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RESEARCH ARTICLE

ciRS-7 circular RNA overexpression in plasma cells is a promising molecular biomarker of unfavorable prognosis in multiple myeloma

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Abstract

Several non-coding RNAs are known to be associated with the pathobiology and progression of multiple myeloma (MM). ciRS-7 (also known as CDR1-AS), a key oncogenic circular RNA (circRNA) that sponges miR-7-5p and other cancer-related microRNAs, was recently found to be downregulated in malignant plasma cells resistant to immunomodulatory drugs. Considering that various circRNAs have a strong potential as molecular biomarkers, we aimed to investigate the expression of ciRS-7 in plasma cell disorders, assess its prognostic importance in MM, and compare these findings with those of individuals with smoldering MM (SMM) and monoclonal gammopathy of unknown significance (MGUS). This study included 171 patients (110 newly diagnosed MM, 34 SMM, and 27 MGUS cases), from which bone marrow aspirate samples were collected for CD138+ plasma cell selection. Total RNA was reversely transcribed using random hexamer primers, and the expression levels of ciRS-7 were quantified using an in-house-developed protocol that includes pre-amplification and real-time quantitative polymerase chain reaction. ciRS-7 levels were found to significantly differ among CD138+ plasma cells of MM, SMM, and MGUS patients. ROC analysis indicated that ciRS-7 expression effectively distinguishes between MM and SMM patients. Moreover, high levels of ciRS-7 were associated with unfavorable prognosis in MM, independently of MM patients' age and Revised International Staging System stage. Additionally, in silico analysis predicted the binding of 85 microRNAs to ciRS-7. In conclusion, this study provides novel insights into the role of ciRS-7 as a promising molecular marker able to distinguish MM from SMM and predict prognosis in MM.

KEYWORDS
CDR1-AS, circRNA, microRNA, molecular biomarker, non-coding RNA, plasma cell dyscrasia

1 | INTRODUCTION

As part of a range of disorders termed monoclonal gammopathies, multiple myeloma (MM) is characterized by clonal plasma cells that infiltrate the bone marrow and by the presence of monoclonal protein in the serum of patients [1]. In the past few years, researchers have deemed it necessary to successfully differentiate symptomatic MM, which requires treatment, from its precursor stages, monoclonal gammopathy of unknown significance (MGUS) and smoldering MM (SMM) [2]. Moreover, the establishment of accurate molecular biomarkers that predict the prognosis of subgroups of MM patients has been the focus of many research efforts [3]. The underlying genetic or epigenetic abnormalities of MM that alter mRNA and non-coding RNA expression are highly responsible for the clinical heterogeneity of MM [4]. A prominent example is circular RNAs (circRNAs), a class of RNA molecules with regulatory potential [5, 6]. They constitute covalently closed loops and are resistant to exonucleolytic decay, therefore having a longer half-life than linear RNAs and being more stable [5, 7]. Their most well-investigated function is that of microRNA (miRNA) sponging, where they act as competing endogenous RNAs [8].

Recent breakthroughs in experimental design and data analysis have revealed that circRNAs possess key roles in both physiological and pathological processes. Among the circRNAs that are implicated in cancer development and progression, ciRS-7 (*CDR1-AS*) is the most well-studied so far [9]. Its most prevalent function is the sponging of miR-7-5p, for which it possesses more than 60 binding sites in its sequence [10]. The implication of ciRS-7 in the malignant behavior of cancer cells affects cell proliferation, migration, invasion, metastasis, angiogenesis, and epithelial-mesenchymal transition [10, 11]. Its prognostic potential is also evidenced in a variety of tumors, including cervical cancer, renal cell carcinoma, lung cancer, and colorectal cancer [12–15]. Despite its established role in solid tumors, the involvement of ciRS-7 in hematological malignancies has not been examined so far, except from a recent study where ciRS-7 was proven to be downregulated in MM patients having developed tolerance to immunomodulatory drugs [16]. Moreover, various circRNAs have established prognostic potential in this malignancy, such as circITCH, circMYC, and circCCT3 [17–19].

Prompted by these facts, we aimed to investigate the expression levels of ciRS-7 in plasma cell neoplasm cell lines and patients' samples at different stages of MM and evaluate its prognostic significance. Our results indicate that ciRS-7 is differentially expressed between the plasma cells of MGUS, SMM, and MM patients. Moreover, high ciRS-7 expression levels predict an unfavorable prognosis in MM, independently of the Revised International Staging System (R-ISS) stage and age. Combining our findings with the limited literature regarding the involvement of ciRS-7 in this malignancy, it becomes evident that further research is needed to understand its biological significance in MM.

2 | METHODS

2.1 | Sample collection

Bone marrow aspirate (BMA) samples were collected, at the time of diagnosis, from 171 adult patients with plasma cell disorders at the Department of Clinical Therapeutics of the "Alexandra" General Hospital of Athens, Greece. Patients who had already received any kind of treatment or had suffered from any other concomitant malignancy were excluded from this study.

This study was approved by the Ethics Committee of the "Alexandra" General Hospital of Athens (protocol code 859/24-10-2017 and date of approval: 25 October 2017) and was conducted according to the principles of the 1964 Declaration of Helsinki. Written informed consent was obtained from each participant of this study.

2.2 | CD138+ plasma cell selection

CD138+ cell selection among mononuclear cells was performed to select only the plasma cells from BMA samples of all patients. The BMA mononuclear cells were first separated using the Ficoll-Paque method, and then CD138+ plasma cells were positively selected using anti-CD138-coated magnetic beads (Miltenyi Biotech).

2.3 | Propagation of plasma cell neoplasm cell lines, RNA extraction, and reverse transcription

The L-363, U266, and H929 cell lines were propagated according to the American Type Culture Collection instructions. Total RNA was isolated from each cell line and from CD138+ plasma cells. Then, 125 ng of each total RNA extract was subjected to reverse transcription.

