samples. Using DIANA-miR-Path, we first identified 46 genes targeted by Let-7f (Table 2), and then performed GO annotation and gene enrichment analyses of target genes. This analysis showed that a significant number of Let-7f target gene products are localized in the nucleoplasm and nucleus, and are part of intracellular RNP complexes (Fig. 5a). We next annotated Let-7f target genes based on their biological function, which included regulation of splicing processes, mRNA transcription, and mRNA 3' end processing (Fig. 5a). Furthermore, target gene enrichment analyses identified 4 signaling pathways affected by Let-7f, most prominently mRNA processing pathways (Fig. 5b).

We then evaluated impact of Let-7f on expression of 47 targeted genes in 20 patients with MGUS, 33 patients with high-risk sMM, 41 patients with MM, and 5 HD PCs. Unsupervised clustering analyses showed that 83% (34 of 41) samples clustered together and formed a subnode with 85% (30 of 33) MGUS samples, while 83% (28 of 33) sMM samples clustered with HD PC samples (Fig. 5b). Among the 47 deregulated genes, 17 are significantly downregulated and 9 are significantly upregulated ($P < 0.05$) in MM patients. These analyses showed that 68% genes are downregulated in MM and MGUS patients and share the same gene expression profiles, which are distinct from those detected in sMM patients. The downregulated gene list includes splicing factors which regulate spliceosome biogenesis, as well as splicing molecules which are involved in mRNA polyadenylation and mRNA surveillance pathways (Fig. 5a).

To validate whether Let-7f regulates predicted target genes, we used LNA antagomir Let-7f to downregulate this miR in OPM2 cells, which express high levels of Let-7f. Genome-wide expression profiling (Fig. 5b) in transfected cells showed that Let-7f target genes that were significantly downregulated in MM, MGUS, and sMM patients were upregulated after Let-7f inhibition, suggesting direct regulation of these genes by Let-7f (Fig. 5c marked). Annotation analyses showed that downregulated gene products are localized in the nucleus, nucleoplasm, chromosome, cytoplasm, and cytosol. They are involved in RNA splicing via trans-esterification reaction, mRNA processing, RNA metabolic processes, negative regulation of RNA splicing, and transcription, as well as in other processes described in Supplementary Table 4. Of note, the downregulated genes include a core splicing factor *PTBP3*, which binds to a polypyrimidine tract of splicing (PPT) a component of the PPT binding complex (*KHSRP*); and *EFTUD2*, a component of U5-snRNP. Importantly, these proteins regulate intron excision during splicing [40–42]. Alteration of this process results in intron retention, a novel epigenetic characteristic of the MM genome. Our studies, therefore, suggest that intron retention in MM patients can be modulated via targeting Let-7f and other deregulated miRNAs in MM.

We then went on to validate the role of Let-7f in vitro and in vivo on MM tumor burden. We tested Let-7f alone and in combination with miR-155, because modulation of these miRs

was observed most frequently in the Group A patient cohort, which includes patients with high and intermediated cytogenetic-risk profile. Based on DIANA-miRPath, we identified 19 genes targeted by miR-155 including: DDX39B, which is an essential splicing factor required for association of the U2 protein complex with pre-mRNA; SRSF2 protein, which is required for formation of the earliest splicing complex and is involved in bridging the 5- and 3-splice sites and in U1 and U2 spliceosome assembly; and heterogeneous nuclear ribonucleoprotein A3, that plays a role in cytoplasmic trafficking of RNA. We found these target genes to be downregulated in MM patients, which suggests translational repression of target genes by miR-155.

We next evaluated the role of let-7f and miR-155 in MM cell line OPM2 by gain- and loss-of function experiments. We used LNA anti-miR probes for loss of function and pre-miR-155 for gain of function studies, alone or in combination. Although manipulation of these miRs induced 20–25% change in OPM2 cell proliferation and/or induction of apoptosis, the combination of antagomir Let-7f with pre-miR-155 significantly decreased MM cell proliferation and induced apoptosis (Fig. 6a, b).

