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Functional Analysis of microRNA in Multiple Myeloma

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Abstract

MicroRNAs (miRNAs) are short non coding RNAs that regulate the gene expression and play a relevant role in physiopathological mechanisms such as development, proliferation, death, and differentiation of normal and cancer cells. Recently, abnormal expression of miRNAs has been reported in most of solid or hematopoietic malignancies, including multiple myeloma (MM), where miRNAs have been found deeply dysregulated and act as oncogenes or tumor suppressors. Presently, the most recognized approach for definition of miRNA portraits is based on microarray profiling analysis. We here describe a workflow based on the identification of dysregulated miRNAs in plasma cells from MM patients based on Affymetrix technology. We describe how it is possible to search miRNA putative targets performing whole gene expression profile on MM cell lines transfected with miRNA mimics or inhibitors followed by luciferase reporter assay to analyze the specific targeting of the 3′untranslated region (UTR) sequence of a mRNA by selected miRNAs. These technological approaches are suitable strategies for the identification of relevant druggable targets in MM.

Keywords: microRNA, miRNA, Microarray profiling, miRNA replacement, miRNA inhibition, Transfection

1 Introduction

Multiple myeloma (MM) is an incurable hematologic disorder in which malignant plasma cells accumulate in the bone marrow. MM is characterized by a range of genetic aberrations leading to clinical heterogeneity in the patient population. The specific mechanisms leading to the development of plasma cell disorders and progression to symptomatic MM have not been fully elucidated. Treatment options for patients with MM have significantly improved within the past decade, resulting in enhanced response rates and survival; treatment options outside of clinical trials are currently limited to combinations of bortezomib, thalidomide and its analogs (IMiDs), chemotherapy and steroids (dexamethasone, prednisone). High-dose melphalan with stem cell transplantation is considered in eligible patients. Although these agents are effective in the treatment of a majority of MM patients, the clinical outcome is worst for patients which can be defined as high-risk. Specifically, the

treatment results are clearly unsatisfactory for MM patients bearing the t(4;14) translocation which represents therefore a substantial challenge (1).

microRNAs (miRNAs) are small evolutionary conserved noncoding RNAs that bind to the 3′-untranslated region (UTR) of target mRNAs, resulting in translation repression or mRNA degradation, and play important roles in cellular processes such as proliferation, development, differentiation, and apoptosis. The dysregulation of these tiny and important molecules in different types of cancers including MM are widely described and make it promising target for new therapeutic approach (2–9). Moreover, recent promising findings are supporting the idea of miRNA-based personalized therapeutic strategies in MM (2, 4, 10–19). Advances in the oligonucleotide chemistry and formulation of miRNA mimics or antisense oligonucleotides (miRNA inhibitors) have added a new value in the enhancement of their therapeutic and commercial potential (7, 20).

We recently reported that new molecular-based targeted approaches, including the use of specific mimics or inhibitors, have provided an example of personalized treatments of MM. For instance, miR-29b replacement strategies are effective against MM in preclinical models (10, 12, 21). Moreover, we described that p53-mutated MM may have benefit by treatment with miR-34a mimics (15) if delivered with appropriate nanovectors (13, 19). We also demonstrated that the use of miR-221 inhibitors may provide benefit for patients with t(4;14) MM representing a potential targeted and personalized therapeutic approach. Based on our preclinical data, it is conceivable that these therapies may benefit a subset of patients with the t(4;14) translocation, particularly those with increased expression of miR-221/222 (4, 14, 22). miR-21 (16) and miR-125a-5p (17) inhibitors were also found to be promising anti-MM therapeutic agents when delivered in vitro and in vivo in MM models.

During last decade, microarray technologies have emerged as a flexible method for analyzing large numbers of nucleic acid fragments in parallel. Based on base-pairing rules microarrays provide a medium for matching known and unknown DNA targets, as well automating the process of identifying the unknowns. This technique accommodates parallel analysis for gene expression, as well as, allows gene discovery and therefore provides simultaneously information on several thousand of genes. Moreover, noncoding (nc) RNA microarray platforms have been recently developed, revealing differential expression patterns of microRNA, as well as of long ncRNA.

