

Biological and clinical relevance of miRNA expression signatures in primary plasma cell leukemia

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Supplementary Methods

Patient samples

PPCL patients included in this study belonged to a national, multicenter pilot clinical trial including 23 patients (12 males, 11 females; median age at diagnosis: 60 ys, range: 44-80 ys). This study was aimed to evaluate safety and anti-tumor activity of lenalidomide and dexamethasone combination in previously untreated pPCL (*RV-PCL-PI-350*, registered at www.clinicaltrials.gov as NCT01553357) (1); in particular, enrolled patients received lenalidomide at a dose of 25 mg/d for 21 days and oral dexamethasone at 40 mg on days 1, 8, 15, and 22 for each 28-day cycle. After 4 cycles, responding patients not eligible for autologous stem cell transplantation (ASCT) continued until 8 cycles of full-dose LD, if tolerated, followed by a maintenance dose of single agent lenalidomide equal to 10 mg/d on days 1-21 of each 28-day cycle. Patients responding after 4 cycles and eligible for ASCT proceeded according to single Centre transplant policy. Patients not responding after 4 cycles or progressing during this treatment were considered off-study. The primary endpoint was the response rate according to International Uniform Criteria (2, 3); secondary endpoints were safety, progression free survival (PFS) and overall survival (OS): for PFS, subjects were considered treatment failures at the time of progression or death from any cause; OS was defined as time from the date of diagnosis to the date of death or last contact. PFS and OS were measured over a median follow-up of 23 months. The description of safety, efficacy and side effects of

the treatment, together with a comprehensive description of the clinical trial, is beyond the scope of this work and will be treated elsewhere (Musto *et al.*, manuscript in preparation).

MiRNA expression profiling

The total RNA extraction and quality assessment were performed as previously described (4). Samples were profiled using the one-color technique in accordance with the manufacturer's instructions on the Agilent Human miRNA Microarray V2 (Agilent Technologies, Santa Clara, CA), which consist of 60-mer DNA probes synthesized *in situ* that represent 723 human and 76 human viral miRNAs from the Sanger miRBase (v10.1) (5). Expression values were extracted according to procedures described in Agilent Feature Extraction Software version 10.1 manual. Non-human probes, miRNAs flagged as "absent" (i.e. expressed below background levels) throughout the whole dataset and miRNAs expired according to Sanger miRBase Release 15 were discarded, and quantile normalization was applied on raw data using the *aroma.light* package for Bioconductor. The data were then converted to obtain positive values throughout the dataset, at a minimum value of 1, and \log_2 transformed. The raw and normalized miRNA data are available through GEO accession number GSE37053. Hierarchical agglomerative clustering of the samples was performed using 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 two-fold from the mean across the dataset; *P* value threshold for sample enrichment was set at 0.0005. Supervised analyses were carried out using *samr* package for Bioconductor. The threshold for significance (at a *q* value of 0) was determined by tuning the Δ parameter on the false discovery rate

and controlling the q value of the selected probe list. Enrichment analysis of miRNA categories was performed using TAM (Tool for Annotations of MicroRNAs, V2; <http://cmbi.bjmu.edu.cn/tam>).

The differentially expressed miRNAs were validated using an independent MM cohort (GEO series GSE17306) (6) generated on Agilent Human microRNA Microarray V1, including fifty-two MM patients and two healthy donors. To this aim, a comprehensive dataset was generated including PC specimens from our pPCL cohort and the fifty-two MM samples of the series GSE17306; quantile normalization was applied on the expression values of the 276 miRNAs represented on both platforms and expressed in at least one sample of our series. All samples passed the relative-log expression (RLE) evaluation (namely, the 25th and 75th percentile values required to fall between -1 and 1, respectively), made to prevent biases possibly due to inter-cohort analysis. Supervised analysis was carried out using the same approach (SAM, q -value=0) applied in the proprietary series.

Gene expression profiling (GEP) and data analysis

The transcriptional profiles of the patients were generated using GeneChip® Gene 1.0 ST Array (Affymetrix Inc., Santa Clara, CA). Preparation of DNA single-stranded sense target, hybridization and scanning of the arrays (7G Scanner, Affymetrix Inc.) were performed according to the manufacturer's protocols. Log₂-transformed expression values were extracted from CEL files and normalized using Transcript Cluster Annotations, Release 31 and robust multi-array average (RMA) procedure in Expression Console software (Affymetrix Inc.). The expression values of transcript cluster ID specific for *loci* representing naturally occurring read-through transcriptions or mapped to more than one chromosomal location were summarized

as the median value for each sample.

References

1. Musto P, D'Auria F, Petrucci MT, Levi A, Cascavilla N, Falcone A, et al. Final Results of a Phase II Study Evaluating Lenalidomide in Combination with Low Dose Dexamethasone As First Line Therapy for Primary Plasma Cell Leukemia. *Blood* 2011;118:2925.
2. van de Donk NW, Lokhorst HM, Anderson KC, Richardson PG. How I treat plasma cell leukemia. *Blood* 2012;120:2376-89.
3. Bird JM, Owen RG, D'Sa S, Snowden JA, Pratt G, Ashcroft J, et al. Guidelines for the diagnosis and management of multiple myeloma 2011. *Br J Haematol* 2011;154:32-75.
4. Lionetti M, Agnelli L, Mosca L, Fabris S, Andronache A, Todoerti K, et al. Integrative high-resolution microarray analysis of human myeloma cell lines reveals deregulated miRNA expression associated with allelic imbalances and gene expression profiles. *Genes Chromosomes Cancer* 2009;48:521-31.
5. Lionetti M, Biasiolo M, Agnelli L, Todoerti K, Mosca L, Fabris S, et al. Identification of microRNA expression patterns and definition of a microRNA/mRNA regulatory network in distinct molecular groups of multiple myeloma. *Blood* 2009;114:e20-e26.
6. Zhou Y, Chen L, Barlogie B, Stephens O, Wu X, Williams DR, et al. High-risk myeloma is associated with global elevation of miRNAs and overexpression of EIF2C2/AGO2. *Proc Natl Acad Sci U S A* 2010;107:7904-9.