Identification of microRNA expression patterns and definition of a microRNA/mRNA regulatory network in distinct molecular groups of multiple myeloma

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Running title: miRNA deregulated pathways in multiple myeloma

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Abstract

To date, little evidence of miRNA expression/deregulation in multiple myeloma (MM) has been reported. To characterize miRNA in the context of the major MM molecular types, we generated miRNA expression profiles of highly-purified malignant plasma cells from 40 primary tumors. Furthermore, transcriptional profiles, available for all patients, were used to investigate the occurrence of miRNA/predicted target mRNA pairs anti-correlations, and the miRNA and genome-wide DNA data were integrated in a subset of patients to evaluate the influence of allelic imbalances on miRNA expression. Differential miRNA expression patterns were identified, which were mainly associated with the major \textit{IGH} translocations: particularly, t(4;14) patients showed specific over-expression of \textit{let-7e}, \textit{miR-125a-5p} and \textit{miR-99b} belonging to a cluster at 19q13.33. The occurrence of other lesions, i.e. 1q gain, 13q and 17p deletions, and hyperdiploidy, was slightly characterized by specific miRNA signatures. Furthermore, the occurrence of several allelic imbalances or loss-of-heterozygosity was found significantly associated with the altered expression of miRNAs located in the involved regions, such as \textit{let-7b} at 22q13.31 or \textit{miR-140-3p} at 16q22. Finally, the integrative analysis based on computational target prediction and miRNA/mRNA profiling defined a network of putative functional miRNA-target regulatory relations supported by expression data.
Introduction

Multiple myeloma (MM) is a malignant proliferation of bone marrow (BM) plasma cells (PCs), characterized by a profound genomic instability involving both numerical and structural chromosomal aberrations of potential prognostic relevance. Nearly half of MM tumors are hyperdiploid (HD) with multiple trisomies of non-random odd-numbered chromosomes and a low prevalence of chromosomal translocations involving the immunoglobulin heavy chain (\textit{IGH}) locus at 14q32 and chromosome 13 deletion; the others are nonhyperdiploid (NHD) tumors often showing chromosome 13 deletion, 1q gain and \textit{IGH} translocations, with the most frequent partners being 11q13, 4p16, 16q23, 20q11 and 6p21. The deregulation of at least one of the cyclin D genes is observed in almost all MM cases and, in combination with recurrent \textit{IGH} translocations, has been proposed for a molecular classification of MM called Translocation/Cyclin (TC) classification. The occurrence of specific transcriptional patterns associated with the molecular subgroups and major genetic lesions of MM has been extensively described in several studies by us and others.

MiRNAs are endogenous ∼22 nt RNAs that play important regulatory roles in animals and plants by targeting mRNAs for cleavage or translational repression. It has been suggested that chromosomal abnormalities and other types of genetic or epigenetic alterations might contribute to miRNA deregulation in cancer.

There are still few published data concerning miRNA expression in MM. Loffler et al. have shown that IL-6 regulates \textit{miR-21} transcription in IL-6-dependent human myeloma cell lines (HMCLs) through a STAT-3-related mechanism, and that ectopic \textit{miR-21} expression can sustain their growth in the absence of IL-6. Pichiorri et al. have reported a set of miRNAs that can be associated with neoplastic...
transformation and progression in MM. Our group has identified a set of deregulated miRNAs in HMCLs that correlate with copy number (CN) alterations or gene expression patterns. More recently, Roccaro et al. reported the down-regulation of miR-15a and miR-16 in a small cohort of relapsed-refractory MM patients, showing that they may regulate the proliferation and growth of MM cells in vitro and in vivo.

We here describe the miRNA expression profiling of MMs in the context of the major recurrent molecular alterations of the disease. Furthermore, in an attempt to define the consequences of differential miRNA expression, we searched for functional targeting relationships by means of the definition of a global miRNA/mRNA regulatory network.