Aberrant miRNA expression in MM as shown by microarray analysis has been reported (23), indicating that miRNA dysregulation characterizes the progression of the disease, modulates important pathways involved in MM cell survival and reflects the

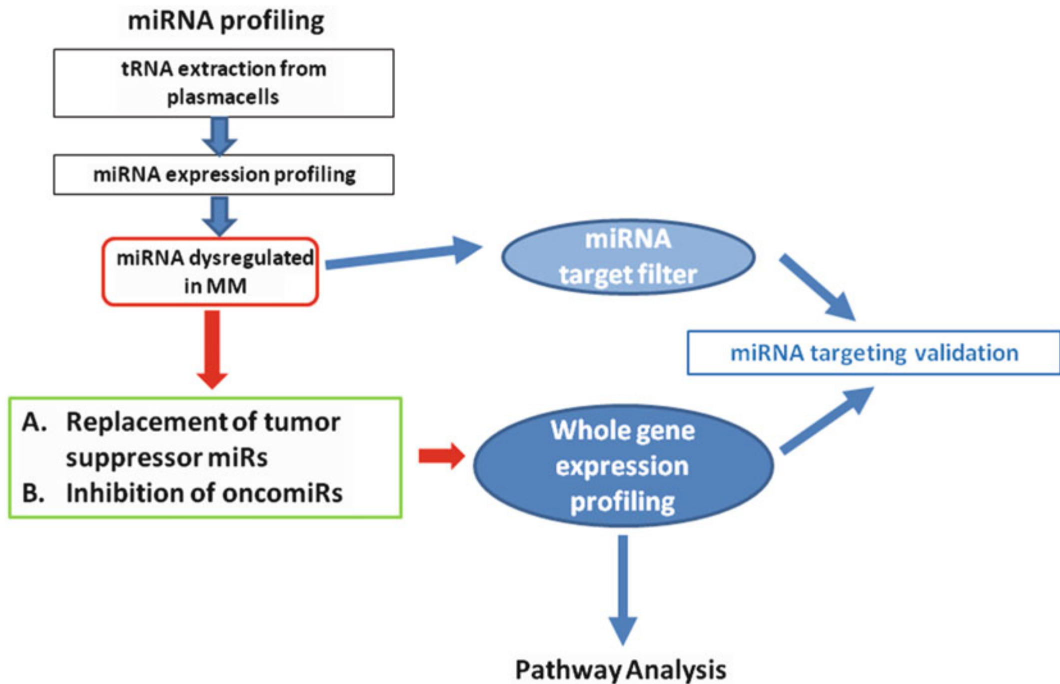

Fig. 1 The figure shows the workflow of procedures for miRNA functional analysis. First the detection of dysregulated miRNAs by differential miRNA profiling. Then the replacement of downregulated miRNAs or specific inhibition of upregulated miRNAs, and the validation of miRNA-specific targeting by (1) the luciferase reporter assay, (2) the whole gene expression analysis by microarray, (3) the study of specific targets at mRNA level by real-time PCR and (4) at protein level by Western Blot analysis

different MM genetic subtypes (23). Global miRNA expression profiling data in MM further underlined the role of miRNA dysregulation in cancer and their potential use in therapy, and gives a solid rationale for the development of effective tools for selective delivery of miRNAs and anti-miRNAs to myeloma cells (9).

At this aim the Affymetrix platform represents a comprehensive tool for microarray technology. The experimental workflow of miRNA functional analysis for our approach is represented in Fig. 1, that schematically shows our strategy for the detection of dysregulated miRNAs in MM samples as compared to normal plasma cells. We then briefly describe how to replace in MM cells downregulated miRNAs or to use specific inhibitors for upregulated miRNAs, which potentially act as tumor suppressors or as oncomiRs, and how we validate a miRNA-specific targeting by luciferase reporter assay. Our flow includes the evaluation of miRNA replacement/inhibition on whole gene expression by microarray analysis, the study of specific targets at mRNA level by real-time PCR and at protein level by Western Blot analysis.

