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ARTICLE

Exploring the molecular biomarker utility of circCCT3 in multiple myeloma: A favorable prognostic indicator, particularly for R-ISS II patients

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Abstract

Circular RNAs (circRNAs) are associated with the pathobiology of multiple myeloma (MM). Recent findings regarding circCCT3 support its involvement in the development and progression of MM, through microRNA sponging. Thus, we aimed to examine the expression of circCCT3 in smoldering and symptomatic MM and to assess its clinical importance. Three cell lines from plasma cell neoplasms were cultured and bone marrow aspirate (BMA) samples were collected from 145 patients with MM or smoldering MM. Next, $CD138^+$ enrichment was performed in BMA samples, followed by total RNA extraction and reverse transcription. Preamplification of circCCT3 and *GAPDH* cDNA was performed. Finally, a sensitive assay for the relative quantification of circCCT3 using nested real-time quantitative polymerase chain reaction was developed, optimized, and implemented in the patients' samples and cell lines. MM patients exhibited significantly higher intracellular circCCT3 expression in their $CD138^+$ plasma cells, compared to those from SMM patients. In addition, MM patients overexpressing circCCT3 had longer progression-free and overall survival intervals. The favorable prognostic significance of high circCCT3 expression in MM was independent of disease stage (either International Staging System [ISS] or revised ISS [R-ISS]) and age of MM patients. Interestingly, circCCT3 expression could serve as a surrogate molecular biomarker of prognosis in MM patients, especially those of R-ISS stage II. In conclusion, our study sheds new light on the significance of circCCT3 as a promising molecular marker for predicting MM patients' prognosis.

INTRODUCTION

Circular RNAs (circRNAs) are a subtype of noncoding RNAs that have recently gained increasing attention as crucial transcriptional and posttranscriptional regulators of gene expression.[1,2] Unlike linear RNAs, circRNAs form a covalently closed continuous loop structure that lacks 5′ and 3′ ends, through a mechanism called back-splicing.[3] Owing to their structure, circRNAs are more stable than linear RNAs, as well as more resistant to exonucleolytic activity.[4] Their most well-investigated function is the regulation of gene expression, mostly by sponging microRNAs (miRNAs) and RNA-binding proteins.[5] According to recent research, circRNAs have been linked to several biological processes, including cell proliferation, differentiation, apoptosis, and migration.[5–7] Moreover, circRNAs are intricately implicated in the initiation and development of cancer.[7] In particular, numerous circRNAs have been found to control cancer-related pathways like the phosphoinositide 3-kinase/AKT serine/threonine kinase 3 (AKT3), mitogen-activated protein kinase/extracellular-signal-regulated kinase, and nuclear factor-κB pathways, and to be up- or downregulated in various cancer types, including multiple myeloma (MM).[6,8] As a result, circRNAs have been suggested as potential diagnostic and prognostic biomarkers for cancer, including MM, as well as therapeutic targets.[9,10]

MM is a hematological malignancy that arises from the clonal expansion of malignant plasma cells in the bone marrow.[11] During the course of the disease, monoclonal immunoglobulin builds up in the blood and urine, ultimately leading to complications, such as

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hypercalcemia, renal impairment, bone disease, and immunosuppression.[12] To diagnose MM, clinical manifestations, blood results, imaging tests, and bone marrow biopsy are utilized.[12,13] The precursor states of MM include two stages: monoclonal gammopathy of undetermined significance (MGUS) and smoldering MM (SMM). MGUS is a benign condition where the monoclonal protein is present, but not any myeloma-defining symptoms. Whereas SMM also lacks myeloma-defining events, it exhibits monoclonal gammopathy and higher levels of plasma cells than MGUS.[14,15] While neither condition has a high risk of progression to MM, SMM is more likely than MGUS to develop into active MM.[16] Treatment of MM is currently based on chemotherapy, immunomodulatory agents, proteasome inhibitors, stem cell transplantation, and radiation therapy.[17] However, MM is regarded as an incurable malignancy, and patients frequently encounter relapses and drug resistance leading to a poor prognosis, despite the fact that these treatments can lead to remission.[15] Therefore, to better manage MM and increase patients' survival, it is necessary to identify reliable biomarkers. Sensitive and accurate MM biomarkers can support early diagnosis, prognosis, monitoring, and therapy decision-making.

