Circulating microRNA expressions can predict the outcome of lenalidomide plus low-dose dexamethasone treatment in patients with refractory/relapsed multiple myeloma

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(Supplementary Data)

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Supplementary Methods

Patients and Treatment Procedures

A total of 100 RRMM patients who started salvage treatment with Len-dex between Jan 2014 and Dec 2015 were included in this study. As previously described,[1] the therapy regimen consisted of lenalidomide 25mg orally once daily on days 1-21 of each 28-day cycle plus low-dose dexamethasone (40 mg per day) weekly, and dose modification was performed according to the recommendations.[2] Low-dose aspirin was used in all patients for thrombosis prophylaxis. Treatment responses were assessed according to the criteria from the International Myeloma Working Group (IMWG) and European Group for Blood and Marrow Transplantation (EBMT)[3,4] and classified as complete response (CR), VGPR, PR, minimal response (MR), stable disease (SD) and progressive disease (PD).

All clinical data, including treatment response, patient characteristics, and survival were prospectively collected. To identify biomarkers related to the treatment response, blood samples were collected at baseline prior to the Len-dex treatment. Cytogenetic and international staging system (ISS) findings were taken from the data established at the time of diagnosis. Cytogenetic risks were assessed in 70 patients (70%). Patients with a deletion of chromosome 13 or hypodiploidy determined by conventional cytogenetic study, or t(4;14), t(14;16), and 17p- established by fluorescent *in situ* hybridization (FISH) of bone marrow

(BM) samples at diagnosis were stratified as high risk.[3] Written informed consent was obtained from each patient before participation in this study. This study was approved by the Institutional Review Board of each participating institution and conducted in accordance with the Declaration of Helsinki.

TaqMan Low Density miRNA Array experiments

This study was conducted according to the REMARK guideline.[5] In the discovery phase, 381 miRNAs were examined from 38 RRMM serum samples (19 Len-dex good responders and 19 poor responders) using TaqMan miRNA ABC Purification Kit (Human Panel A; Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instruction. CR and VGPR were defined as good responders and PR, MR, SD and PD as poor responders. The megaplex reverse transcription reactions and pre-amplification reactions were performed to increase the quantity of cDNA for miRNA expression analysis using Megaplex PreAmp Primers Human Pool A and TaqMan PreAmp Master Mix (Thermo Fisher Scientific). TaqMan Low Density miRNA Array (TLDA) panel A v2.0 (Thermo Fisher Scientific) was performed with the ViiA7 real-time PCR system (Thermo Fisher Scientific) to evaluate the expression of miRNAs. Raw data were processed using ExpressionSuite Software v1.0.4 (Thermo Fisher Scientific) to determine Ct values for each miRNA.

Data analysis for TLDA

The TLDA data were normalized against the miR-320, which was used as endogenous control since this miRNA had the smallest standard deviation among samples and showed no statistical difference in its expression levels between good and poor responders. MiRNAs with a Ct value > 35 were considered as undetectable. We excluded miRNAs that were unreliably quantified or expressed <20% in the discovery set from further analysis. The mean

miRNA expression level of good responders was used as calibrator. The expression level of each miRNA target was defined as $2^{-\Delta\Delta Ct}$, where ΔCt is the difference in threshold cycles for the sample in question, normalized against the endogenous control gene (miR-320) and expression level relative to the value obtained by the calibrator (individual/calibrator) as described elsewhere.[6] MiRNAs with fold changes (poor responder/good responder) ≥ ±2 were considered to be different between two groups.