2 Materials

2.1 Screening by miRNA Profiling

1. CD138 MicroBeads human (Miltenyi Biotec, catalog no. 130-051301).
2. RNeasy® Mini kit (Qiagen, catalog no. 74104).
3. TRIzol reagent (US Patent No. 5,346,994). Ready-to-use reagent for the homogenization or cell lysis that maintains the integrity of the RNA.
4. Affymetrix® FlashTag™ Biotin HSR RNA Labeling Kit (catalog no. 901910, sufficient for 10 reactions).
5. Affymetrix® miRNA 2.0, 3.0, or 4.0 Array (catalog no. 901753, 902017, or 902411, respectively, contains 2 arrays), designed to interrogate a different miRBase Release up to all mature miRNA sequences in miRBase Release 20, as for 4.0 Array.

Equipment

1. NanoDrop 1000 Spectrophotometer (www.nanodrop.com).
2. Microarray Affymetrix® workstation (hybridization oven, fluidic station, Scanner; www.affymetrix.com).

2.2 Synthetic miRNA Overexpression or Inhibition

2.2.1 Transient Transfection

1. miRNA mimics (miRVana™ catalog no. 4464070, 5 nmol lyophilized pellet) are small, chemically modified double-stranded RNAs that mimic endogenous miRNAs and enable miRNA functional analysis by upregulation of miRNA activity.
2. miRNA inhibitors (miRVana™ catalog no. 4464066, 5 nmol lyophilized pellet) are small, chemically modified single-stranded RNA molecules designed to specifically bind to and inhibit endogenous miRNA molecules and enable miRNA functional analysis by downregulation of miRNA activity.
3. Negative control (Life Technologies).
4. Neon® Transfection System 100 μL kit (Invitrogen™), catalog no. MPK10025. The Neon® Transfection System 100 μL Kit includes 1 mL resuspension buffer R, 1 mL resuspension buffer T, 75 mL E electrolytic buffer, 25 reaction delivery tips, five electroporation tubes.
5. Exponentially growing MM cell lines.
6. Six-well plates.
7. RPMI-1640 medium.
8. Fetal bovine serum.

Equipment

1. Neon® Transfection System (Catalog Number MPK5000).
2. Neon® Pipette (Catalog Number MPP100).

3. Neon® Pipette Station (Catalog Number MPS100).
4. Equipment for cell culture: CO_2 Incubator, laminar flow cabin.

2.2.2 miRNA Quantitative Analysis

1. tRNA isolation: TRIzol® Reagent (Life Technologies), chloroform, isopropanol, 75 % ethanol, and nuclease-free water.
2. TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems).
3. TaqMan® MicroRNA Assays (Applied Biosystems).
4. NanoDrop 1000 Spectrophotometer.
5. Optical 96-well reaction plates with barcode (Applied Biosystems).
6. Viia7 Dx real-time PCR system (Applied Biosystems).

2.3 Analysis of Target Modulation

2.3.1 Gene Profiling

1. RNeasy® Mini kit (Qiagen, catalog no. 74104).
2. TRIZOL reagent (US Patent No. 5,346,994). Ready-to-use reagent for the homogenization or cell lysis that maintains the integrity of the RNA.
3. Affymetrix® GeneChip WT PLUS Reagent Kit (catalog no. 902309, sufficient for 10 reactions).
4. Affymetrix® GeneChip Hybridization, Wash, and Stain Kit (catalog no. 900720, sufficient for 30 reactions).
5. Affymetrix® GeneChip Hybridization Control Kit (catalog no. 900454, sufficient for 30 reactions) including Control Oligo B2, 3 nM (catalog no. 900301).
6. Affymetrix® GeneChip Human Transcriptome Array 2.0 (catalog no. 902233, contains 2 arrays), designed to empower next-generation expression profiling studies, this array provides the ability to go beyond gene-level expression profiling by providing the coverage and accuracy required to detect all known transcript isoforms produced by a gene. This high-resolution array design contains an unprecedented >6.0 million probes covering coding transcripts and noncoding transcripts. 70 % of the probes on this array cover exons for coding transcripts, and the remaining 30 % of probes on the array cover exon–exon splice junctions and noncoding transcripts. The unparalleled coverage of this array provides the deepest insight into all coding and noncoding transcripts available.