We went on to evaluate efficacy of these miRs in an in vivo murine xenograft model of human MM to determine their therapeutic potential. Human MM-bearing mice were treated intraperitoneally for four consecutive days with the LNA antagomir Let-7f and pre-miR-155 probes, alone and in combination, and with scramble miR. We observed that the single LNA anti- Let-7f and pre-miR-155 treatment reduced tumor size by 36% after 7 days treatment (Fig. 6c). More significant tumor size reductions were achieved when animals were treated with combinations; for example, 58% reduction in tumor after antagomir Let-7f plus pre-miR-155 treatment. We did not observe any significant systemic toxicity in the animals. Our results, therefore, suggest identify a biological role for Let-7f and miR-155 both in MM in both in vitro and in vivo models, and suggest potential of novel therapeutics targeting these miRs.

Discussion

Multiple transcriptome modifiers play a critical role in determining growth and survival of cancer cells, including MM cells [5, 6]. These modifiers include small non-coding RNAs such as miRs, and a large repertoire of molecules involved in pre-mRNA/mRNA processing [9, 11, 43, 44]. In this study, we delineate the interplay between these two processes in MM cells. We evaluated miR expression profiles in MM patient and HD-PC samples, since miRs are modulators of gene expression that influence malignant cell growth by controlling protein expression. The majority of miRs identified in our studies were downregulated in MM compared to HD-PCs, consistent with prior reports in MM as well other hematologic malignancies and solid tumors [11, 45].

We next investigated target genes and identified pathway signatures affected by miRs that were significantly ($P < 0.05$) deregulated in MM samples. We focused on pathways that control pre-mRNA processing, since these pathways together with miRs control global gene expression. To delineate interactions between miR expression and mRNA processing, specifically splicing, we evaluated whether deregulated miRs in MM patients target SF or

SM (splicing enhances and/or silencers). Importantly, gene and pathway enrichment analyses suggested that 78% deregulated miRs target genes involved in pre-mRNA splicing (Fig. 2). Moreover, most deregulated miR target genes mapped to Ch-1, -6, and -17. Of note, some deregulated miRs in MM are encoded by genes originating from introns. For example, in MM patients we identified 13 (60%) intronic miRs among 22 deregulated miRs. These intronic miRs, unlike intragenic or intergenic miRs, are processed from the introns of their host gene and share common regulatory mechanisms, and consequently expression patterns. These analyses, therefore, demonstrated that deregulated miRs in MM patients control the expression of genes on Ch1q and Ch17p, both of which contribute to disease pathogenesis and may confer poor prognosis.

Our analyses also showed that genes targeted by the upregulated miRs in MM are enriched for genes encoding proteins in the spliceosome complex; involved in trans-esterification reactions, mRNA capping, and polyadenylation; and regulating mRNA export from the nucleus (Fig. 2c). Furthermore, deregulated miR target gene enrichment analyses identified those pathways that control U2 and U6 spliceosome complex assembly, mRNA stability and transport. Our validation studies in MM, MGUS, sMM and HD-PC transcriptomes identified a distinct miR target gene expression pattern in MM patient samples that is a reflection of the actual miR expression patterns (Fig. 4). These findings, therefore, suggest that the effects of deregulated miRs may play role in regulating the mRNA processing network, specifically splicing, and that these alterations contribute to the aberrant splicing mechanisms identified in MM patients.

Pre-mRNA-splicing is a complex process catalyzed co-transcriptionally by the spliceosome; it is accomplished via two trans-esterification reactions. Spliceosome assembly and the formation of complexes E, A, and B complexes characterizes the first trans-esterification reaction, while C complex promotes catalysis of this reaction [46]. The E, A, and B complexes are formed as a result of an extensive rearrangement of U1, U2, U4, U5, and U6 small nuclear ribonucleoproteins (snRNP) that must recognize the exon-intron boundaries [46–48]. The 3' end of any given intron consists of two conserved sequence elements, a splicing branch point (BP) and a polypyrimidine tract (PPT) of splicing. These classical splicing elements are sites where snRNPs are assembled as a spliceosome complex. At the end of the first trans-esterification reaction, the 2'-hydroxyl group of the branch point adenosine residue “attacks” the phosphate group of the 5' splice site, leading to cleavage of the 5' splice site from the adjacent exon; then the 5' splice site is ligated to the BP [46, 47]. The first trans-esterification reaction results in a detached 5' exon and the 3'exon/intron in a lariat structure and can be influenced by the differential expression of snRNPs. Here, we found that upregulated miRs target U1 and U2 snRNPs and cause their downregulation (Fig. 4). In the same group of patients, we found downregulated miRs targeting U6 snRNP. Thus, our studies further suggest that formation of E, A, and B splicing complexes are altered in MM patients, which could lead to aberrant splicing including partial exon skipping and/or intron retention characteristic of MM cells. Furthermore, normal intron processing, a detachment of the intron from the 3'end of exon, can be influenced by splicing enhancer or suppressor snRNPs that bind to introns during assembly of spliceosome complexes. It is well established that intronic splicing can be caused by splicing enhancer and suppressor snRNPs that bind to BP and PPT of splicing elements on given pre-mRNA transcripts [49].