There are numerous categories of biomarkers, including protein-based, genetic, and RNA-based ones; circRNAs are considered a class of biomolecules with promising potential for cancer detection and prognosis, among RNA-based biomarkers.[18–21] Especially in MM, circRNAs are often considered preferable biomarkers, due to their stability in blood and other bodily fluids, as well as to their capacity to reflect the individualized disease biology and therapy response.[8,22] Many circRNAs are deregulated in MM and may serve as novel biomarkers for this malignancy. Among these, circCCT3 is such an example. This circRNA is produced from the chaperonin containing TCP1 subunit 3 (*CCT3*) gene; it was found upregulated in MM patients' samples compared to normal samples.[23] Mechanistically, it enhances MM proliferation and metastatic potential by sponging miR-610, ultimately leading to the upregulation of the AKT3. However, apart from its role as a competitive endogenous RNA, the prognostic utility of circCCT3 in MM has not been examined yet.

To assess the clinical importance of this circRNA, we set out to examine the expression levels of circCCT3 in the $CD138^+$ plasma cells of patients with MM and compare them to those of patients with SMM. For this reason, MM cell lines were cultured and 145 bone marrow aspirate (BMA) samples were collected from patients with plasma cell disorders, as well. Quantification of circCCT3 was performed via a sensitive assay of nested real-time quantitative polymerase chain reaction (qPCR) using divergent primers. Our study demonstrates that plasma cells from MM patients have significantly higher levels of circCCT3, compared to SMM patients. Additionally, circCCT3 functions as a favorable prognostic factor in MM, independently of the International Staging System (ISS), revised ISS (R-ISS), and age of MM patients. Further investigation and validation of these results are required, to prove the clinical value of circCCT3 and incorporate it into standard clinical practice.

METHODS

Patient samples and data collection

At the Department of Clinical Therapeutics of the "Alexandra" General Hospital of Athens (Athens, Greece), 145 BMA samples were collected from adult patients with plasma cell dyscrasias, at the time of diagnosis. Patients who had previously undergone any type of treatment or who had any other concurrent malignancy were not included in this study. To ascertain whether cytogenetic aberrations including del(17p), t(4;14), t(14;16), t(11;14), and (+1q) were present, fluorescence in situ hybridization was used. Whole-body low-dose computed tomography was used to assess the degree of osteolysis. Each participant in this study provided written informed consent. The study was conducted according to the guidelines of the Declaration of Helsinki and 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).

Propagation of cell lines

Three cell lines derived from plasma cell neoplasms were purchased from the American Type Culture Collection (ATCC®) and propagated at 37°C and 5% CO_2 concentration. More specifically, the H929 (plasmacytoma), U266 (MM), and L-363 (plasma cell leukemia) cell lines were cultured in RPMI-1640 supplemented with 2 mM L-glutamine, 100 U/mL penicillin/streptomycin, and 10% fetal bovine serum, except the L-363 cell line for which the fetal bovine serum was higher (15%). All supplies for cell culture were purchased from Biowest.

$CD138^+$ plasma cell isolation

Given that the expression of the cell surface antigen syndecan-1 (CD138) is an important marker of plasma cells in the bone marrow,[24] $CD138^+$ selection among mononuclear cells was carried out, to isolate most of the plasma cells from the BMA samples of all patients included in this study. For this purpose, 10 mL of BMA from each subject was collected in ethylenediaminetetraacetic acid (EDTA). The $CD138^+$ enrichment procedure was immediately performed. Thus, Ficoll-Paque separation of the BMA mononuclear cells was followed by the selection of $CD138^+$ plasma cells using anti-CD138-coated magnetic beads (Miltenyi Biotech).

RNA isolation and first-strand complementary DNA (cDNA) synthesis

The H929, U266, and L-363 cell lines were harvested when grown to the suggested ATCC® cell concentration, and total RNA was extracted from the three cell lines and the $CD138^+$ plasma cells of patients by using the TRI Reagent® (Molecular Research Center Inc.). Agarose gel electrophoresis was used to determine any degree of degradation of each RNA extract, and a Qubit 2.0 Fluorometer (Invitrogen, Thermo Fisher Scientific Inc.) was used to calculate the concentration. Then, using the MMLV reverse transcriptase (Invitrogen, Thermo Fisher Scientific Inc.), 125 ng of the total RNA extracts were subjected to first-strand cDNA synthesis with random hexamer primers as per the manufacturer's instructions.

Quantification of circCCT3 expression using nested real-time qPCR

To selectively amplify and quantify circCCT3, we designed two pairs of divergent primers that were specific for this circRNA. Thus, we ensured that only circular transcripts could be amplified, and not linear counterparts; the accurate amplification of circCCT3 was also achieved by designing a primer that is complementary to the back-splice junction of this circRNA. Moreover, convergent primers were designed for *GAPDH* mRNA (internal control). The sequences of all primers used in these assays are shown in Table 1. Then, a SYBR Green-based real-time qPCR assay with a preamplification step was developed and optimized to measure the expression of circCCT3. Next, the cDNAs from all cell lines

TABLE 1 The primer pairs were used for the preamplification and the nested qPCR assays were for the amplification of circCCT3 and *GAPDH* cDNAs