MiRNA specific quantitative reverse transcription PCR

To validate the miRNAs significantly associated with the response to Len-dex treatment, quantitative reverse transcription PCR (qRT-PCR) targeting each miRNA was performed using the TaqMan microRNA Assay (miR-26a-5p-000405, miR-29c-3p-000587, miR-30b-5p-000602, miR-30c-5p-000419, miR-193a-5p-002281 andmiR-331-3p-000545) and the ViiA7 real-time PCR system according to the manufacturer's protocol. The TaqMan microRNA Assay of miR-320 (miR-320-002277) was used as endogenous control. In brief, 5ul of RNA was converted to first-strand cDNA with miRNA-specific primers using TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher Scientific), followed by real-time PCR with TaqMan probes. The expression level of each miRNA target was defined as $2^{-\Delta\Delta Ct}$ as described above. qRT-PCR reactions for all the samples were carried out in triplicate. Student's t-test was used to verify the statistical significance.

Treatment response modeling and cross validation

The prediction model for Len-dex treatment response was constructed by Support Vector Machine (SVM) with radial basis function (RBF) kernel by LIBSVM[7] using the set of features which consist of 18 clinical variables and expression levels of six miRNAs. Multiple models were generated along with changing input features. Some clinical variables were

simplified into categorical values and the combination of input features for a SVM could include numerical and/or categorical values derived from one clinical variable. We measured the accuracy of each model by the 20-fold cross validation (CV)[8] which LIBSVM provides. In addition to the model, the other two models were also selected: one using only clinical variables, and the other using only miRNA markers. After the selection, their performances were evaluated by leave-one-out CV.

Statistical analysis

Pearson's chi-squared test or Fisher's exact test were used for categorical variables. Student's t-test was used for continuous variables. The relationships between expression levels of the miRNA markers and treatment responses were evaluated using ANOVA with post-hoc Tukey's honest significance test. The receiver operating characteristic (ROC) curve and area under curve (AUC) were used to assess the predictive values of miRNA markers for Len-dex treatment. For survival analysis, time-to-event variables were defined as duration from the initiation date of Len-dex treatment to the date of death from any cause (overall survival [OS]) or to the date of disease progression (time to progression [TTP]). Patient survival was calculated by Kaplan-Meier method and differences in survival rates between groups were tested with the log-rank test. The Cox regression was used to estimate hazard ratios. Statistical analyses were performed using SPSS (version 21, Chicago, IL). GraphPad Prism software (version 6, La Jolla, CA) was used to create graphs. All *P* values less than 0.05 were considered significant in all statistical analyses.

Figure S1. Heatmap of the six differentially expressed miRNAs. 19 good responders and 19 poor responders of Len-dex treatment with RRMM showed a clear discrepancy. Green and red represent the downregulated and up-regulated miRNAs, respectively.

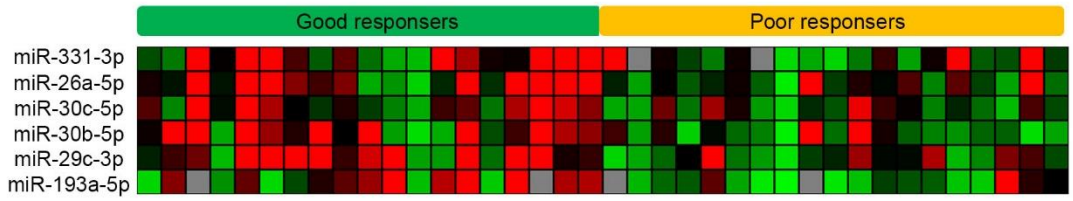

Figure S2. Expression levels of six miRNAs measured by qRT-PCR. (A**)** The relative miRNA expression level of each miRNA was normalized to miR-320. Y-axis represents relative miRNA expression level (fold change) based on the median of good responders as calibrator. (B) The expression levels of the six miRNAs by Len-dex treatment response. The expression levels of the miRNAs were inversely related to the level of response. CR, complete response; VGPR, very good partial response; PR, partial response; MR, minimal response; SD, stable disease; PD, progressive disease