In our validation studies, we found that Let-7f, which is frequently deregulated in MM patients, targets genes that are involved in intron processing; and that their downregulation leads to alterations in this process. The downregulated genes include: a core splicing factor *PTBP3*, which binds to a polypyrimidine tract of splicing (PPT); and *KHSPR*, which is part of the PPT binding complex that binds specifically to an intronic splicing enhancer; both proteins are involved in intron processing [41, 50, 51]. In addition, it is reported that *KHSPR* regulates miR biogenesis [52]. Thus, miR splicing factor regulation can be bidirectional in MM. Here we focused on effects of miRs on RNA processing, and the effects of splicing on miR biogenesis in MM are the object of ongoing studies.

Two other downregulated target genes are: [1] *EFTUD2*, a component of U5 snRNP which is involved in bridging the 5'- and 3'- splice sites and is required in U1 and U2 assembly, the first catalytic reaction of splicing; and [2] *SUPT6H*, which is involved in chromatin organization remodeling, specifically H3-K27 methylation [46, 52]. It has been shown that chromatin structure perturbation via histone depletion results in drastic alterations in the splicing process [53]. Regulation of these splicing factor/molecules by Let-7f in MM is selective, evidenced by knockdown experiments which demonstrate that expression of all four genes (*PTBP3*, *KHSPR*, *EFTUD2*, and *SUPT6H*) is significantly ($P < 0.005$) restored after transfections of OPM2 cells with the anti-Let-7f antagomir. Our studies, therefore, suggest that deregulated miRs in MM modulate expression of genes that encode proteins mediating early splicing mechanisms. Furthermore, our findings indicate that increased intron retention, a novel characteristic of the MM genome, can be regulated and corrected by modulating miR expression using LNA antagomirs and/or pre-miRs to modify core splicing factors and/or the splicing network in MM.

Finally, our gain- and loss- of function ex vivo and in vivo experiments show combined effects of deregulated miRs in MM on tumor burden. These studies showed that both anti-let-7f and pre-miR-155, alone and to a greater extent when combined, inhibited in vivo tumor growth (Fig. 6). Our studies, therefore, provide the framework for targeting deregulated miRs with novel therapeutics to reverse altered splicing and improve patient outcome in MM.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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Fig. 1. miR expression profiling in MM. miR array data were normalized and 384 human mature miRs were filtered using software “R” as described in Materials and methods and the Supplement. For further analysis, we selected 141 miRs identified in MM, HD-PCs, and/or MM cell lines. miRs are clustered along the vertical axis, and samples are on the horizontal axis. The normalized expression value for each miR is indicated by a color, with red representing low expression and blue representing high expression

Fig. 2. Annotation genes targeted by deregulated miRs in MM. Heatmaps of predicted miRNA target genes in MM. a Deregulated miRNA target genes detected by DIANA-miRPath considering different parameters were mapped to the genome. A red to green gradient coloration implies an increased percentage of target genes mapped to the individual chromosome. b, c Represent gene ontology (GO) distributions for deregulated miRNA targets. The DAVID program defines GO under three categories: cellular component (CC), biological process (BP), and molecular function. b A summary of CC. c A summary of BP. The resulting heatmaps display gene-terms affected by up- or down-regulated miRs in Group A (Gp-A), Group B (Gp-B), and MM patients. The significance of gene-term enrichment for each category (CC or BP) was determined based first on p-value enrichment with cut off $p < 0.05$, and second on the magnitude of fold enrichment with cut off > 10 . A white to red gradient coloration implies an increased percentage of gene-term enrichment