2.4 | Quantification of ciRS-7 expression, using an in-house-developed real-time quantitative polymerase chain reaction assay

To quantify the expression of ciRS-7, we developed and optimized an SYBR Green-based quantitative real-time PCR (qPCR) assay, preceded by a pre-amplification step. The sequences of all primers used in these assays are shown in Table S1. The levels of ciRS-7 were determined using the comparative threshold cycle (C_T) method ($2^{-\Delta\Delta Ct}$), and *GAPDH* was used as the reference gene [20, 21].

2.5 | Biostatistics analysis

The Kruskal-Wallis *H* test was employed to compare the expression of ciRS-7 in patients with MGUS, SMM, and MM. In order to examine whether ciRS-7 can effectively distinguish MM from SMM patients, receiver operating characteristic (ROC) analysis was conducted. Kaplan-Meier progression-free survival (PFS) and overall survival (OS) analyses as well as univariate and multivariate Cox regression were also performed, to assess the prognostic significance of ciRS-7 expression in MM. Only *p*-values lower than 0.050 were considered significant. All statistical analyses employed are detailed in the Supporting Information Materials and Methods.

2.6 | In silico functional analysis for ciRS-7 target prediction

We conducted in silico analysis using publicly available bioinformatics tools and algorithms to predict the miRNA targets of ciRS-7 and gain insight into its function in MM. The custom prediction tool of the miRDB database and the "miRNA Target Sites" function of the CircInteractome web tool were used [22, 23], so as to computationally identify potential binding sites for miRNAs within ciRS-7.

2.7 | Kyoto Encyclopedia of Genes and Genomes pathway analysis

From the above analyses, a list of miRNAs that are predicted to interact with ciRS-7 was generated. We selected and grouped several miRNAs with a functionally relevant role; DIANA miRPath v.3.0 was used to predict the putative targets of these miRNAs [24]. Then, the Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Analysis was conducted, attempting to pinpoint regulatory pathways that ciRS-7 might participate in or regulate.

3 | RESULTS

3.1 | Clinical and pathological characteristics of the patients' cohort

The cohort of 171 adult patients included in our study consisted of 110 newly diagnosed MM, 34 SMM, and 27 MGUS cases, according to the updated criteria of the International Myeloma Working Group. The biological and clinicopathological characteristics of the MM, SMM, and MGUS patients are presented in detail in Table 1.

3.2 | Optimized real-time qPCR assays for ciRS-7 quantification and expression analysis of ciRS-7 in plasma cell neoplasm cell lines

Serial dilutions of the L-363 cDNA were used in triplicate to generate relative standard curves for ciRS-7 and *GAPDH*, in order to check amplification efficiencies. The mean C_q values were plotted against the log of relative cDNA quantity (Figure 1A) and the specificity of each amplicon was evidenced by the unique melt curves (Figure 1B). Through the optimized pre-amplification and qPCR assays, we quantified the relative expression levels of ciRS-7 in the L-363, U266, and H929 cell lines. Interestingly, the relative ciRS-7 levels in U266 and H929 cell lines were much lower (2165- and 26,616-fold, respectively) than in L-363 cells.

3.3 | Differential ciRS-7 expression in CD138+ plasma cells of MGUS, SMM, and MM patients

A statistically significant difference was observed in the intracellular ciRS-7 expression levels of CD138+ plasma cells isolated from MM, SMM, and MGUS patients. As shown in Figure 2A, ciRS-7 is downregulated in MM compared to SMM and MGUS patients' samples. The median values of ciRS-7 expression were 2.79 RQU in MGUS patients, 1.74 RQU in SMM patients, and 0.41 RQU for MM patients ($p = 0.006$). Measures of the distribution of ciRS-7 expression in each group of patients are shown in detail in Table 2. Moreover, ROC analysis showed that intracellular ciRS-7 expression levels of CD138+ plasma cells can effectively distinguish MM from SMM patients (area under the curve = 0.64, 95% confidence interval [CI] = 0.55–0.74, $p = 0.012$) (Figure 2B).

Next, MM patients were categorized as having a high or low expression of ciRS-7, based on the optimal cut-off point (1.12 RQU; 63rd percentile of the distribution) determined for prognostic purposes, using the X-tile software. After that categorization, high ciRS-7 expression was found to be significantly associated with high cytogenetic risk ($p = 0.032$) and the presence of del(17p) ($p = 0.006$). On the other hand, no significant association was found between ciRS-7 and MM patients' age, presence of bone disease, ISS, and/or R-ISS stage.

3.4 | High ciRS-7 expression as a molecular indicator of unfavorable prognosis in MM, independently of the R-ISS stage and patients' age

Next, we investigated the potential prognostic significance of intracellular ciRS-7 expression in terms of PFS and OS of MM patients. The median follow-up time was 24 months. Out of 110 MM patients, 70 were considered as ciRS-7(low) and 40 as ciRS-7(high) expressors, based on the aforementioned optimal cut-off threshold. As depicted in the Kaplan-Meier PFS (Figure 3A) and OS curves (Figure 3B), the

TABLE 1 Biological and clinicopathological characteristics of the multiple myeloma (MM), smoldering MM (SMM), and monoclonal gammopathy of unknown significance (MGUS) patients.