Equipment

1. NanoDrop 1000 Spectrophotometer (www.nanodrop.com).
2. Microarray Affymetrix® workstation (hybridization oven, fluidic station, Scanner, www.affymetrix.com).

Additionally support.

Library Files

GeneChip® Human Transcriptome Array 2.0 Analysis (zip, 303 MB).

GeneChip® Human Transcriptome Array 2.0 AGCC Library File Installer (zip, 109 KB).

2.3.2 Luciferase Reporter Assay for miRNA-Target Validation

1. Plasmid constructs (3′ UTR of the gene of interest is cloned in pEZX-MT01 vector, Genecopoeia).
   (a) 400 mL LB liquid medium: 10 g/L tryptone; 5 g/L yeast extract, 10 g NaCl.
   (b) Kanamycin for bacterial selection.
   (c) LB-Kanamycin bacterial plates: LB liquid media plus 8 g/L agar. Autoclave, cool down to about 50 °C in a water bath and add Kanamycin to have the final concentration of 25 μg/mL; mix well and distribute 15–20 mL of medium per 10 cm plate.
   (d) Maxi prep kit (PureLink® HiPure Plasmid Maxiprep Kit, Invitrogen, Life Technologies) for isolation of high purity plasmid from bacteria.
2. MM cell transfection and 3′ UTR luciferase reporter assay.
   (a) Exponentially growing MM cells.
   (b) miRVANA miRNA mimics (Applied Biosystems).
   (c) Six-well plates.
   (d) RPMI-1640 medium.
   (e) Fetal bovine serum (FBS).
   (f) Dual-Glo Luciferase Assay kit (Promega).

Equipment

1. Neon electroporation system (Life technologies).
2. Plate reader for luminesce detection.

3 Methods

3.1 Screening by miRNA Profiling

Plasma cells from human peripheral blood mononuclear cells (PBMCs) bone marrow are isolated at >90 %, purity as determined by flow cytometry, by the use of CD138 magnetic beads sorting according to the manufacturer's instructions (www.miltenyibiotec.com); subsequently total RNA (tRNA) including small RNA fractions is extracted by a modified Qiagen protocol (www.qiagen.com). Briefly, 5×10^4 cells are lysed by 250 μL of TRIzol® solution, then the aqueous phase is loaded on the Qiagen column. After two washings by high speed centrifugation at r.t. the

tRNA is eluted from the column by loading 15 μL of RNase-free water directly on the top of the filter in the column followed by a further 5 min incubation at r.t. The RNA solution is collected and immediately stored at −80 °C.

miRNA expression profiling is carried out according to the Affymetrix (www.affymetrix.com) recommended protocol. Briefly, after quantification of tRNA by NanoDrop spectrophotometry, 300 ng is processed using the FlashTag labeling kit according to the manufacturer's instructions (www.affymetrix.com), which is based on a tailing reaction followed by ligation of the biotinylated signal molecule to the target RNA sample. The 45-min assay, from the extracted RNA sample to the labeled target, does not involve complex reactions such as amplification or purification steps that can reduce the yield or can bias the results by introducing false positives or false negatives. The labeled RNA is then hybridized to Affymetrix GeneChip microRNA arrays which is scanned by the GeneChip Scanner 3000 7G.