**Materials and methods**

**Patient samples**

BM aspirates were obtained during standard diagnostic procedures from 54 untreated patients (48 MM and 6 plasma cell leukemias, PCL), most of whom have been included in our previous reports. All of the patients had given their informed consent in accordance with the Declaration of Helsinki, and all studies were approved by the institutional review board of the University of Milan. Normal control samples (NCs) were obtained from three healthy donors. Patients had been diagnosed on the basis of previously described criteria. PCs were purified from BM biopsies using CD138 immunomagnetic microbeads (MidiMACS, Miltenyi Biotec, Auburn, CA). The purity of the positively selected PCs (≥90%) was assessed by means of flow cytometry. Patients has been stratified according to the previously
described TC classification. Briefly, TC1 group included patients characterized by the t(11;14) or t(6;14) translocation; TC2, those showing low to moderate levels of the CCND1 gene in the absence of any primary IGH translocation; TC3 includes tumors that do not fall into any of the other groups; TC4 cases show t(4;14) translocation; and TC5 either the t(14;16) or t(14;20) translocations.

MiRNA expression profiling

Thirty-eight MM, two PCL and three NC samples were investigated for miRNA expression by means of microarray analysis. The patient samples were selected to be representative of the five TC groups, and the PCL samples with translocated MAF genes were included because of the relatively small number of TC5 MM patients. FISH information regarding the major numerical chromosomal alterations was already available (Table S1). The total RNA extraction and quality assessment were performed as previously described. The samples were profiled on the Agilent Human miRNA Microarray V2 (Agilent Technologies, Santa Clara, CA), consisting of 60-mer DNA probes synthesized in situ that represent 723 human and 76 human viral miRNAs from the Sanger database (v10.1), using the one-color technique in accordance with the manufacturer’s instructions. The human miRNAs data were re-annotated on Sanger Release 12.0 and normalized using the Aroma Light package for Bioconductor. In order to overcome scaling biases due to background subtraction, the data were converted to obtain positive values throughout the dataset, at a minimum value of 1. The raw and normalized microRNA data are available through GEO accession number GSE17498. Hierarchical agglomerative clustering of the selected probe lists was performed using Pearson’s correlation
coefficient and average linkage as distance and linkage metrics, respectively. Supervised analyses were carried out using Significant Analysis of Microarrays (SAM) software version 3.02 as previously described. The significance threshold (at a q value of 0) was determined tuning the Δ parameter on the false discovery rate and controlling the q value of the selected probes. The cross-validation of the identified signatures was performed using the default options of ld() package for Linear Discriminant Analysis in R software, choosing LOOCV (Leave-One-Out Cross-Validation) method and applying a 1000-permutations model on a bootstrapped resampled dataset.

**Quantitative Real-Time PCR (Q-RT-PCR)**

Selected mature miRNAs underwent Q-RT-PCR using TaqMan® microRNA assays (Applied Biosystems, Foster City, CA) as previously described.16

**Gene expression profiling**

The transcriptional profiles of the patients have been generated using Affymetrix GeneChip® HG-U133A arrays as previously described and are available through GEO accession number GSE13591. The raw intensity signals were extracted from CEL files and normalized using the RMA package for Bioconductor and custom GeneAnnot-based Chip Annotation Files version 2.0.1 (CDF), available at the URL http://www.xlab.unimo.it/GA_CDF, thus leading to the definition of 12195 unique well-characterized genes.
**Genome-wide profiling**

Nineteen of the 40 MM/PCL samples profiled for miRNA expression underwent genome-wide DNA analysis using Affymetrix® GeneChip Human Mapping 50K XbaI microarrays, and the copy number values were generated as recently described.²⁰ Briefly, raw CN values for individual SNPs and the probability of loss-of-heterozygosity (LOH) were extracted from CEL files and converted into signal intensities using Affymetrix GTYPE 4.1 and CNAT 4.0.1 software; robust estimate of genotype calling was performed using BRLMM distance classifier. Piecewise constant estimates of the underlying local DNA CN variation were calculated using the DNAcopy Bioconductor package, which looks for optimal breakpoints using circular binary segmentation (CBS), and the estimated profiles were normalized using the self-developed FBN package for R software. LOH was defined applying a 100 kb smoothing window (i.e. two-fold the mean intermarker distance on the arrays) on the CNAT-derived value with a probability higher than 0.95 for the detection of a monoallelic polymorphism.²⁰ The CEL files are available through GEO accession number GSE16121.