Variable	MM patients ($n = 110$)	SMM patients ($n = 34$)	MGUS patients ($n = 27$)
	Median (Range)		
Age (years)	70 (35–93)	68 (49–86)	61 (37–84)
	Number of patients (%)		
Gender			
Male	66 (60.0%)	21 (61.8%)	15 (55.6%)
Female	44 (40.0%)	13 (38.2%)	12 (44.4%)
Serum M-protein isotype			
Heavy chain			
IgA	23 (20.9%)	10 (29.4%)	5 (18.5%)
IgG	63 (57.3%)	24 (70.6%)	22 (81.5%)
IgD	2 (1.8%)	–	–
IgM	2 (1.8%)	–	–
None detected	20 (18.2%)	–	–
Light chain			
Kappa light chain	74 (67.3%)	21 (61.8%)	17 (63.0%)
Lambda light chain	33 (30.0%)	13 (38.2%)	10 (37.0%)
None detected	3 (2.7%)	–	–
Bone marrow plasma cell infiltration			
<20%	8 (7.3%)	20 (58.8%)	27 (100.0%)
20%–40%	18 (16.4%)	9 (26.5%)	–
>40%	84 (76.3%)	5 (14.7%)	–
Serum β_2 microglobulin			
<3.5 mg/L	33 (30.0%)	24 (70.6%)	23 (85.2%)
3.5–5.4 mg/L	31 (28.2%)	9 (26.5%)	4 (14.8%)
≥5.5 mg/L	46 (41.8%)	1 (2.9%)	–
Serum albumin			
<3.5 g/dL	30 (27.3%)	1 (2.9%)	2 (7.4%)
≥3.5 g/dL	80 (72.7%)	33 (97.1%)	25 (92.6%)
Lactate dehydrogenase			
Normal (≤225 U/L)	87 (79.1%)	31 (91.2%)	26 (96.3%)
Elevated (>225 U/L)	23 (20.9%)	3 (8.8%)	1 (3.7%)
2/20/20 risk stratification model for SMM[a]	N/A		N/A
Low risk		17 (50.0%)	
Intermediate risk		13 (38.2%)	
High risk		4 (11.8%)	
Cytogenetic abnormalities		N/A	N/A
del(17p)			
Absence	92 (83.6%)		
Presence	18 (16.4%)		
t(4;14)			
Absence	96 (87.3%)		
Presence	14 (12.7%)		

(Continues)

TABLE 1 (Continued)

Variable	MM patients ($n = 110$)	SMM patients ($n = 34$)	MGUS patients ($n = 27$)
t(14;16)			
Absence	107 (97.3%)		
Presence	3 (2.7%)		
t(11;14) (65/110 patients)			
Absence	47 (72.3%)		
Presence	18 (27.7%)		
(+1q) (75/110 patients)			
Absence	40 (53.3%)		
Presence	35 (46.7%)		
Cytogenetic risk[b]		N/A	N/A
Standard	85 (77.3%)		
High	25 (22.7%)		
ISS stage		N/A	N/A
I	30 (27.3%)		
II	34 (30.9%)		
III	46 (41.8%)		
R-ISS stage		N/A	N/A
I	23 (20.9%)		
II	62 (56.4%)		
III	25 (22.7%)		
WBLDCT osteolysis (82/110 patients)		N/A	N/A
No	28 (34.1%)		
Yes	54 (65.9%)		
Primary treatment		N/A	N/A
Bortezomib, lenalidomide, and dexamethasone	64 (58.1%)		
Bortezomib, thalidomide, and dexamethasone	9 (8.2%)		
Daratumumab, bortezomib, lenalidomide, and dexamethasone	6 (5.5%)		
Isatuximab, bortezomib, lenalidomide, and dexamethasone	12 (10.9%)		
Bortezomib, cyclophosphamide, and dexamethasone	10 (9.1%)		
Bortezomib and dexamethasone	2 (1.8%)		
Lenalidomide and dexamethasone	7 (6.4%)		
Bisphosphonate treatment (77/110 patients)		N/A	N/A
No	36 (46.8%)		
Yes	41 (53.2%)		

Abbreviations: Ig, immunoglobulin; ISS, International Staging System; N/A, not applicable; R-ISS, Revised International Staging System; WBLDCT, whole-body low-dose computed tomography.

[a]The 2/20/20 risk stratification model for SMM is based on 3 factors, each of which is independently associated with increased risk of SMM progression to symptomatic MM: serum M-protein levels > 2 g/dL, involved to uninvolved serum free light-chain ratio > 20, and bone marrow plasma cell infiltration > 20%.

[b]The cytogenetic risk was defined as high based on the presence of del(17p), translocation t(4;14), and/or translocation t(14;16).

cohort of MM patients with ciRS-7(high) expression had lower survival probabilities than the group of MM patients with ciRS-7(low) expression, implying an unfavorable prognostic role for ciRS-7 expression in MM, in terms of both PFS ($p = 0.039$) and OS ($p = 0.006$). Particularly for OS, stratification of MM patients according to their ISS or R-ISS stage showed that MM patients of ISS stage III (Figure 3C) or R-ISS stage III (Figure 3D) overexpressing ciRS-7 in their CD138+ plasma cells have significantly shorter OS time intervals, compared to those of the same disease stage combined with low ciRS-7 expression ($p < 0.001$ for ISS stage III; $p = 0.004$ for R-ISS stage III).