Expression values for miRNAs are extracted from CEL files using Affymetrix miRNA QC tool software (RMA normalized and log2-transformed), for miRNA 2.0 and 3.0 Array, or Affymetrix® Expression Console™ Software (version 1.3.1 and higher) for 4.0 Array, including array quality control. 4.0 Array provides the opportunity of data filtering by the use of analysis options by the following 4 filtering options:

1. Analysis of all organisms.
2. Mouse only.
3. Human only.
4. Rat only.

By selecting either the human, mouse, or rat only analysis option, only the probe sets for the selected organism(s) are reported in the analysis output. In the previously miRNA Array Release (i.e., 2.0 and 3.0) analyzed by QC Tool, the analysis for single organism was performed by different tools. After generating the signal summarization, differential expression analysis can be performed and results can be visualized by Affymetrix® Transcriptome Analysis Console (TAC) Software. Both Expression Console Software and TAC Software can be free downloaded from www.affymetrix.com.

The analyses are performed on $\log_2$ transformed data. First, hierarchical agglomerative clustering of the samples is performed by Pearson's correlation coefficient and average linkage as distance and linkage metrics, respectively, on those probes whose average change in expression levels varied at least twofold from the mean across the dataset; *P* value threshold for sample enrichment is default set at 0.5. Supervised analyses are carried out using DChip software (www.hsph.harvard.edu/cli/complab/dchip). The threshold for significance is determined by fold change analysis applied on the expression values of the miRNAs.

3.2 Synthetic miRNA Overexpression or Inhibition

3.2.1 Transient Transfection

The following procedure refers to the transfection of RPMI-8226 MM cells.

The day before the transfection, MM cells are seeded at 5.0×10^5 cells/mL in RPMI-1640 containing 10 % heath inactivated FBS and 1 % penicillin-streptomycin.

MM cells are transfect by the Neon transfection system (Life Technologies): briefly, 1.0×10^6 cells are used for each transfection point, washed in PBS and resuspended in 100 μL of buffer R. Add 2 μL of 100 μM miRNA mimics or inhibitors or negative control (NC). The mix is resuspend and electroporated by the use of the Neon pipette and 100 μL tips at the following electroporation conditions: 1,050 V, 30 ms, 1 pulse. Transfected cells are seeded into a six-well plate containing 2 mL of pre-warmed growth medium without antibiotics. The plate is incubated into a 37 °C/ 5 % CO_2 incubator and cells collected 24 and 48 h after transfection and analyzed for miRNA and target expression.

3.2.2 miRNA Quantitative Analysis

tRNA Isolation

0.5 mL TRIzol® Reagent is added to 1×10^6 harvested cells; cell sample are lysed by pipetting the cells up and down several times. The homogenized sample is incubated for 5 min at room temperature then 0.2 mL of chloroform are added. After shaking vigorously by hand for 15 s, the tube is incubated for 2 min at room temperature. The sample is then centrifuged at 12,000 × *g* for 15 min at 4 °C. The aqueous phase is now transferred into a fresh Eppendorf tube avoiding to draw any of the interphase or organic layer into the pipette. After adding of 0.5 mL of 100 % isopropanol to the aqueous phase, mix well the sample and incubate for 10 min at room temperature. After centrifugation at 12,000 × *g* for 10 min at 4 °C the supernatant is discarded from the tube, and the RNA pellet washed with 1 mL of 75 % ethanol. After centrifugation at 7,500 × *g* for 5 min at 4 °C, the supernatant is discarded and the RNA pellet air-dried for 5–10 min. The RNA pellet is then resuspended in nuclease-free water by pipetting up and down several times. The concentration is measured by NanoDrop spectrophotometer.

cDNA Generation and qRT-PCR Performance and Analysis

To prepare the RT master mix using the TaqMan® MicroRNA Reverse Transcription Kit (Applied Biosystems) components, the kit components are allowed to thaw on ice. Before use, the RT primer tubes are vortexed. The RT master mix is prepared in a polypropylene tube on ice, gently mixed and then centrifuged.

Note: RT master mix for each sample consists of:

- 100 mM dNTPs (with dTTP): 0.15 μL.
- MultiScribe™ Reverse Transcriptase, 50 U/μL: 1.0 μL.
- 10× Reverse Transcription Buffer: 1.50 μL.
- RNase Inhibitor, 20 U/μL: 0.19 μL.