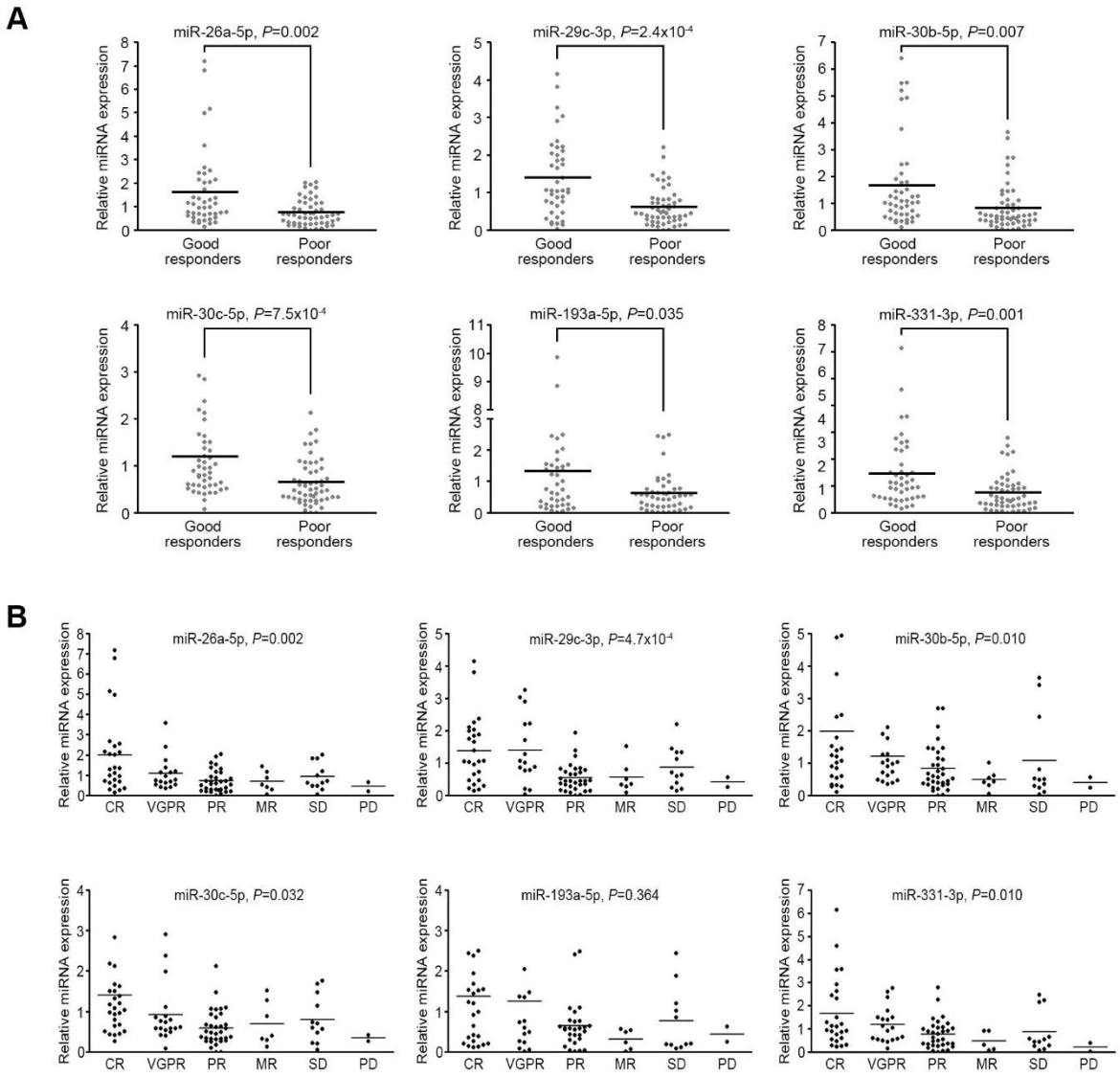

Table S1. Clinicopathologic characteristics of the study subjects

Clinical characteristics	Discovery set (N=38)			Validation set (N=62)		
	Good responder (N=19)	Poor responder (N=19)	*P*	Good responder (N=26)	Poor responder (N=36)	*P*
Age, years; median (range)	59 (43-84)	62 (23-73)	0.469	64 (42-77)	64.5 (29-73)	0.760
Sex						
F	11 (57.9%)	10 (52.6%)	1.000	12 (46.2%)	14 (38.9%)	
M	8 (42.1%)	9 (47.4%)		14 (53.8%)	22 (61.1%)	
Serum M-protein						
IgG	4 (21.1%)	8 (42.1%)	0.230	3 (11.5%)	10 (27.8%)	0.108
IgA	11 (57.9%)	6 (31.6%)		13 (50.0%)	20 (55.6%)	
Light chain disease	3 (15.8%)	5 (26.3%)		8 (30.8%)	6 (16.7%)	
Others	1 (5.3%)	0 (0.0%)		2 (7.7%)	0 (0.0%)	
Durie-Salmon stage						
I	1 (5.3%)	0 (0.0%)	0.220	1 (3.8%)	4 (11.1%)	0.593
II	0 (0.0%)	2 (10.5%)		0 (0.0%)	1 (2.8%)	
III	18 (94.7%)	17 (89.5%)		24 (92.3%)	30 (83.3%)	
NA	0 (0.0%)	0 (0.0%)		1 (3.8%)	1 (2.8%)	
ISS stage						
I	6 (31.6%)	5 (26.3%)	0.588	3 (11.5%)	7 (19.4%)	0.707
II	7 (36.8%)	4 (21.1%)		7 (26.9%)	11 (30.6%)	
III	4 (21.1%)	7 (36.8%)		10 (38.5%)	13 (36.1%)	
NA	2 (10.5%)	3 (15.8%)		6 (23.1%)	5 (13.9%)	
Cytogenetics*						
Standard	8 (42.1%)	9 (47.4%)	0.515	17 (65.4%)	21 (58.3%)	0.109
High	2 (10.5%)	4 (21.1%)		1 (3.8%)	8 (22.2%)	
NA	9 (47.4%)	6 (31.6%)		8 (30.8%)	7 (19.4%)	