A) RNA processing pathway

B) Spliceosome Components

U1	U2	U4/U6	U5
U1snRNA	U2snRNA	U4snRNA	U5snRNA
+ Sm	+ Sm	U6snRNA	+ Sm
U1-70K	+ U2A'	+ Lsm	+ Snu114
U1A	+ U2B''	+ Sm	+ Brr2
U1C	+ SF3a	Prp3	Prp6
U1 related	+ SF3b	Prp4	+ Prp8
+ FBP11	U2 related	CypH	Prp8BP
+ S164	+ U2AF	Prp31	Prp28
p68	PUF60	Snu13	DIB1
CA150	+ SPF30	U4/U6.U5 tri-	
	SPF45	SnRNP assoc.	
	CHERP	+ snRNP27	
	SR140	Sad1	
	+ Prp43	Snu66	
	PAP-1	Snu23	
		+ Prp38	
		PAP-1	

Prp19 Complex	Prp 19 related	EJC/ TREX	Common components
Prp19	SKIP	+ ACINUS	+ CBP80/20
CDC5	Syf	eIFA3	+ hnRNPs
+ SPF27	Isy1	+ Y14	+ SR
PRL1	PPIL1	magoh	
AD002	CypE	+ UAP56	
CTNNBL1	CCDC12	THOC	
+ HSP73	RBM22		
NPW38	G10		
NPW38BP	AQR		

Complex B specific

Fig. 3. Example diagram adapted from the DAVID database that shows the mRNA splicing process, spliceosome components, and splicing complexes. Putative target genes of deregulated miRs involved in the splicing pathway are marked with a red star

Fig. 4.

Deregulated miRNA target gene profiling in MM, MGUS, sMM, and HD-PC. **a, b** Heatmaps show expression patterns of target genes in MM, MGUS, sMM, and HD-PC samples. Profiles were clustered using Euclidean distance in the similarity matrix. The color scale for expression values is shown on the right, and clusters of patient and HD-PC samples are presented as dendrograms on the top of the heatmaps. The gene expression analysis was done using GDS4968 dataset deposited in the NCBI GEO database. **c** A principal component analysis (PCA) of all deregulated miR target gene expression in MM, MGUS, sMM, and HD samples demonstrates the difference between the groups. Furthermore, there was a clear separation between the HD-PC and patient samples with respect to targeted gene expression, with a few outlier samples. We identified the minimal overlap between the MM, sMM, and MGUS clusters. Specifically, the overlap was detected between MM and sMM, or between sMM and MGUS. The PCA analyses of the groups which more clearly show the

extent of overlap between MM and sMM, or between sMM and MGUS are reported as a
Supplementary Fig. 3

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Fig. 5. Effects of Let-7f deregulation. **a** GO enrichment analyses of Let-7f target genes according to the cellular component (CC) and biological processes (BP) are summarized as pie charts; target gene-related KEGG and BIOCARTA pathways are shown as a table. Enrichment analyses considered p -value enrichment with a cut off of $p < 0.05$, and the fold enrichment with a cut off > 10 . **b** The left heatmap shows the expression pattern of Let-7 target genes in MM, MGUS, sMM, and HD samples. For unsupervised clustering analysis, a GO4896 dataset was used. The color scale for log expression values is shown at the bottom, while sample clustering is presented as a dendrogram on the top of the heatmap. **c** The right heatmap shows the relative expression of Let-7f target genes in MM, MGUS, and sMM patient groups as compared to HD-PC, and OPM2 cells transfected with a Let-7f antagonist compared to OPM2 cells transfected with a scrambled antagonist. Let-7f target genes are listed on the left; on the right of the heatmap, a dendrogram shows sub-nodes of Let-7f

target genes; red subnodes include target genes selectively affected by Let-7f; the color scale for expression values is shown at the bottom. MM, MGUS, sMM, and OPM2 clustering are presented as dendrograms on the top of the heatmap

Fig. 6.

