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

Patients with relapsed/refractory multiple myeloma (RRMM) presents a therapeutic challenge. Among the diverse combinations of therapeutic agents, lenalidomide plus low-dose dexamethasone (Len-dex) therapy is a standard of care for RRMM.[1] However, since RRMM patients tend to progress despite treatment, it is essential to discover pathways underlying therapeutic resistance or relapse and to identify biomarkers for predicting treatment response and prognosis. Recently, circulating miRNA has been suggested as a minimally invasive prognostic marker for multiple myeloma (MM) including relapse, which may spare traditional invasive tumor cell examination by bone marrow biopsy.[2-5] However, circulating miRNA markers associated with the outcomes of Len-dex therapy have not been well studied. A better understanding of these can improve prediction of treatment outcome and selection of therapeutic options in the clinical setting. For this purpose, we investigated the expression of serum miRNAs and explored their predictive values in RRMM patients receiving Len-dex.

To identify miRNAs associated with the Len-dex treatment outcome, 38 RRMM patients (19 good responders and 19 poor responders) were analyzed as a discovery set using TaqMan Low Density miRNA Array (TLDA). Based on the previous reports,[6] we categorized complete response (CR) and very good partial response (VGPR) into good responders and partial response (PR), minimal response (MR), stable disease (SD) and progressive disease (PD) into poor responders. Details of TLDA analysis procedures and clinicopathological features of the samples are available in the *Online Supplementary Methods* and *Online Supplementary Table S1*. Thirty-four miRNAs were differentially expressed between two groups (*Online Supplementary Table S2*), but only six miRNAs (miR-26a-5p, miR-29c-3p, miR-30b-5p, miR-30c-5p, miR-193a-5p and miR-331-3p) were significantly down-regulated in poor responders (Table 1). Their heatmap showed a clear discrepancy between good and poor responders (*Online Supplementary Figure S1*). Among the clinical variables, a higher number of previous therapies, lower hemoglobin level and lower platelet count were significantly associated with poor responder status for higher number of previous therapies [OR 1.84 (95%CI: 1.10-3.06); *P*=0.019], for lower hemoglobin [OR 1.27 (95%CI: 1.01-1.62); *P*=0.046], and for lower platelet values [OR 1.01 (95%CI: 1.00-1.01); *P*=0.017] (*Online Supplementary Table S3*).

To validate the six candidate miRNAs, we performed qRT-PCR with a larger independent validation set of 62 RRMM patients (good responders n=26; poor responders n=36) (*Online Supplementary Table S1*). Five miRNAs (miR-26a-5p, miR-29c-3p, miR-30b-5p, miR-30c-5p, and miR-331-3p) were significantly down-regulated in poor responders in a consistent manner (Table 1). One exception was miR-193a-5p, which was also down-regulated in poor responders, but not significantly. When we combined the data from the discovery and validation sets (100 RRMM patients; good responders n=45, poor responders n=55), the differences in expression of all six miRNAs between the two groups were consistently significant (Table 1 and *Online Supplementary Figure S2A*). Of the six miRNAs, miR-29c-3p showed the highest significance level ($P=2.4\times10^{-4}$). When we evaluated the relationships between expression levels of the six miRNA markers and treatment responses, the expressions of the miRNAs were inversely related to treatment responses (*Online Supplementary Figure S2B*). These data suggest the reliability of the candidate miRNA markers in prediction of Len-dex treatment response under our criteria. In ROC analyses, predictive values of area under the curve (AUC) for the miRNA markers showed a range of 0.647-0.734 (*Online Supplementary Table S4*). Notably, all miRNA markers showed superior AUC values to any of the clinical factors (AUC 0.593-0.645).

To evaluate prognostic implications of the six miRNAs, survival analysis was performed with the combined set. Median duration of follow up was 15.6 months (range 0.4-33.6) from the start of Len-dex treatment. The 2-year time to progression (TTP) and overall survival (OS) rates were 22.7% and 64.2%, respectively. Lower expression of the six miRNAs were significantly associated with shorter TTP (miR-26a-5p, *P*=0.002; miR-29c-3p, *P*=0.021; miR-30b-5p, *P*=0.001; miR-30c-5p, *P*=0.002; miR-193a-5p, *P*=0.032; miR-331-3p, *P*=0.032) (Figure 1A). Six clinical variables were also significantly associated with shorter TTP [International Staging System (ISS) stage III, *P*=0.034; previous number of therapies, *P*=0.042; lower hemoglobin, *P*=0.001; lower white blood cell (WBC) count, *P*=0.001; lower platelet count, $P=9.3\times10^{-5}$; lower albumin level, *P*=0.001] (*Online Supplementary Table S3*).