The prognostic value of ciRS-7 expression in MM, regarding both PFS and OS of patients, was further confirmed by

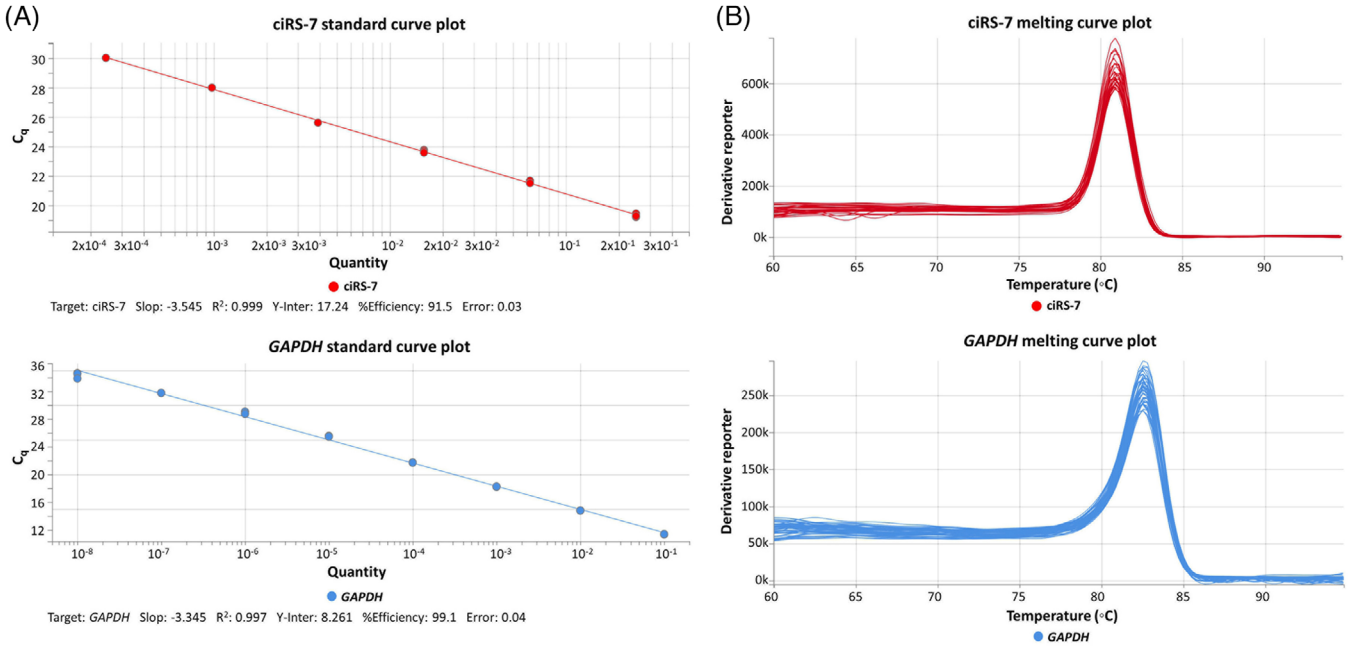

FIGURE 1 Results of the optimized quantitative real-time PCR (qPCR) assays for the relative quantification of ciRS-7. (A) Standard curve plots for ciRS-7 and the selected reference gene (*GAPDH*). Both amplicons are generated and amplified with optimal reaction efficiency (90%–100%), as evidenced by the slope of each standard curve. (B) Melting curves of ciRS-7 and *GAPDH* amplicons, showing the uniqueness of each qPCR product, generated following pre-amplification of both targets.

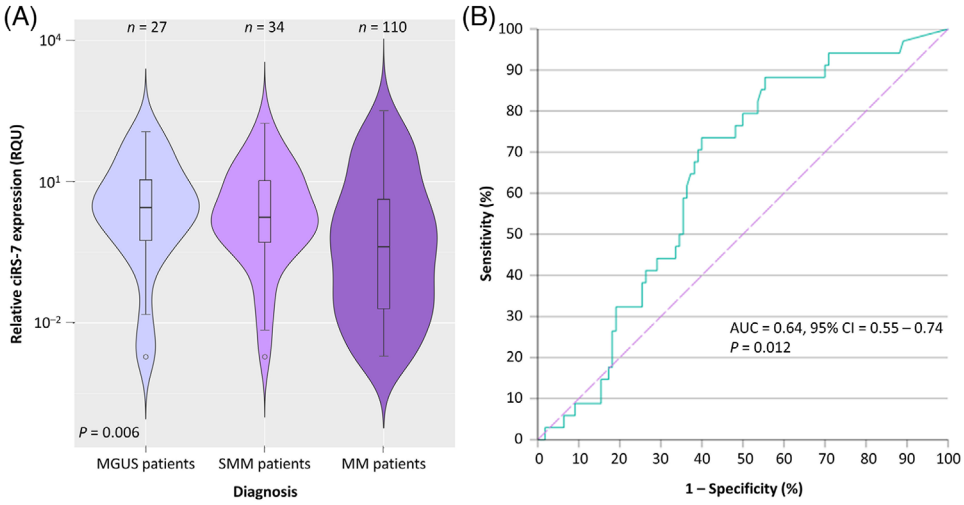

FIGURE 2 Differences observed in ciRS-7 expression between patients with plasma cell disorders, and its ability to distinguish symptomatic from asymptomatic (smoldering) multiple myeloma (MM). (A) Violin plots and box plots showing the distribution of ciRS-7 expression in CD138+ plasma cells of monoclonal gammopathy of unknown significance (MGUS), smoldering MM (SMM), and MM patients. The median value of each distribution is depicted as a bold line inside the respective boxplot, each box represents the interquartile range, and the horizontal thin lines show the approximate 2nd and 98th percentiles of each distribution. The scale of the vertical axis is logarithmic. (B) A receiver operating characteristic (ROC) curve illustrating the ability of ciRS-7 expression to efficiently distinguish MM from SMM patients.

Cox regression analyses (Table 3). Thus, ciRS-7(high) expressors were at higher risk of exhibiting MM progression (hazard ratio [HR] = 1.82, bias-corrected and accelerated [BCa] 95% CI = 1.04–3.19, bootstrap p = 0.032) and succumbing to MM (HR = 3.38, BCa 95% CI = 1.43–10.91, bootstrap p = 0.008). Interestingly, multivariate Cox regression analysis showed that this significantly elevated risk of MM patients with ciRS-7(high) expression was independent of their age and R-ISS stage, again with regard to PFS and OS (Table 3).