– Nuclease-free water: 4.16 μL.
– Each 15-μL RT reaction consists of 7 μL master mix, 3 μL of 5× RT primer, and 5 μL RNA sample diluted at 10 ng/L.

qRT-PCR

The stock cDNA is 15 times diluted to the working concentration using nuclease-free water.

Then the following components are combined into one reaction: 5 μL of diluted cDNA, 1 μL of 20× TaqMan probe, 4 μL of nuclease free water, and 10 μL TaqMan Universal Master Mix without UNG AmpErase. The reaction mixture is transferred to each well of an optical 96-well PCR microplate which is then sealed with an ultra clear sealing film. The plate is briefly centrifuged and the RT-PCR run is performed on a real-time PCR system, such as the Viia7 Dx instrument (Applied Biosystems) with the following conditions: 95 °C, 10 min (enzyme activation); 95 °C, 15 s (denaturation) and 60 °C, 1 min (annealing/ extension), for 40 cycles. The results, analyzed by the comparative Ct method or 2-ΔΔCt method (24) for miRNAs relative expression (corrected to a reference miRNA such as RNU44 or RNU6), can be reported as the relative fold change versus the control sample.

3.3 Analysis of Target Modulation

To identify mRNA targets which can contribute to explain the role of miRNAs in MM, Affymetrix gene expression profiling is performed. Target mRNAs are then evaluated as possible miRNA targets by luciferase reporter assay by the use of the 3′ UTR mutated and wild type sequence of the miRNA-target gene cloned in pEZX-MT01 vector (Genecopoeia).

3.3.1 Gene Profiling

Total RNA (tRNA) including small RNA fractions, is extracted from transfected MM cells using a modified protocol from Qiagen as above described. tRNA samples consist of a combination of ribosomal RNA (rRNA), messenger RNA (mRNA), transfer RNA (tRNA), and other small RNA species with the rRNA fraction constituting the vast majority (non-rRNA depleted). Gene expression profiling (GEP) is then carried out according to the Affymetrix recommended protocol. Briefly, after quantification of tRNA by NanoDrop Spectrophotometry, 100 ng is processed using the GeneChip WT PLUS Reagent Kit according to manufacturer's instructions (www.affymetrix.com), which uses a reverse transcription priming method that primes the entire length of each RNA transcript, including both poly-A and non-poly-A mRNA to provide complete transcriptome. By the use of 100 ng of tRNA without rRNA depletion 10 μg of cRNA is obtained to be carried into the second cycle. The cRNA concentration is measured by UV spectrophotometry (NanoDrop) following beads purification. Total cRNA yield is calculated by multiplying the measured concentration by the

estimated elution volume (10.5 μL). Generally total cRNA yield generates sufficient cRNA for the following reaction. Ten micrograms of cRNA is in fact used to generate second cycle, first strand cDNA. In cases where cRNA concentration is low, the volume of cRNA could be reduced by SpeedVac to a maximum of 6.5 μL. The second strand (ss) cDNA yield is, as previously, measured by UV Spectrophotometry (NanoDrop). The total yield of single stranded cDNA was calculated by multiplying the measured concentration by the estimated elution volume (28 μL). The 100 ng of RNA input starting amounts (without rRNA reduction) generated sufficient single stranded cDNA in the second cycle to generate a hybridization cocktail containing 5.5 μg of labeled target. The yield of single stranded cDNA is fairly consistent across all samples as is expected since we use a constant mass (10 μg) of cRNA as input into this step. Target preparation was carried out exactly as described in the Whole Transcript (WT) Sense Target Labeling Assay Manual. 5.5 μg of single-stranded cDNA is then fragmented, labeled, and hybridized. The kit generates amplified and biotinylated sense-stranded DNA targets, coupled with GeneChip® WT Terminal Labeling and Controls Kit (Affymetrix) is adequate for preparing hybridization-ready targets. Hybridization cocktail is prepared according to manual instruction (www.affymetrix.com) using 5.2 μg of labeled RNA. A total of 200 μL were loaded on each Affymetrix Human Transcriptome Array (HTA) 2.0. Hybridized arrays were labeled and washed using the Hybridization, Wash and Stain Kit on a GeneChip® Fluidics Station 450 with the appropriate fluidics scripts (see manual for details; Expression Wash, Stain and Scan User Manual for Cartridge Arrays). Arrays are subsequently scanned on a GCS3000 7G Scanner. CEL file are generated after quality control of array scan.