Table 1. Discovery and validation of six miRNAs.

miRNA	Event*	Discovery set (TLDA)			Validation set (qRT-PCR)			Overall			
		GR# (average)	PR# (average)	*P*	GR# (average)	PR# (average)	*P*	GR# (average)	PR# (average)	Fold change	*P*
miR-26a-5p	Down	1.884	0.797	0.028	1.403	0.715	0.007	1.603	0.835	0.51	0.002
miR-29c-3p	Down	1.541	0.762	0.013	1.322	0.524	0.013	1.446	0.646	0.43	2.4×10^{-4}
miR-30b-5p	Down	1.935	0.819	0.018	1.439	0.818	0.023	1.712	1.000	0.47	0.007
miR-30c-5p	Down	1.467	0.724	0.023	1.001	0.598	0.015	1.324	0.741	0.48	7.5×10^{-4}
miR-193a-5p	Down	1.164	0.529	0.025	1.450	0.671	0.358	2.032	0.98	0.57	0.035
miR-331-3p	Down	1.784	0.888	0.046	1.201	0.659	0.003	1.476	0.859	0.47	0.001

Discovery set: 38 multiple myeloma (MM) patients (19 good responders and 19 poor responders). Validation set: 62 MM patients (26 good responders and 36 poor responders). Overall: 100 MM patients (45 good responders and 55 poor responders). *Down: relatively down-regulated (poor responders/good responders). #GR: good responders; PR: poor responders.

Regarding the OS, lower expression of the four miRNAs were significantly associated with poorer OS (miR-30b-5p, P=0.008; miR-30c-5p, P=0.031; miR-193a-5p, P=0.033; miR-331-3p, P=0.013) (Figure 1B). Five clinical variables were also significantly associated with poorer OS (shorter time since diagnosis, P=0.038; lower platelet count, P=0.009; higher total protein level, P=0.045; lower albumin level, P=0.032; higher lactate dehydrogenase (LDH) level, P=1.1x10^{-4}) (*Online Supplementary Table S3*). To explore the additive effect of the miRNAs on the prognosis, we scored each patient according to expression levels of the miRNAs: 1 was assigned to lower expression and 0 to higher expression based on the optimal cut off for each miRNA. Patients with high scores (score sum ≥3) showed significantly shorter TTP and poorer OS than those with low scores (score sum <3) (P=4.2x10^{-4} and P=6.6x10^{-5}, respectively) (Figure 1C).

Consistent with our findings, Zhao *et al*. reported that the members of miR-30 family including miR-30b and miR30c were down-regulated in MM.[7] They also demonstrated that miR-30s were inversely related to BCL9 expression, which suggests that downregulation of miR-30s may induce upregulation of BCL9 and consequent proliferation of MM cells by activating the Wnt signaling pathway.[7] In the study by Navarro *et al*. exploring miRNA signatures associated with CR and progression after