TABLE 2 Distribution of ciRS-7 expression in CD138+ plasma cells of patients with monoclonal gammopathy of unknown significance (MGUS), smoldering MM (SMM), or multiple myeloma (MM).

Patient diagnosis	ciRS-7 expression[a] (Mean ± SEM)	Range	Percentiles		
			25th	50th (Median)	75th
MGUS ($n = 27$)	14.5 ± 5.33	0.002–113	0.53	2.79	15.0
SMM ($n = 34$)	13.1 ± 5.47	0.002–171	0.44	1.74	11.4
MM ($n = 110$)	15.7 ± 4.35	0.002–313	0.020	0.41	4.45

Abbreviations: MGUS, monoclonal gammopathy of unknown significance; MM, multiple myeloma; SEM, standard error of the mean; SMM, smoldering multiple myeloma.

[a]Measured in relative quantification units (RQU).

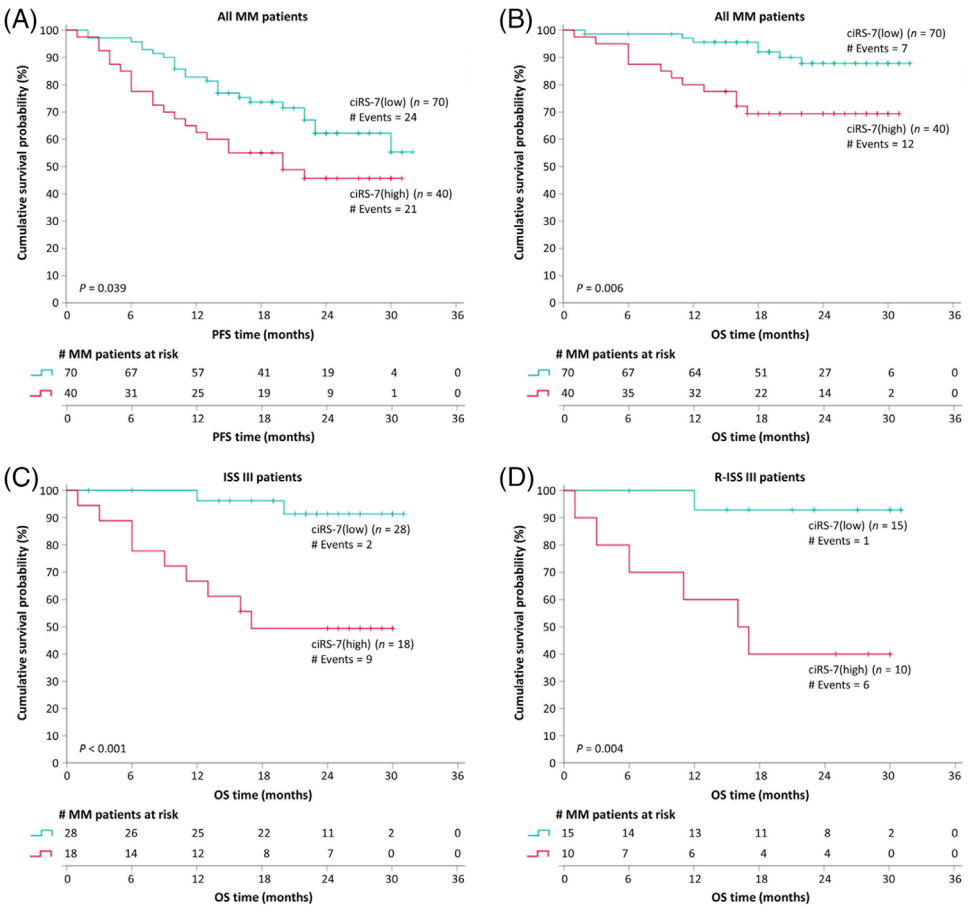

FIGURE 3 Kaplan–Meier survival curves of multiple myeloma (MM) patients, grouped according to ciRS-7 expression (high vs. low). Elevated expression of this circular RNA (circRNA) is related to shorter survival intervals, regarding both progression-free survival (PFS) and (A) and overall survival (OS) (B) of MM patients. Stratification of patients according to the International Staging System (ISS) (C) or Revised International Staging System (R-ISS) (D) classification showed that ciRS-7 overexpression may further separate MM patients of these stages into two groups with significantly different cumulative survival probabilities.

3.5 | miRNAs predicted to be sponged by ciRS-7 and their functional roles

According to our bioinformatic analysis, ciRS-7 possesses putative binding motifs of 71 miRNAs. Following an extensive literature review, three groups of miRNAs were formed: six miRNAs are validated target of ciRS-7, six miRNAs have an established functional role in MM onset and/or progression, and 12 miRNAs have regulatory roles in human malignancies (Figure 4A).

In addition, 25 miRNAs were predicted to be targeted by ciRS-7 according to CircInteractome. By combining the results from miRDB and CircInteractome, 11 common miRNAs arise by the intersection of these two lists (Figure 4B). Out of these 11 miRNAs, miR-7-5p, miR-1299, and miR-1270 are experimentally verified targets of ciRS-7 with

TABLE 3 Cox regression analyses, regarding multiple myeloma (MM) patients' progression-free survival (PFS) and overall survival (OS).