Target	Assay	Primer direction	Primer sequence (5′ → 3′)	T_a (°C)
circCCT3	Preamplification	Sense	TCTGCTCGTCTTCCAACATC	58
		Antisense	GCTCAGGATTATCTGGAAGACC	
	Nested qPCR	Sense	TACCCAGTCTTCCATCAACTGG	60
		Antisense	ACACAGGTGCCATCGGAAAC	
GAPDH	Preamplification	Sense	CCACATCGCTCAGACACCAT	60
		Antisense	TGACAAGCTTCCCGTTCTCA	
	Nested qPCR	Sense	ATGGGGAAGGTGAAGGTCG	60
		Antisense	GGGTCATTGATGGCAACAATATC	

Abbreviations: qPCR, quantitative polymerase chain reaction; T_a, annealing temperature.

and samples were subjected to a first-round regular PCR. Following this, nested qPCR assays were performed using an inner (second) pair of primers for each amplicon and 0.5 μL of diluted PCR products as a template. All first-round PCR experiments were carried out in an Applied Biosystems MiniAmp Thermal Cycler, and a QuantStudio 5 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific Inc.) was used for all qPCR assays.

The qPCR products of circCCT3 from the three cell lines were purified using spin columns and the Gel and PCR Clean-up kit (Macherey-Nagel GmbH & Co. KG). Then, two-sided Sanger sequencing was performed on the purified qPCR products, to verify the circCCT3 amplicon sequence. For the analysis of sequencing results, the determined sequences were manually annotated. The comparative threshold cycle (C_t) method ($2^{-\Delta\Delta C_t}$) was used to calculate the relative levels of circCCT3[25,26]; as aforementioned, *GAPDH* served as a reference gene and the L-363 cell line as a calibrator. In each patient's sample, the normalized circCCT3 expression was measured in relative quantification units (RQUs).

Biostatistical analysis

The SPSS® statistical software suite (version 28) was used to conduct extensive biostatistical analysis. The nonparametric Mann–Whitney *U* test was used to compare circCCT3 expression between MM and SMM patients as well as between MM patients' subgroups since the distributions of circCCT3 expression levels were nonnormal. Additionally, χ^2 tests were used to examine possible associations between the expression status of circCCT3 and other categorical factors; Fisher's correction was performed where appropriate. To determine whether circCCT3 can efficiently distinguish between patients with MM and SMM, receiver operating characteristic (ROC) analysis was conducted, and the Hanley and McNeil method was used to assess the area under the ROC curve (AUC).

After that, survival analysis was performed, to evaluate the prognostic potential of circCCT3 expression in MM. For this purpose, the 110 MM patients were categorized in either of two groups, based on the expression levels of circCCT3 (high vs. low circCCT3 expression). The X-tile software, which generates the ideal prognostic cut-off point using the least *p* value approach,[27] was used to establish the best cut-off point. Next, univariate and multivariate Cox regression analyses were carried out; the univariate and multivariate Cox regression models were bootstrapped with 1000 samples, and the bias-corrected and accelerated (BCa) 95% confidence interval (CI) of each hazard ratio (HR) was estimated. Bootstrapping was considered necessary in our study since it provides more stable and accurate estimates of statistical parameters and ensures that the conclusions are not overly influenced by potential outliers. Moreover, Kaplan–Meier survival analysis was performed after stratification of MM patients in subgroups based on ISS and R-ISS staging, and the respective survival curves were built. Differences between the Kaplan–Meier curves were assessed using the Mantel–Cox (log-rank) test.

Only *p* values lower than 0.050 were considered significant in all statistical analyses.

RESULTS

Baseline characteristics of the MM and SMM patients

The MM patient cohort consisted of 110 newly diagnosed cases. The median age of MM patients at diagnosis was 70 years, ranging from 35 to 93 years. Regarding the 35 SMM patients included in our study, they had a median age of 68 years at diagnosis, ranging from 49 to 86 years. Table 2 summarizes the baseline clinicopathological characteristics of MM and SMM patients included in this study, as well as the treatment regimens of the MM patients.

Development of a nested real-time qPCR assay for the relative quantification of circCCT3 expression

The circCCT3 amplicon sequence was verified by Sanger sequencing and its back-splice junction was annotated (Figure 1A). Then, a sensitive, nested real-time qPCR assay, preceded by a preamplification step, was designed and optimized for the accurate quantification of circCCT3 in samples of low total RNA mass. For this purpose, we performed standardization experiments concerning cDNA input, primer concentration, annealing temperature, $MgCl_2$ concentration, number of thermal cycles during the first PCR, and dilution of the preamplified template used in the nested real-time qPCR. Next, triplicate reactions of serial dilutions of the L-363 cDNA were used to generate specific melt curves for circCCT3 and *GAPDH* amplicons, to validate the qPCR efficiencies. The unique melt curve of each amplicon served as evidence of the specificity of the assay, and the mean C_t values were plotted against the log of L-363 cDNA quantity (Figure 1B).