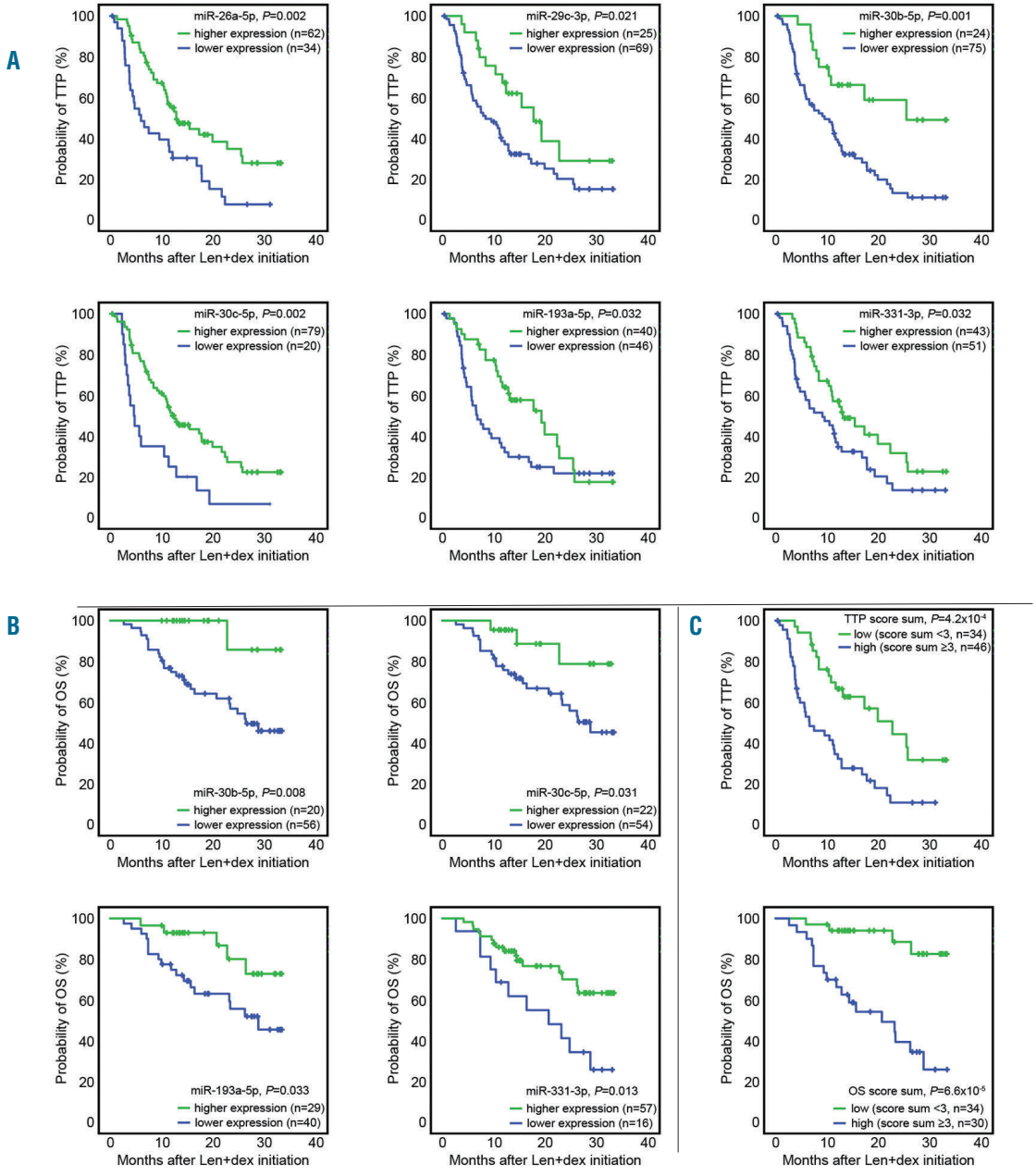

Figure 1. Kaplan-Meier curves for time to progression (TTP) and overall survival (OS) by miRNA expression level. (A) Patients with lower expression (blue) of each miRNA in all six markers showed significantly poorer TTP than those with higher expression (green). (B) Patients with lower expression (blue) of each miRNA in four markers showed significantly poorer OS than those with higher expression (green). (C) As the sum of the lower expression marker increased, prognosis became significantly worse in a dose-dependent manner (upper plot: TTP; lower plot: OS).

autologous stem cell transplantation in MM, lower expression of miR-331 was significantly associated with poor progression-free survival.[5] Biological implications of miR-26a, miR-29c and miR-193a have not been studied in MM, but these miRNAs were also suggested as tumor suppressors in hematologic malignancies. For example, miR-193a represses the expression of KIT and its restoration reduces the mRNA and protein levels of KIT in acute myeloid leukemia (AML).[8] Downregulation of miR-26a was also frequently reported in AML.[9,10] Downregulation of miR-29c was reported to be associated with treatment-free survival and OS in chronic lymphocytic leukemia.[11] These reports support the biological and clinical implications of the six miRNA markers associated with the Len-dex treatment response. A recent genome-wide association study provided evidence of common genetic susceptibility to immunoglobulin light chain amyloidosis and MM, in which some significant SNPs were mapped in RNA genes or miRNAs known to be associated with organ involvement in MM,[12] suggesting the importance of exploring gene-regulatory mechanisms behind pathogenesis and treatment-refractoriness in RRMM.