**MicroRNA target prediction**

miRNA targets have been predicted applying miRanda algorithm²² on human miRNA and transcript sequences of miRBase Release 12.0²³ and ENSEMBL Release 52, respectively. The score threshold of miRanda, associated with each predicted miRNA-transcript targeting relationship and depending on the sequence alignment
and thermodynamic stability of the RNA duplex, has been set at 160. Finally, the correspondence between ENSEMBL_transcript and EntrezGene_ID was defined.

**Integrative analysis of miRNA and gene expression profiles to reconstruct a miRNAs/genes regulatory network**

The post-transcriptional regulatory network of miRNA and genes in MM has been defined as a directed, bipartite graph in which miRNA-mRNA relationships are supported by both targeting predictions and expression data. Specifically, the network has been reconstructed using the subset of miRNAs and genes characterized by i) a regulatory relationship according to miRanda predictions and ii) expression profiles strongly anti-correlated. Indeed, since miRNAs tend to down-regulate target mRNAs, the expression profiles of genuinely interacting pairs are expected to be anti-correlated. Thus, for each miRNA/gene pair scored as potentially interacting on the basis of miRanda prediction, the Pearson correlation coefficient of the respective expression vectors in MM samples was calculated and used as an estimator of the functional activity of the miRNAs on the target genes. Genes were considered genuine miRNA targets when included within the top 3% of all pairs ordered on their anti-correlation score. This selection gave rise to the final adjacency matrix $S$ of regulatory relations supported by MM expression data and the associated miRNAs/genes regulatory network. The matrix $S$ defined a bipartite directed network with two types of nodes (miRNAs and mRNAs) connected by directed edges, each representing a probably functional regulatory effect of a miRNA on a target gene. The post-transcriptional network allows identifying groups of genes regulated by the same miRNA/s, and of miRNAs regulating specific groups of genes.
of functional relevance. The networks were drawn using Cytoscape\textsuperscript{25} and the functional enrichment analysis was performed using DAVID (Database for Annotation, Visualization and Integrated Discovery) 2008 Tool (http://david.abcc.ncifcrf.gov/).

**Statistical analysis**

Conventional statistical procedures were applied using standard packages for R software (Kendall’s $\tau$ correlations, Wilcoxon rank-sum tests, $t$-tests, Fisher’s exact tests).

**Results**

**Global miRNA expression profiling in MM patients**

MiRNA profiles were analyzed by means of high-density microarrays, specific for 723 human miRNAs, in 40 patients representative of the five TC groups (Supplemental Table A1), and in PCs from three NCs. To determine whether global miRNA profiling could distinguish the molecular groups, we performed an unsupervised analysis using conventional hierarchical agglomerative clustering: the 43 samples were described by 74 miRNAs whose average change in expression levels varied at least two-fold from the mean across the dataset.\textsuperscript{19} The most striking finding was that all of the TC4 patients were tightly clustered (Figure 1, grey cluster, $P < 10^{-5}$), as were 4/5 TC5 cases (red cluster, $P = 2 \times 10^{-4}$). The TC2 cases were partially grouped (blue cluster, $P = .0046$), whereas the TC1 and TC3 samples were scattered along the dendrogram. The NCs were clearly grouped in a distinct sub-branch.
Next, multiclass analysis allowed identifying a set of 26 miRNAs showing highly significant differential expression (at \( q \)-value=0) across the five TC groups (Table S2). As shown in Figure 2A, all of the TC groups except TC3 were characterized by the up-regulation of specific miRNAs. In particular, ten (38\%) miRNAs (\( \text{miR}-150, \text{miR}-133b, \text{miR}-99a, \text{miR}-133a, \text{miR}-155, \text{miR}-125b, \text{let}-7c, \text{miR}-1, \text{miR}-155^* \text{and} \text{miR}-34b^* \)) were expressed at higher levels in TC5 than in the other classes, seven (27\%) in TC4 (\( \text{miR}-125a-5p, \text{let}-7e, \text{miR}-99b, \text{miR}-222, \text{miR}-221, \text{miR}-221^*, \text{and} \text{miR}-365 \)), six (23\%) in TC2 (\( \text{miR}-874, \text{miR}-1237, \text{miR}-512-3p, \text{miR}-940, \text{miR}-933, \text{and} \text{miR}-1226^* \)), and three (11\%) in TC1 (\( \text{miR}-361-3p, \text{miR}-582-5p, \text{miR}-30e^* \)). Notably, \( \text{miR}-125a-5p, \text{let}-7e \) and \( \text{miR}-99b \), which were associated with the highest scores in the supervised analysis and over-expressed specifically in the TC4 samples, belong to a cluster at 19q13.33, whereas \( \text{mir}-99a, \text{let}-7c \) and \( \text{mir}-125b-2 \), highly expressed in the TC5 cases, belong to a paralogous cluster at 21q21.1. Figure 2B shows the 40 MM samples clustered according to the expression profiles of the 26 miRNAs, suggesting their capacity to drive TC distribution into separate branches. To test the correctness of the obtained signature and its capability to discriminate the five TC groups, we additionally tested the predictive power of the 26 miRNAs using linear discriminant analysis for classification of multivariate observations. The procedure led to confirm the accuracy of the identified signature, at a percentage of an overall classification rate of 89.3\%: specifically, all TC1, TC4 and TC5 samples were classified correctly, whereas TC2 and TC3 cases showed a misclassification error of 32.8\% and 11.1\%, respectively.