Data Processing and Analysis

All resulting CEL files for each array type are processed as a single group via the Affymetrix Power Tools (APT) package using sketch normalization and the RMA algorithm to summarize probeset signal. QC metrics from the APT report file shows, the mean perfect match (PM) and mean background intensity for all of the samples included in the analysis.

Expression values for gene are extracted from CEL files using Affymetrix® Transcriptome Analysis Console (TAC) software. Different GeneChip® Array have been developed in the last decade by the company. For the older GeneChip Whole Transcriptome (WT) Arrays, $\log_2$-transformed expression values are extracted from CEL files and normalized using Transcript Cluster Annotations, and robust multi-array average (RMA) procedure in Expression Console (EC) software (Affymetrix Inc.). The analyses are then performed on $\log_2$ transformed data generated from EC. After hierarchical *clustering* of the samples, enabled to group either genes, specimens or both with similar expression patterns,

supervised analyses is carried out using DChip software (www.hsph.harvard.edu/cli/complab/dchip). The threshold for significance is determined by fold change analysis applied on the expression values of the transcript.

These microarray experiment procedures offer the unique possibility to select a subset of differentially expressed genes between two classes of samples (i.e., transfected versus control) and thus providing a global picture of the modification induced by miRNA mimics or inhibitors treatment on the expression pattern across the entire genome of the myeloma cell.

New miRNA targets could be identified by GEP analysis and then will validated by in vitro and in vivo experiments to delineate the mechanism of action of miRNA mimics or inhibitors in MM.

In addition it could be suggested the development of novel therapies by targeting molecules involved in cell survival, migration, as well as drug resistance pathways or for the use of miRNA mimics or inhibitors in combination therapy regimen.

Then pathway analysis could be applied by the use of the fold change genes list, for example, loading into Ingenuity Pathway Analysis (IPA®) software. This tool will be of support to reveal biological pathways modulated by miRNAs overexpression or silencing. For example after miR-221/222 knockdown, we identified by IPA modulation of canonic pathways involved in cell proliferation signals and activation of immune response (20).

The information derived from molecular profiling technologies will translate into the definition of the whole transcriptome perturbation induced by miRNA replacement or inhibition and pathway analysis will define the scenario by integrating the experimental data with previous stored knowledge.

This approach will offer the opportunity for the identification of therapeutical targets as well as pathways involved in crucial cellular function as angiogenesis or bone marrow drive survival or drug resistance.

All these finding will be finally validated by protein analysis, which at present mostly rely on flow cytometry, immunofluorescence or western blotting analysis.

3.3.2 Luciferase Reporter Assay for miRNA-Target Validation

The following procedure is based on the use of 3′ UTR sequence of the selected mRNA cloned in pEZX-MT01 vector, specifically designed by and purchased from Genecopoeia. The 3′ UTR of interest—or a deletion mutant lacking the predicted miRNA target sequence(s)—is specifically cloned in such vector containing both Firefly and Renilla luciferase reporters.

Amplification of Plasmid DNA

50 ng of plasmid DNA containing the wild type 3′ UTR sequence of the gene of interest cloned in pEZX-MT01 (Genecopoeia)—or a deletion mutant lacking the miRNA-target sequence—is added to 50 μL of competent cells (DH5α, Life Technologies). After 20 min

of incubation on ice, the cells underwent heat shock at 42 °C for 40 s and immediately placed on ice for 3 min. 250 μL of S.O.C. medium are added to the cells and incubated at 37 °C for an hour. 100 μL of the bacterial culture are then spread on a pre-warmed (37 °C) culture plate containing kanamycin and incubated overnight at 37 °C.