In order to make a more reliable prediction of the response to Len-dex treatment, we designed a model called Len-dex treatment response prediction (LdTRP) by adopting support vector machine (SVM). For optimal feature selection, 2,468,732 SVMs were constructed along with changing combinations of 18 clinical variables and six miRNAs (*Online Supplementary Table S5*). We selected the best SVM as LdTRP model which uses the input features composed of two miRNAs (miR-26a-5p and miR-193a-5p) and five clinical variables (cytogenetics, ISS stage, hemoglobin, WBC and calcium). Although the question as to whether prognostic markers from newly diagnosed MM such as cytogenetic data are relevant for relapsed disease remains a matter of debate, prognostic stratification based on baseline data is known to be associated with OS as well as with progression-free survival,[13,14] which may indicate that their prognostic significance is not restricted to front-line therapy. In receiver operating characteristic (ROC) analyses, the LdTRP model showed much improved stratification power (AUC=0.933) compared with SVMs composed of either clinical variables only (AUC=0.670) or miRNAs only (AUC=0.675) (Figure 2). Sensitivity, specificity and accuracy of the model were 90.0%, 77.8% and 85.4%, respectively, based on the probability score threshold being set at 0.53. (Our model is available online at *http://ircgp.com/RRMM/index.html*). With this model, users can calculate the probability score for treatment response before starting the Len-dex treatment. Taken together, these data suggest that using both clinical variables and miRNA expressions is essential for predicting treatment response to Len-dex treatment. To our knowledge, this is a unique approach to combine microRNAs and clinical factors to build a prediction model in RRMM. In addition, this model can easily be applied in the clinical setting, since we have suggested the specific score threshold to predict treatment response.

Although significant miRNA markers were identified and our prediction model performed well, there are several limitations to our study. First, some well-known prognostic miRNA markers of MM[2-5,15] such as miR-19a were not identified. Since we focused on RRMMs with bortezomib failure and used the miRNA-array which contains just part (381 miRNAs) of the known human miRNAs, there is a possibility that some significant miRNA markers were missed. In addition, a relatively small sample size may increase the possibility of false negative results. Second, due to the limited sample size, for survival analysis and model building, we used the combined set of samples without further external validations. External validation with a larger independent set

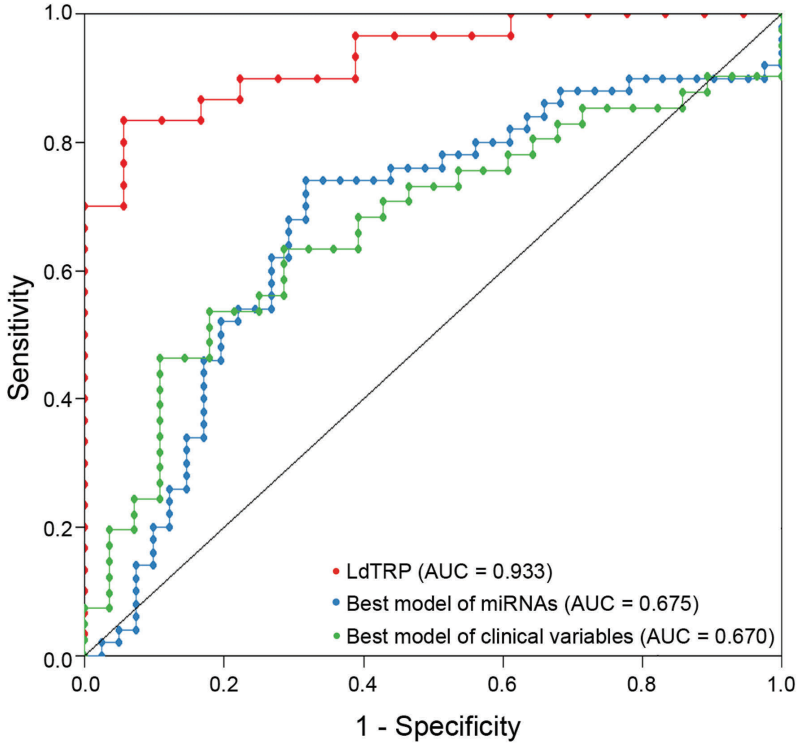

Figure 2. Receiver Operating Characteristic (ROC) curves for the lenalidomide+low-dose dexamethasone (Len-dex) treatment response prediction (LdTRP) model. ROC curves for the LdTRP model composed of five clinical variables [cytogenetics, International Staging System (ISS) stage, hemoglobin, white blood cell (WBC) count, and calcium] and two miRNAs (miR-26a-5p and miR-193a-5p) (red), and other support vector machines (SVMs) with either clinical variables only (green) or miRNAs only (blue) are shown. The LdTRP model showed much superior stratification power [area under the curve (AUC)=0.933] to other SVMs, especially in terms of specificity.

will add validity to our conclusion. Third, cytogenetics data at the time of diagnosis, one of the clinical variables for LdTRP modeling, was available for only 70% of the samples. Therefore, the study size became smaller, which may introduce additional uncertainty in our model.

In summary, our results suggest the potential of using circulating miRNAs as minimally invasive markers for predicting treatment response and prognosis in RRMM patients, which may replace traditional invasive tumor cell examination by bone marrow biopsy. In addition, since our LdTRP model suggests a specific score threshold for predicting treatment response, it can easily be applied in the clinical setting to select treatment regimens.

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