We also investigated the differential miRNA expression on the basis of the occurrence of other recurrent chromosomal alterations, such as 1q gain and 13q and
17p deletions, and identified a number of differentially expressed miRNAs, none of which was located in the involved chromosomal region. Similarly, comparison of the HD and NHD cases revealed a set of upregulated miRNAs only in the latter. Some of the miRNAs identified in these analyses were the same as those found in IGH-translocated patients, in all likelihood because of their representativeness within 1q gain, del(13), del(17) and NHD cases (Figure 3).

Q-RT-PCR validation of differentially expressed miRNAs

To validate the microarray data, the levels of some miRNAs (miR-99b, miR-582-5p, miR-155, miR-30e*, miR-125b, miR-133b, miR-221, miR-222, miR-125a-5p and miR-99a) were quantified by means of Q-RT-PCR in all of the 43 samples. As shown in Table S3, linear correlation analysis indicated a very good correspondence between the two techniques.

Furthermore, the expression of miR-99b, miR-582-5p, miR-133b and miR-125a-5p was also evaluated in 14 additional cases: four cases of which carrying t(11;14), seven t(4;14), and three t(14;16) or t(14;20). Overall, in the 54-sample panel, miR-99b and miR-125a-5p were confirmed as showing statistically significant and specific overexpression in the t(4;14) group; miR-133b in the t(14;16)/t(14;20) group; and miR-582-5p in the t(11;14) group ($P < 10^{-5}$ for all miRNAs; Figure 4).

Integrative analysis of miRNA expression profiles with genome-wide copy number variations and occurrence of LOH

To evaluate the influence in miRNA expression of the allelic imbalances detected at a genome-wide level, we performed an integrative analysis of mature miRNA
expression levels and their inferred DNA CN values, available in 19 of 40 cases (Table S1). The correlation analysis identified 49 mature miRNAs ($P < .05$) (Table S4), mainly localized on chromosome 1 (8/49, 16%), followed by chromosome 19 (6/49, 12%), chromosome 17 (4/49, 8%), and chromosomes 3, 5, 11, 13, 14 and 15 (3/49 each, 6%). Noteworthy, miR-520a-5p, miR-518d-5p, miR-498 and miR-520g belong to a cluster at 19q13.41, and miR-17, miR-20a and miR-20a* belong to the mir-17~92 cluster at 13q31.3.

Next, we tested the correlation between miRNA expression and the occurrence of LOH, as defined in our previous report. We identified the downregulation of 14 miRNA genes in the presence of LOH (Table S5): let-7b, mapped to 22q13.31 ($P=.017$), and miR-662 (16p13.3, $P=.0117$) were the most statistically significant. We also highlighted the intronic miRNA miR-140-3p ($P=.022$), which maps to 16q22.1; the miR-19b and miR-20a belonging to the mir-17~92 cluster at 13q31.3 ($P=.0215$ and .0289, respectively); and miR-137 ($P=.0234$) which maps to 1p21.3.