Myeloma bone disease on plain radiographs						
Yes	16 (84.2%)	16 (84.2%)	1.000	23 (88.5%)	25 (69.4%)	0.144
No	3 (15.8%)	3 (15.8%)		3 (11.5%)	11 (30.6%)	
Time since diagnosis, months; median (range)	52.2 (16.7-216.6)	40.7 (9.4-103.0)	0.230	32.6 (4.4-167.2)	43.3 (4.9-293.0)	0.594
Previous number of therapies†, median (range)	3 (1-6)	2 (1-6)	0.061	2 (1-7)	2 (1-4)	0.090
Previous ASCT						
Yes	10 (52.6%)	16 (84.2%)	0.080	10 (38.5%)	16 (44.4%)	0.833
No	9 (47.4%)	3 (15.8%)		16 (61.5%)	20 (55.6%)	
Previous therapy before Len-dex						
Bortezomib-based regimens	10 (52.6%)	5 (26.3%)	0.184	17 (65.4%)	16 (44.4%)	0.170
Both Bortezomib- and thalidomide-based regimens	9 (47.4%)	14 (73.7%)		9 (34.6%)	20 (55.6%)	
Laboratory data (mean ± SEM)						
Hb, g/dL	11.5 ± 1.5	11.0 ± 1.8	0.283	11.6 ± 1.7	10.8 ± 2.0	0.093
WBC, $x10^6$/L	5549.5 ± 2255.8	4079.5 ± 884.1	**0.014**	5475.4 ± 1750.0	5388.3 ± 2240.6	0.870
Platelet, $x10^9$/L	178.6 ± 62.4	130.6 ± 57.3	**0.018**	194.0 ± 84.0	161.0 ± 75.7	0.114
Total protein, g/dL	7.8 ± 1.5	7.7 ± 1.5	0.734	7.6 ± 1.1	7.7 ± 1.2	0.742
Albumin, g/dL	3.9 ± 0.4	3.8 ± 0.6	0.479	4.0 ± 0.4	3.7 ± 0.5	**0.050**
Ca, mg/dL	9.1 ± 0.5	9.0 ± 0.4	0.689	9.6 ± 1.0	9.0 ± 1.1	0.056
Cr, mg/dL	1.0 ± 0.2	1.6 ± 1.8	0.142	1.4 ± 1.4	1.5 ± 1.7	0.752
LDH, U/L	409.2 ± 176.3	451.3 ± 176.6	0.467	418.9 ± 105.7	414.9 ± 140.2	0.902