Culture bacterial colonies: one individual colony is touched with a sterile pipette tip which is then released in a sterile culture tube containing 3 mL of liquid LB medium + kanamycin. Place the tubes with the bacterial culture in a 37 °C incubator with agitation. After 6 h, transfer 200 μL into 400 mL of LB broth containing kanamycin and leave overnight. The isolation of plasmid DNA is performed by PureLink® HiPure Plasmid Maxiprep Kit (Life Technologies) according to the manufacturer's protocol.

MM Cells Transfection

The following procedure refers to the transfection of RPMI-8226 MM cells.

The day before the transfection, MM cells are seeded at 5.0×10^5 cells/mL in RPMI-1640 containing 10 % FBS and 1 % penicillin-streptomycin. The transfection of MM cells is obtained by the Neon transfection system (Life Technologies): briefly, use 1.0×10^6 cells, washed in PBS and resuspended in 100 μL of buffer R. 2 μL of 100 μM miRNA mimics and 2.5 μL of the wild type 3′ UTR plasmid concentrated at 1 μg/μL are then added. The Neon pipette and 100 μL tips are used for electroporation at the following conditions: 1,050 V, 30 ms, 1 pulse. The transfected cells are seeded into a six-well plate containing 2 mL of pre-warmed growth medium without antibiotics. The same procedures is performed using a deletion mutant lacking the miRNA-target sequence. The plate is incubated in 37 °C/5 % CO_2 incubator for 24 h.

Note: prepare the following controls:

(a) Mix 2.5 μg of empty vector (pEZX-MT01) with either 100 nM miRNA mimic or negative control (NC).

(b) 2.5 μg of empty vector (pEZX-MT01) alone.

(c) 2.5 μg of the 3′ UTR-pEZX-MT01 plasmid alone.

(d) 2.5 μg of the mutated 3′ UTR-pEZX-MT01 plasmid alone.

3′ UTR Luciferase Reporter Assay

24 h after the transfection, MM cells are collected and lysed with 200 μL of passive lysis buffer contained into the Dual Luciferase Reporter Assay System (Promega). After incubation at r.t. for 10 min the cells are centrifuged at 2,320 rcf for 5 min and the supernatants collected. The Luciferase Assay Reagent II and the Stop & Glo buffer (Dual-Glo Luciferase Assay Kit) are thawed at r.t. and the content of one bottle of Luciferase Assay Reagent II is

transfered to the bottle of Luciferase Assay Substrate to create the *Dual-Glo Luciferase Reagent*. The solution is then mixed thoroughly by inversion.

Measuring Firefly luciferase: 30 μL of cell lysates are incubated with 100 μL of the luciferase substrate for 10 min into a white 96-well plate appropriate for luminescence and the Firefly luminescence is measured by reader set up for 96-well plates (GloMax, Promega).

Measuring Renilla luciferase activity: 100 μL of Dual-Glo Stop & Glo Reagent are added to each well and, after mix, incubated for at least 10 min at room temperature and the Renilla luminescence measured in a plate reader.

The ratio of luminescence from the experimental reporter (Firefly) to the control reporter (Renilla) is calculated for each well, and the average of at least three replicates is determined. The results are normalized for the specific miRNA mimic versus the control treatment.

The final aim of the luciferase assay is to validate the mRNA as a specific target of the miRNA under investigation. It is important to make clear that the UTR microRNA target can be predicted by several software like TargetScan. Such prediction needs molecular validation by luciferase assays in order to formally prove the real miRNA targeting, because the biological activity is not regulated by the identified nucleotide sequence alone. It has also to be considered that miRNA luciferase assay measures the mRNA stability and are different from the traditional luciferase assays aimed to measure the transcriptional activity of a DNA sequence.

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