Integrative analysis of miRNA/mRNA expression and reconstruction of a regulatory network in MM

The integrative analysis of miRNA/mRNA expression profiles allows reconstructing a network of functional interactions occurring in MM from the panel of potential regulatory relationships predicted from sequence information. Our integrative approach assumes that the final effect of a truly functional interaction between a miRNA and its predicted mRNA targets can be seen as a pair of anti-correlated expression profiles. Thus, the set of MiRanda predicted targeting relationships was
refined selecting those more strongly supported by the miRNA and mRNA expression data. The entire procedure led to the identification of 23,729 regulatory relationships, namely anti-correlation, involving 628 miRNAs and 6,435 predicted target genes, as about 47% of the genes associated with an expression profile were not targets of any of the considered miRNAs, and 93 miRNAs (13%) were not significantly anti-correlated with any target gene. The data from the integrative analysis were used to reconstruct a bipartite direct miRNAs/mRNAs regulatory network. The number of target genes per miRNA ranged from 1 to 440 (average 34; with a mean 3.7 miRNAs per gene) (Table S6).

Various sub-networks can be derived from the global identified network, as those accounting for the targeting relationships of specific miRNA signatures associated with distinct TC groups. In Figure S1 is reported the sub-network of the t(4;14) miRNA signature, i.e. one of the most consistent and specific sub-groups of our dataset. The network of t(4;14) consists of seven miRNAs and 289 anti-correlated targets, with the number of targets per miRNA ranging from 1 to 113, and approximately 29% of the genes being targeted by at least two miRNAs. Interestingly, three genes are commonly regulated by five miRNAs (CBFA2T2, core-binding factor, runt domain, alpha subunit 2, translocated to, 2; PPP1R16B, protein phosphatase 1, regulatory inhibitor subunit 16B; and GOSR2, Golgi SNAP receptor complex member 2). Functional enrichment analysis of anti-correlated targets revealed various over-represented biological processes, including chromosome segregation, protein polyubiquitination, cell cycle regulation, and unfolded protein response (Table S7). Interestingly, a comparison between the identified supported miRNA target genes and mRNAs down-regulated in the t(4;14) cases (as identified
in a larger proprietary dataset of 132 MMs, data not shown) highlighted that the miRNA targets were significantly enriched in down-regulated genes ($P < 10^{-5}$) (Table S8). Similarly, sub-networks were derived considering the sets of miRNAs differentially expressed in TC5 and TC1 groups (Table S9).

**Discussion**

In this study, the integrative analysis of the different types of genomic data (i.e., miRNA and mRNA expression levels and genome-wide CN profiles) allowed the definition of distinct patterns of miRNA deregulation and the prediction of the miRNA/mRNA regulatory networks in molecular subtypes of multiple myeloma. Particularly, our results highlighted that specific patterns of miRNA expression may differentiate MMs with distinct and well-known genetic alterations. Specific signatures were found to be associated with t(4;14) or translocated MAF genes and, to a lesser extent, with t(11;14) and TC2 group (expressing moderate levels of CCND1 in the absence of IGH translocation). This scenario is reminiscent of that previously described at mRNA level in the context of the TC classification. Other chromosomal abnormalities recurrently observed in MM samples (i.e. 13q deletion, 1q gain/amplification, 17p deletion and the hyperdiploid status) appeared to be associated to less strong miRNA signatures, a finding that needs to be clarified in larger series.