ASCT, autologous stem cell transplantation; Ca, calcium; F, female; Hb, hemoglobin; Len-dex, lenalidomide and low-dose dexamethasone; LDH, lactate dehydrogenase; M, male; NA, not available.

*High-risk cytogenetics is defined as hypodiploidy or deletion of chr13 on conventional cytogenetics or presence of t(4;14), t(14;16), -17p on fluorescent *in situ* hybridization and/or conventional cytogenetics. All other cytogenetic abnormalities were considered standard risk.

†Induction + ASCT was considered as one therapeutic line.

Table S2. List of the 34 differentially expressed miRNAs between good responders and poor responders in discovery cohort

Up-regulated miRNAs			Down-regulated miRNAs		
miRNA	*P*-value	Fold	miRNA	*P*-value	Fold
hsa-miR-20b-001014	0.358	2.237	hsa-miR-16-000391	0.611	$5.9x10^{-5}$
hsa-miR-363-001271	0.879	7.831	hsa-miR-15b-000390	0.170	$6.6x10^{-5}$
hsa-miR-146b-001097	0.889	31.737	hsa-miR-19b-000396	0.395	0.001
hsa-miR-21-000397	0.825	309.949	hsa-miR-155-002623	0.220	0.037
			hsa-miR-19a-000395	0.073	0.070
			hsa-miR-122-002245	0.233	0.101
			hsa-let-7g-002282	0.327	0.104
			hsa-miR-345-002186	0.354	0.105
			hsa-miR-520e-001119	0.741	0.185
			hsa-miR-574-3p-002349	0.327	0.208
			hsa-miR-186-002285	0.081	0.229
			hsa-miR-708-002341	0.516	0.233
			hsa-miR-20a-000580	0.241	0.245
			hsa-miR-376a-000565	0.613	0.252
			hsa-miR-192-000491	0.808	0.274
			hsa-miR-486-3p-002093	0.746	0.294
			hsa-miR-324-3p-002161	0.902	0.316
			hsa-miR-215-000518	0.291	0.321
			hsa-miR-885-5p-002296	0.333	0.358
			hsa-miR-518b-001156	0.503	0.359
			hsa-miR-26a-000405	**0.028**	0.423
			hsa-miR-30b-000602	**0.018**	0.423
			hsa-miR-193a-5p-002281	**0.025**	0.455
			hsa-miR-509-5p-002235	0.119	0.472
			hsa-miR-17-002308	0.579	0.475
			hsa-miR-27a-000408	0.688	0.478
			hsa-miR-145-002278	0.760	0.493
			hsa-miR-30c-000419	**0.023**	0.494
			hsa-miR-29c-000587	**0.013**	0.494
			hsa-miR-331-000545	**0.046**	0.498

Bold: P<0.05

Table S3. Univariate analyses of predictive factors for response (≥ VGPR), time to progression, and overall survival