This figure summarizes *in vitro* and *in vivo* effects on MM cells of Let-7f and miR-155 alone and in combination. Cell proliferation and apoptosis were measured 72 h after transfection of OPM2 cells with scramble (SC), antagomir Let-7f (anti-Let-7f), pre-miR-155, and anti-Let-7f + pre-miR-155. Cell proliferation was determined by MTT assay, absorbance was recorded at 570 nm (a), and cell apoptosis was evaluated by Annexin V5 and caspase apoptosis assays (b). Although after transfection experiments, Let-7f and miR-155 alone induced 20–25% change in MM cell proliferation and/or induced apoptosis, a combination of anti-miR-Let7f with pre-miR-155 had dramatic effects on OPM2 cell, over 60% underwent apoptosis (a, b). Figure c demonstrates *in vivo* activities of the Let-7f antagomir and pre-miR-155 in an MM xenograft model established after subcutaneous injection of sublethally irradiated (2 Gy) female NOD/SCID mice with an OPM2 MM cell line. The animals were randomized when a median tumor of 200–250 mm³ was reached in the cohort (4 mice/group). Then, an appropriate group of mice was treated with the anti-Let-7f antagomir, pre-miR-155, or anti-Let-7f + pre-miR-155; a control group of mice was treated with scrambled probes (SC). All animals were treated via tail vein injection and treatments were administered on days 1–4, and days 8–11 after animals were randomized. Tumor volume was evaluated by caliper measurement. Differences between the groups were

evaluated by standard *t*-tests (GraphPad Software). Animal handling methods are consistent with the recommendations of the Panel on Euthanasia of the American Veterinary Medical Association

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Table 1

Signaling pathways affected by miRs deregulated in MM

Pathway name	# of genes	% of genes	Fold enrichment	P-Value
<i>MM-U</i>				
Spliceosome	20	61	45	9.80E-31
<i>MM-D</i>				
Spliceosome	44	36	31	4.00E-57
mRNA surveillance	19	15	19	9.60E-19
RNA polymerase	5	4	14	3.50E-04
Herpes simplex infection	9	7	5	7.00E-04
RNA transport	8	7	4	2.30E-03
Pyrimidine metabolism	5	4	4	2.50E-02
<i>Gp-A-U</i>				
Spliceosome	32	39	36	2.10E-44
mRNA surveillance	6	7	10	2.90E-04
RNA polymerase	3	4	14	1.80E-02
Herpes simplex infection	5	6	4	3.00E-02
<i>Gp-A-D</i>				
Spliceosome	27	35	33	2.50E-35
mRNA surveillance	10	13	18	2.50E-09
Influenza A	5	6	5	2.10E-02
<i>Gp-B-U</i>				
Spliceosome	4	25	30	1.30E-04
mRNA surveillance	3	19	33	2.50E-03
<i>Gp-B-D</i>				
Spliceosome	33	38	33	8.50E-44
mRNA surveillance	11	13	16	7.80E-10
RNA polymerase	4	5	17	1.60E-03
Herpes simplex infection	7	8	5	2.10E-03
RNA transport	5	6	4	3.70E-02

KEGG signaling pathways affected by up- (U) or down- (D) regulated miRs in Group A (Gp-A), Group B (Gp-B), and MM patients. Enrichment analyses were done by considering a *p*-value enrichment with a cut off of $p < 0.05$, and a fold enrichment with a cut off > 2

Table 2

Let-7f target genes and their chromosomal locations

Gene symbol	Ch	Gene symbol	Ch
HNRNPR	1	HSPA8	11
HNRNPU	1	SF3B2	11
PRPF38B	1	THOC5	12
SF3B4	1	MBNL2	13
WBP11	1	PAPOLA	14
ZNF326	1	SYNCRIP	15, 19
CPSF3	2	FUS	16
POLR2D	2	CDK12	17
SF3B1	2	EFTUD2	17
SNRNP200	2	NSRP1	17
HNRNPA0	5	POLR2A	17
PPP2CA	5	PRPF8	17
SREK1	5	SRSF1	17
SF3B5	6	SUPT6H	17
SYNCRIP	6	HNRNPM	19
HNRNPA2B1	7	HNRNPUL1	19
RBM28	7	KHSRP	19
CCAR2	8	SCAF1	19
ESRP1	8	SNRPA	19
PTBP3	9	RBM11	21
RBMX	9, X	SON	21
HNRNPF	10	HNRNPL	22
CPSF7	11	SMC1A	X