Most of the 26 miRNAs significantly discriminating the TC groups have previously been found to be involved in solid and hematological tumors. The most extensively investigated are miR-155, miR-221 and miR-222, and the let-7 family. MiR-155 is involved in many biological processes, including hematopoiesis,
inflammation and immunity, and its deregulation has been found to be associated with certain types of solid and hematological tumors, in which it is predominantly over-expressed and acts as an oncomir. Notably, miR-155 has been very recently found deregulated in Waldenström macroglobulinemia (WM), suggesting a role in the proliferation and growth of WM cells acting on signaling cascades including MAPK/ERK, PI3/AKT, and NF-kB pathways. In addition, miR-155 knockdown leads to significant increase of WM cells in G1 phase, and to the downregulation of cyclin-dependent kinases and cyclins D and the simultaneous upregulation of p53 expression, suggesting a critical role in the regulation of cell-cycle proteins responsible for G1 arrest. MiR-221 and miR-222 have also been found to be up-modulated in many tumors and described to target the C-KIT, p27 and p57 genes. Finally, many human let-7 genes, which are known to target RAS genes and oncogenes involved in the cell cycle, such as HMGA2, MYC, CDK6 and CDC25, map to regions frequently deleted in human tumors, indicating that they may function as tumor suppressors. The most striking finding was the very specific expression of three miRNAs (miR-99b, let-7e and miR-125a-5p), encoded in a conserved genomic cluster, in the t(4;14) cases. They are coordinately up-regulated during metamorphosis in Drosophila, where they are co-transcribed as a single polycistronic transcript. Although their involvement has been suggested in various tumors, to our knowledge this is the first evidence of their coordinated deregulation in cancer.

The differential miRNA expression associated with distinct genetic subgroups is a novel finding in MM. Of note, it has already been reported in other hematologic malignancies, such as acute myeloid leukemia (AML) and chronic lymphocytic
leukemia (CLL). As regards this latter neoplasia, in which the role of miRNA has been extensively investigated, the presence of miRNA signatures associated with the major specific genetic lesions (trisomy 12 and 13q14, 11q23 and 17p13 deletions) has been reported very recently. Interestingly, as found in our study, the discriminating miRNAs were not localized in the chromosomal regions specific for the corresponding cytogenetic abnormalities. Overall, the identification of specific miRNA patterns may help not only to distinguish distinct MM genetic subgroups known to show differences in term of response to therapy and survival, but also to provide a better understanding of their pathogenesis.

We also evaluated the impact in miRNA expression of allelic imbalances occurring in MM. Our integrative analysis identified the occurrence of a gene dosage effect on the expression of a number of miRNAs, as has previously been reported in HMCLs. Interestingly, some of the miRNA genes map to hot-spot altered regions in MM: e.g. mir-17 and mir-20a which belong to a cluster at 13q31, which was deleted in almost 40% of our patients; furthermore, a number are located in odd-numbered chromosomes involved in hyperdiploidy, particularly chromosomes 3, 5, 7, 9, 11 and 19, and three (mir-1231, mir-205 and mir-215) belong to the long arm of chromosome 1, which was gained at a frequency of more than 30%. Using analogue integrative approach, we evaluated the impact of LOH on miRNA expression, based on the data described in our recently published study. Among the identified miRNA whose expression correlated with the occurrence of LOH, let-7b has been previously reported as the most discriminatory miRNA between acute lymphoid leukemia (ALL) cases when compared with acute myeloid leukemia cases (significantly down-regulated in ALL). Moreover, it has been demonstrated that let-7b correlated with
the cytogenetic prognostic risk associated to the samples, being low in the favourable groups and high in intermediate and adverse acute myeloid leukemia cases. Of note, we also identified a significant correlation between miR-140-3p expression and the occurrence of LOH at 16q22.1-q23.1, which has been described as recurrent in MM by others and us. We have also demonstrated that LOH at 16q22.1 significantly correlated with the expression of WWP2, host gene of miR-140-3p involved in ubiquitination processes, suggesting that LOH (associated with monoallelic loss or other mechanisms such as uniparental disomy), may also represent an important mechanism in the coordinated control of miRNA and gene expression in MM.