variables[†]	Response (≥ VGPR)		TTP		OS	
	Odds ratio (95% CI)	***P***	**Hazard ratio (95% CI)**	***P***	**Hazard ratio (95% CI)**	***P***
Age, years, continuous	1.00 (0.96-1.05)	0.828	1.02 (0.99-1.05)	0.143	1.03 (0.99-1.08)	0.139
Sex, (Male vs Female)	1.31 (0.59-2.89)	0.509	0.72 (0.45-1.16)	0.178	0.93 (0.44-1.99)	0.857
Serum M-protein (Others vs Light chain only)	0.79 (0.30-2.06)	0.628	1.25 (0.72-2.17)	0.424	1.00 (0.40-2.48)	0.996
ISS stage, (III vs I-II)	0.87 (0.36-2.10)	0.748	0.57 (0.34-0.96)	**0.034**	0.62 (0.27-1.41)	0.253
Cytogenetics, (High vs standard)	3.45 (0.87-13.62)	0.077	1.52 (0.79-2.92)	0.212	1.23 (0.50-3.03)	0.660
Myeloma bone disease on plain radiographs, (Yes vs No)	2.27 (0.82-6.98)	0.126	0.91 (0.50-1.66)	0.751	0.85 (0.32-2.25)	0.744
Previous number of therapies, continuous	1.84 (1.10-3.06)	**0.019**	1.17 (1.01-1.37)	**0.042**	0.96 (0.75-1.23)	0.729
Previous ASCT (Yes vs No)	0.55 (0.24-1.22)	0.143	0.95 (0.59-1.52)	0.831	0.88 (0.41-1.90)	0.742
Time since diagnosis, months, continuous	1.00 (0.99-1.01)	0.864	1.00 (0.99-1.00)	0.688	0.99 (0.97-1.00)	**0.038**
Hb, g/dL, continuous	1.27 (1.01-1.62)	**0.046**	0.77 (0.67-0.89)	**0.001**	0.80 (0.63-1.01)	0.064
WBC, $x10^6$/L, continuous	1.00 (1.00-1.00)	0.151	1.00 (1.00-1.00)	**0.001**	1.00 (1.00-1.00)	0.060
Platelet, $x10^9$/L, continuous	1.01 (1.00-1.01)	**0.017**	0.99 (0.99-1.00)	**$9.3x10^{-5}$**	0.99 (0.99-1.00)	**0.009**
Total protein, g/dL, continuous	1.01 (0.74-1.38)	0.951	1.18 (0.96-1.43)	0.111	1.43 (1.01-2.02)	**0.045**
Albumin, g/dL, continuous	2.46 (1.04-6.47)	0.051	0.40 (0.23-0.69)	**0.001**	0.33 (0.12-0.91)	**0.032**
Ca, mg/dL, continuous	1.57 (0.98-2.76)	0.082	0.80 (0.58-1.11)	0.179	1.17 (0.80-1.71)	0.422
Cr, mg/dL, continuous	0.84 (0.56-1.12)	0.278	1.03 (0.87-1.21)	0.767	1.01 (0.79-1.29)	0.936
LDH, U/L, continuous	1.00 (0.99-1.01)	0.662	1.00 (1.00-1.00)	0.073	1.01 (1.00-1.01)	**$1.1x10^{-4}$**

Ca, calcium; CI, confidence interval; Hb, hemoglobin; TTP, time to progression; VGPR, very good partial response

Table S4. Performance of each genetic and clinical factors for predicting Len-dex treatment response

Factor	AUC*	*P*-value	95% CI	Sensitivity	Specificity	Accuracy
Genetic factors						
miR-26a-5p	0.679	0.003	0.572-0.786	67.9%	63.6%	66.0%
miR-29c-3p	0.734	<0.001	0.628-0.840	73.6%	69.0%	71.6%
miR-30b-5p	0.647	0.012	0.539-0.755	60.0%	64.4%	62.0%
miR-30c-5p	0.700	<0.001	0.598-0.802	61.8%	66.7%	64.0%
miR-331-3p	0.671	0.004	0.564-0.778	65.4%	65.9%	65.6%
Clinical factors#						
Cytogenetics	0.593	0.193	0.458-0.727	-	-	-
Previous number of therapies	0.635	0.021	0.525-0.745	40.7%	71.1%	54.5%
Hb	0.622	0.038	0.512-0.732	60.0%	59.3%	59.6%
Albumin	0.607	0.069	0.495-0.718	66.7%	55.6%	60.6%
Ca	0.628	0.029	0.519-0.737	68.9%	48.1%	57.6%
Platelet	0.645	0.013	0.536-0.754	60.0%	63.0%	61.6%

* AUC, area under the curve

Six clinical variables with $P<0.1$ were selected to evaluate the AUC.