	Covariate	Univariate analysis					Multivariate analysis				
		HR	95% CI	*p*- Value[a]	BCa bootstrap 95% CI	Bootstrap *p*-value[a]	HR	95% CI	*p*- Value[a]	BCa bootstrap 95% CI	Bootstrap *p*-Value[a]
PFS	**ciRS-7 expression**										
	Low	1.00					1.00				
	High	1.82	1.02–3.28	0.044	1.04–3.19	0.032	1.88	1.03–3.40	0.038	1.03–3.81	0.041
	ISS stage					0.005					
	I	1.00									
	II	0.82	0.31–2.12	0.68	0.27–2.28	0.67					
	III	2.47	1.16–5.23	0.019	1.22–6.56	0.016					
	R-ISS stage					0.14					0.16
	I	1.00					1.00				
	II	1.93	0.79–4.72	0.15	0.92–6.47	0.11	1.76	0.72–4.33	0.21	0.80–6.03	0.17
	III	2.64	1.01–6.88	0.047	1.15–9.47	0.031	2.52	0.97–6.58	0.058	0.99–9.40	0.041
	Age	1.01	0.99–1.04	0.31	0.98–1.05	0.33	1.41	0.72–2.77	0.32	0.75–2.97	0.32
OS	**ciRS-7 expression**										
	Low	1.00					1.00				
	High	3.38	1.33–8.59	0.011	1.43–10.91	0.008	3.35	1.28–8.74	0.014	1.36–10.48	0.004
	ISS stage					0.29					
	I	1.00									
	II	0.98	0.25–3.94	0.98	0.18–7.37	0.99					
	III	2.05	0.65–6.45	0.22	0.68–10.94	0.18					
	R-ISS stage					0.19					0.19
	I	1.00					1.00				
	II	2.23	0.49–10.19	0.30	$0.64–9.17 \times 10^4$	0.15	1.96	0.43–9.02	0.39	$0.56–9.45 \times 10^4$	0.27
	III	4.01	0.83–19.32	0.084	$1.06–1.40 \times 10^5$	0.035	3.75	0.78–18.11	0.10	$0.91–1.33 \times 10^5$	0.061
	Age	1.02	0.98–1.07	0.30	0.99–1.07	0.26	1.05	0.40–2.76	0.91	0.39–3.15	0.92

Abbreviations: BCa, bias-corrected and accelerated; CI, confidence interval; HR, hazard ratio; ISS, International Staging System; OS, overall survival; PFS, progression-free survival, R-ISS, Revised International Staging System.

[a] Italics indicate a statistically significant *p*-value ($p < 0.050$).

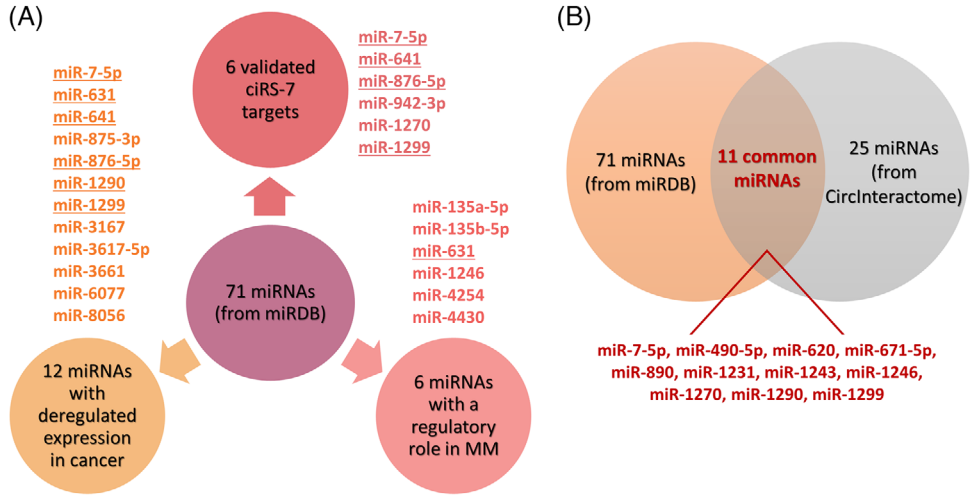

FIGURE 4 The microRNAs (miRNAs) predicted to be sponged by ciRS-7. The bioinformatic analysis was conducted using the custom prediction tool of the miRDB database and the "miRNA Target Sites" function of the CircInteractome web tool. (A) According to an extensive review of the existing literature, several of the 71 miRNAs resulting from queries in miRDB can be further categorized into three subgroups of particular interest. Underlined miRNAs are found in more than one subgroup. (B) Combination of our bioinformatic analyses revealed 85 miRNAs as putative targets of ciRS-7, with 11 of them being predicted by both web tools.

a multifaceted role in cancer [25–27], while miR-1246 is an established diagnostic and prognostic biomarker in MM [28], and miR-1290 is intricately implicated in the regulation of tumor cell proliferation, apoptosis, and invasion [29]. Detailed results from the two databases are provided in Tables S2 and S3.

3.6 | Significantly enriched KEGG pathways

For the three miRNA groups that were created following the bioinformatic analysis from miRDB and DIANA miRPath, KEGG pathway analysis was conducted (Figure 5). Each enrichment score was considered significant when the *p*-value was less than 0.050. Regarding the miRNAs that are experimentally validated targets of ciRS-7, 36 significantly enriched KEGG pathways were obtained, namely the P53, WNT, and Hippo signaling pathways, which possess important roles in the pathogenesis and treatment of MM, as well as the apoptotic pathway and signaling pathways regulating pluripotency of stem cells. For the miRNAs that possess a regulatory role in MM, 24 enriched pathways were obtained, among which the PI3K-AKT, Hippo, and P53 signaling pathways, pathways in cancer, as well as the cell cycle and the mRNA surveillance pathways. Lastly, 21 enriched pathways were obtained regarding the miRNAs with a functional role in other malignancies, with the most prevalent being the Hippo, FOXO, and NF-kappa B (NF-κB) signaling pathways, as well as the cell cycle and apoptosis. Moreover, members of all three miRNA groups are heavily involved in distinct solid and hematological malignancies.