The computational prediction of miRNA targets currently presents a number of significant challenges because all of the most widely used tools (miRanda, TargetScan, PicTar, PITA and RNAhybrid) are characterized by a significant proportion of false positive interactions that are partly due to the fact that post-transcriptional regulation is context-dependent. On the basis of increasing experimental evidence supporting the hypothesis that miRNAs can act through target degradation, it has been proposed that target predictions could be integrated with miRNA and gene expression profiles to select functional miRNA/mRNA relationships. This can be done adopting a variational Bayesian model and software, or simply using a non heuristic method based on miRNAs/mRNAs anti-correlations. We applied the latter to our data set, which allowed the reconstruction of a general miRNA/mRNA regulatory network that represents the putative functional regulatory effects (as supported by expression data) of all of these miRNAs on their targets in MM.
On the basis of the target genes identified here, a number of the miRNAs differentially expressed in *IGH* translocated cases may play important roles in the biology of MM PCs. With regard to the t(4;14)-miRNA signature, five miRNAs target *CBFA2T2*, a nuclear repressor homologous to *ETO* that binds to the AML1-ETO complex and may play a role in hematopoietic differentiation.\(^{43,44}\) Furthermore, *let-7e* targets *PTPRE*, a positive regulator of osteoclast function\(^ {45}\) and a selective inhibitor of IL-6- and IL-10-induced JAK-STAT signaling.\(^ {46}\) Interestingly, the expression of the tumor suppressor gene *PDCD4* (programmed cell death 4),\(^ {47}\) a supported target of *miR-221* based on our analysis, has recently been found to depend on the levels of MMSET, which is deregulated by the t(4;14).\(^ {48}\) *ING4*, a tumor suppressor frequently mutated or down-regulated in human cancers which was recently described to exert an inhibitory effect on MM-induced angiogenesis,\(^ {49,50}\) is a supported target of *miR-365*. Concerning the TC5 signature, *miR-133a* targets *DMTF1*, a putative tumor suppressor which activates the ARF-p53 pathway, leading to cell growth arrest or apoptosis; notably, it maps at 7q21, often deleted in human malignancies.\(^ {51}\) Finally, among the miRNAs up-regulated in t(11;14), *miR-361-3p* and *miR-30e*\(^ *\) target *PPP2R4*, an activator subunit of PP2A which play an important role in the survival and growth of MM cells since it dephosphorylates the GP130 subunit of the IL-6 receptor, thus preventing its degradation and allowing the activation of IL-6 signaling.\(^ {52,53}\)

Taken together, our findings strongly suggest that understanding the molecular biology of myeloma requires considering the miRNome in the context of the genomic and transcriptomic features of malignant PCs. Based on this integrated
approach, our data may provide an important contribution to future investigations aimed at characterizing the role of specific miRNAs in MM pathogenesis.

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**Authorship**

Contribution: M.L. performed the research, performed miRNA profiling and Q-RT-PCR, analyzed the data, and wrote the manuscript; M.B. and G.S. generated and analyzed the microRNA/mRNA network data; L.A. performed transcriptional, genotyping, statistical and integrative analyses, and wrote the manuscript; K.T. generated gene expression profiling data; L.M. generated genotyping data; S.F. and G.L.D. collected patient samples; L.L. designed research and revised the manuscript; S.B., S.B. and A.N. designed research and wrote the manuscript.
References


FIGURE LEGENDS

Figure 1. Unsupervised analysis of miRNA expression profiles. Hierarchical clustering of the samples using the 74 most variable miRNAs (patients in columns, miRNAs in rows). The color scale bar represents the relative miRNA expression changes normalized by the standard deviation. The patients’ molecular characteristics are shown above the matrix; n indicates unavailable information. Specific characteristics are enriched in colored sub-branches (see text).

Figure 2. Identification of miRNA signatures characterizing TC classes. Heatmap of the differentially expressed miRNAs in MM patients stratified into the five TC groups (A); dendrogram of the 40 MM samples clustered according to the expression profiles of the 26 miRNAs (B).

Figure 3. Identification of miRNA signatures characterizing distinct MM genetic subgroups. Supervised analyses identifying the miRNAs that are differentially expressed in MM patients harboring: (A) gain/amplification of the 1q arm; (B) del(13q14); (C) deletion of 17p; and (D) hyperdiploidy.

Figure 4. Q-RT-PCR validation of miRNA expression. Box plots of miRNAs quantified in Q-RT-PCR in the broadened panel of 54 MM-PCL cases, whose expression significantly correlated with the presence of a specific IGH translocation. Expression levels are given as $2^{-\Delta\Delta Ct}$. 
Figure 1
Figure 3
Figure 4

Box plots showing the expression levels of miR-99b, miR-125a-5p, miR-133b, and miR-582-5p in different cell lines. The box plots compare the expression levels between different conditions, as indicated by the notation below each plot.
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