Ca, calcium; Hb, hemoglobin; CI, confidence interval

Table S5. Clinical and genetic variables for Len-dex treatment response prediction model

	Clinical variables	Description	Cutoff value
1	Sex	Categorical	Male vs Female
2	Age, years	Continuous	-
3	Serum M protein	Categorical	Others vs Light chain only
4	ISS stage	Categorical	III vs I-II
5	Cytogenetics	Categorical	High vs standard
6	Myeloma bone disease on plain radiographs	Categorical	Yes vs No
7	Previous number of therapies (PNT)	Continuous	-
8	PNT Group	Categorical	2 times
9	Previous ASCT	Categorical	Yes vs No
10	Time since diagnosis, month	Continuous	-
	Time since diagnosis Group	Categorical	60 month
11	Hb, g/dL	Continuous	-
	Hb Group	Categorical	8.5 g/dL
12	WBC, $x10^6$/L	Continuous	-
	WBC Group	Categorical	4000 $x10^6$/L
13	PLT, $x10^9$/L	Continuous	-
	PLT Group	Categorical	250 $x10^9$/L
14	Total protein, g/dL	Continuous	-
	Total protein Group	Categorical	8 g/dL
15	Albumin, g/dL	Continuous	-
	Albumin Group	Categorical	3.5 g/dL
16	Ca, mg/dL	Continuous	-
17	Cr, mg/dL	Continuous	-
18	LDH, U/L	Continuous	-
	LDH Group	Categorical	480 U/L
	Genetic variables	**Description**	**Cutoff value**
1	miR26a-5p	Delta Ct value	4.56
2	miR29c-3p	Delta Ct value	6.93
3	miR30b-5p	Delta Ct value	2.25
4	miR30c-5p	Delta Ct value	4.03
5	miR193a-5p	Delta Ct value	5.35
6	miR331-3p	Delta Ct value	3.78

Supplementary references

1. Lee SE, Lim JY, Ryu DB, et al. Circulating immune cell phenotype can predict the outcome of lenalidomide plus low-dose dexamethasone treatment in patients with refractory/relapsed multiple myeloma. Cancer Immunol Immunother. 2016;65(8):983-994.

2. Dimopoulos MA, Palumbo A, Attal M, et al. Optimizing the use of lenalidomide in relapsed or refractory multiple myeloma: consensus statement. Leukemia. 2011;25(5):749-760.

3. Kyle RA, Rajkumar SV. Criteria for diagnosis, staging, risk stratification and response assessment of multiple myeloma. Leukemia. 2009;23(1):3-9.

4. Blade J, Samson D, Reece D, et al. Criteria for evaluating disease response and progression in patients with multiple myeloma treated by high-dose therapy and haemopoietic stem cell transplantation. Myeloma Subcommittee of the EBMT. European Group for Blood and Marrow Transplant. Br J Haematol. 1998;102(5):1115-1123.

5. McShane LM, Altman DG, Sauerbrei W, et al. Reporting recommendations for tumor marker prognostic studies (REMARK). J Natl Cancer Inst. 2005;97(16):1180-1184.

6. Jung SH, Yim SH, Hu HJ, et al. Genome-wide copy number variation analysis identifies deletion variants associated with ankylosing spondylitis. Arthritis Rheumatol. 2014;66(8):2103-2112.

7. Chang CC, Lin CJ. LIBSVM: A Library for Support Vector Machines. Acm T Intel Syst Tec. 2011;2(3):

8. Stone M. Cross-Validatory Choice and Assessment of Statistical Predictions. Journal of the Royal Statistical Society Series B (Methodological). 1974;36(2):111-147.