4 | DISCUSSION

Multiple myeloma is a complex and heterogeneous disease with diverse molecular profiles and clinical outcomes [30]. Despite advances in therapy, MM remains an incurable disease, highlighting the need for novel biomarkers that can assist in early diagnosis, prognostication, and treatment stratification [31, 32]. circRNAs have recently emerged as potential cancer biomarkers due to their stability, tissue specificity, and deregulation in cancer cells; in fact, circRNAs may serve as MM biomarkers, according to a number of studies [33]. Among the various circRNAs, ciRS-7 has been reported as a cancer biomarker in several malignancies, including hepatocellular carcinoma and renal cell carcinoma [34, 35]. However, the clinical significance of ciRS-7 in MM remains unknown.

Prompted by these, we aimed to investigate the clinical significance of ciRS-7 in MM and its potential as a molecular prognostic biomarker. First, we observed that ciRS-7 expression was significantly different among the 3 plasma cell neoplasm cell lines; in particular, ciRS-7 levels were much higher in the L-363 cell line. This finding provides insight into the natural variation of ciRS-7 expression among different cell types, which could reflect varying disease states or molecular subtypes. Next, we observed that ciRS-7 expression was significantly different between CD138+ plasma cells of MM patients versus SMM and MGUS patients, with the lowest levels being observed in MM patients. Furthermore, ciRS-7 expression effectively discriminated between MM and SMM patients, indicating its potential as a biomarker for differential diagnosis between these plasma cell dyscrasias. ciRS-7 expression levels could potentially aid in the early detection and timely intervention against MM, ultimately improving outcomes for patients with this challenging malignancy, including those with high-risk SMM.

Through survival analysis, we demonstrated that high ciRS-7 expression levels in CD138+ plasma cells are significantly associated with poor prognosis regarding both PFS and OS, as shown in the Kaplan-Meier curves. Additionally, the unfavorable prognostic value of high ciRS-7 levels particularly concerns patients with ISS III or R-ISS III stage MM. This is quite encouraging, considering the urgent need for a better stratification system capable of predicting the disease

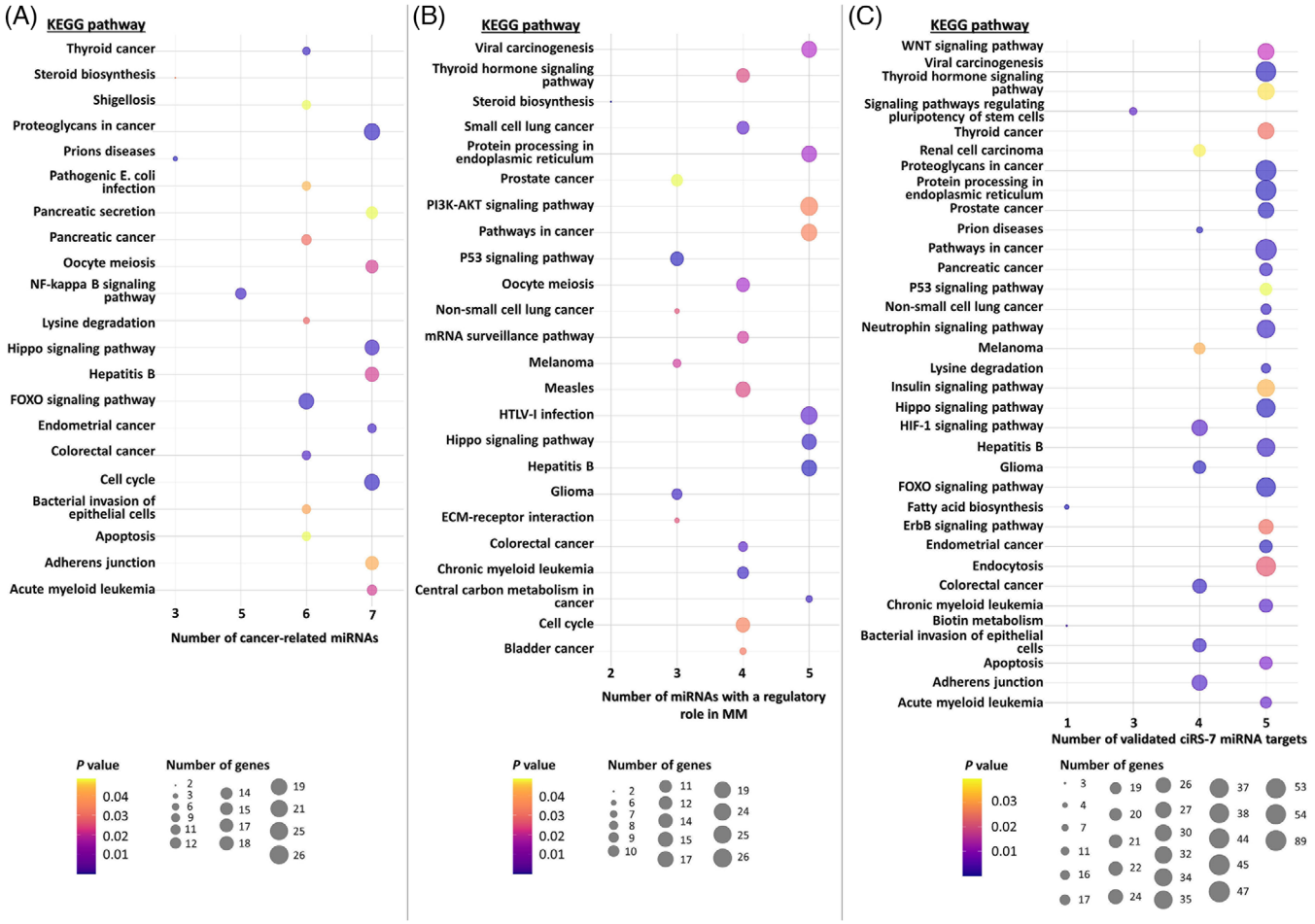

FIGURE 5 The significantly enriched the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathways revealed by the in silico functional analysis. This computational analysis included cancer-related microRNAs (miRNAs) from the query in miRDB, and more particularly those with deregulated expression in cancer (A), those with an established regulatory role in multiple myeloma (MM) (B), and those having previously been experimentally validated to be sponged by ciRS-7 (C). The size of each bubble shows the number of miRNA-targets of ciRS-7 related to each pathway, while the color of each bubble represents the *p*-value.

progression of patients in this stage. Notably, high ciRS-7 expression was associated with unfavorable prognosis independently of other established prognostic factors, such as R-ISS stage and age, which highlights its potential as a prognostic molecular biomarker. This finding might seem conflicting with the low levels of ciRS-7 in MM compared to MGUS and SMM. However, this is also the case with other unfavorable molecular biomarkers. For example, in colorectal adenocarcinoma, high mRNA levels of heat shock protein beta 3 (*HPSB3*) and clusterin (*CLU*) predict a poor prognostic outcome, and at the same time, they presented reduced expression levels in colorectal adenocarcinoma tissues compared with paired noncancerous tissues [36, 37]. Therefore, the elucidation of the functional role of ciRS-7 in MM progression is crucial in order to better comprehend the biological processes that lead to adverse outcomes in this disease.

The clinical utility of ciRS-7 as a biomarker in MM may be attributed to its role in cancer progression. By sponging miRNAs, ciRS-7 functions as a competing endogenous RNA controlling the expression of downstream target genes. Although the molecular mechanisms by which ciRS-7 promotes MM progression remain unclear, we performed functional in silico analysis, aiming to explore the potential targets and pathways affected by ciRS-7 in MM. Through this analysis, we found that ciRS-7 possesses binding motifs for several miRNAs, with 11 of them predicted by both the miRDB custom prediction tool and the CircInteractome web tool. Several of these miRNAs affect cell viability, migration, invasion, and drug resistance in MM. Some are also promising biomarkers; a prominent example is circulating miR-1246, which is found in exosomes and can be exploited for improved MM diagnosis [28]. Most miRNAs that have modulatory roles in cancer regulate signaling cascades (e.g., miR-876-5p and miR-641) [38, 39], are involved in therapy resistance (e.g., miR-6077) [40], or may serve as non-invasive molecular biomarkers in liquid biopsies since they are enriched in serum (e.g., miR-631) [41].

For these miRNAs that are implicated in MM or other human malignancies and the validated miRNA-targets of ciRS-7, significantly enriched KEGG pathways were obtained, including the PI3K/AKT, P53, FOXO, NF-κB, and Hippo signaling pathways. These signaling pathways possess important roles in the pathogenesis and/or treatment of MM [42–46]. Preclinical investigations have recently shown the critical

implication of the Hippo pathway in regulating apoptosis and mediating resistance in MM and other hematological malignancies; moreover, it is linked to the pathophysiology of MM-related bone disease [44]. The potential implication of ciRS-7 in regulating the Hippo signaling pathway has not been investigated yet. In this regard, this is a promising approach to understanding the underlying molecular interactions in MM. Moreover, findings from recent studies suggest that miRNAs are involved in the suppression of P53 in MM [42], and investigating the role of ciRS-7 in this interaction would aid in elucidating the physiologic impact of deregulated P53 levels in MM pathogenesis and treatment resistance. It is known that certain non-coding RNAs, even at low levels, can exert regulatory effects on gene expression [47–49]. Considering that the enriched KEGG pathways found in this study are consistent with prior studies on MM, ciRS-7 may be a promising target to affect the activity of these pathways.

Future applications of ciRS-7 as a molecular biomarker in MM are highly promising. The incorporation of a ciRS-7 assay into standard clinical practice is a possibility that could be achieved through the integration of the assessment of ciRS-7 expression levels into existing gene expression panels. However, to assess the feasibility of this proposal, it is essential to validate and optimize the assay in larger and more diverse patient cohorts. Additionally, conducting long-term follow-up studies will confirm the prognostic value of ciRS-7 and its usefulness in guiding treatment decisions over prolonged periods. Moreover, a larger cohort of MGUS and SMM patients in future studies would strengthen the results of the ROC analysis and the role of ciRS-7 as a biomarker of differential diagnosis. Furthermore, understanding the ciRS-7 function and its interaction with potential target miRNAs in MM may provide insights into the biology underlying this condition.

AUTHOR CONTRIBUTIONS

Maria Papatsirou contributed to the experimental design, performed experiments, analyzed and discussed data, and wrote the manuscript; Christos K. Kontos supervised the study, contributed to the experimental design, analyzed and discussed data, performed the biostatistical analysis, and edited the manuscript; Ioannis Ntanasis-Stathopoulos collected patients' clinical information and survival data, and edited the manuscript; Panagiotis Malandrakis, Foteini Theodorakakou, Christine-Ivy Liacos, Nefeli Mavrianou-Koutsoukou, Despina Fotiou, Maria Gavriatopoulou, Efstathios Kastritis, and Meletios A. Dimopoulos, provided tissue specimens; Andreas Scorilas provided reagents; Evangelos Terpos supervised the study, provided reagents and tissue specimens, and edited the manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data supporting the findings of this study are available upon reasonable request from the corresponding authors.

ETHICS STATEMENT

This study was approved by the Ethics Committee of the "Alexandra" General Hospital of Athens (protocol code 859/24-10-2017 and date of approval: October 25, 2017) and was conducted according to the principles of the 1964 Declaration of Helsinki.

PATIENT CONSENT STATEMENT

All patients provided written informed consent to participate in the study.

CLINICAL TRIAL REGISTRATION

The authors have confirmed clinical trial registration is not needed for this submission.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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