The expression levels of circCCT3 differ significantly between MM and SMM patients' $CD138^+$ plasma cells

A notable upregulation of intracellular circCCT3 expression in the $CD138^+$ plasma cells was observed between patients with MM and

TABLE 2 Biological and clinicopathological characteristics of the MM and SMM patients, and treatment of the MM patients

Variable	Number of patients (%) MM	SMM
Gender		
Male	66 (60.0)	22 (62.9)
Female	44 (40.0)	13 (37.1)
M-protein isotype		
Heavy chain		
IgA	23 (20.9)	10 (28.6)
IgG	63 (57.3)	25 (71.4)
IgD	2 (1.8)	–
IgM	2 (1.8)	–
None detected	20 (18.2)	–
Light chain		
Kappa light chain	74 (67.3)	22 (62.9)
Lambda light chain	33 (30.0)	13 (37.1)
None detected	3 (2.7)	–
Bone marrow plasma cell infiltration		
<20%	8 (7.3)	20 (57.1)
20%–40%	18 (16.4)	10 (28.6)
>40%	84 (76.3)	5 (14.3)
Serum β_2 microglobulin (mg/L)		
<3.5	33 (30.0)	25 (71.4)
3.5–5.4	31 (28.2)	9 (25.7)
≥5.5	46 (41.8)	1 (2.9)
Serum albumin (g/dL)		
<3.5	30 (27.3)	1 (2.9)
≥3.5	80 (72.7)	34 (97.1)
Lactate dehydrogenase		
Normal (≤225 U/L)	87 (79.1)	32 (91.4)
Elevated (>225 U/L)	23 (20.9)	3 (8.6)
2/20/20 risk stratification model for SMM[a]	N/A	
Low risk		17 (48.6)
Intermediate risk		14 (40.0)
High risk		4 (11.4)
Cytogenetic abnormalities		N/A
del(17p)		
Absence	92 (83.6)	
Presence	18 (16.4)	
t(4;14)		
Absence	96 (87.3)	
Presence	14 (12.7)	
t(14;16)		
Absence	107 (97.3)	
Presence	3 (2.7)	
Cytogenetic risk		N/A
Standard	85 (77.3)	
High	25 (22.7)	
ISS stage		N/A
I	30 (27.3)	
II	34 (30.9)	
III	46 (41.8)	
R-ISS stage		N/A
I	23 (20.9)	
II	62 (56.4)	
III	25 (22.7)	
WBLDCT osteolysis (82/110 patients)		N/A
No	28 (34.1)	
Yes	54 (65.9)	
Frontline therapy		N/A
Anti-CD38	18 (16.4)	
Immunomodulatory imide drug (IMiD)	7 (6.4)	
Proteasome inhibitor (PI)	12 (10.9)	
Combined IMiD and PI	73 (66.3)	
Bisphosphonate treatment (77/110 patients)		N/A
No	36 (46.8)	
Yes	41 (53.2)	

Abbreviations: ISS, International Staging System; MM, multiple myeloma; 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 three 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%.

those with SMM ($p = 0.022$) (Figure 2A). In more detail, the median value of circCCT3 expression in MM patients' plasma cells was fivefold higher than in SMM patients' plasma cells (Table 3). In accordance with these findings, the ROC curve analysis demonstrated that circCCT3 expression may successfully distinguish MM from SMM patients (AUC = 0.63, 95% CI = 0.51–0.74, $p = 0.022$) (Figure 2B). On the other hand, circCCT3 expression did not differ significantly among the three tested cell lines (H929, U266, and L-363).

Next, MM patients were categorized into two groups, according to the expression of circCCT3 in their $CD138^+$ plasma cells: high versus low. The cut-off value was set at 11.8 RQU, namely the 44th percentile, which was shown to be the optimal one, based on the X-tile software. Using the χ^2 test, circCCT3 expression status was shown not to be significantly associated with any tested cytogenetic abnormality, including del(17p), t(4;14), t(14;16), t(11;14), and (+1q), as well as with the occurrence of osteolytic lesions, the presence of bone disease, or the front-line treatment of MM patients (data not shown).

Considering that the *CCT3* gene that produces circCCT3 is located on chromosome 1, we also checked whether its expression is associated with (+1q). According to the Mann–Whitney *U* test, the difference in circCCT3 levels between MM patients with (+1q) and those not having this cytogenetic abnormality was rather not statistically significant; moreover, circCCT3 levels were lower in MM patients with (+1q) (Supporting Information S1: Table 1).

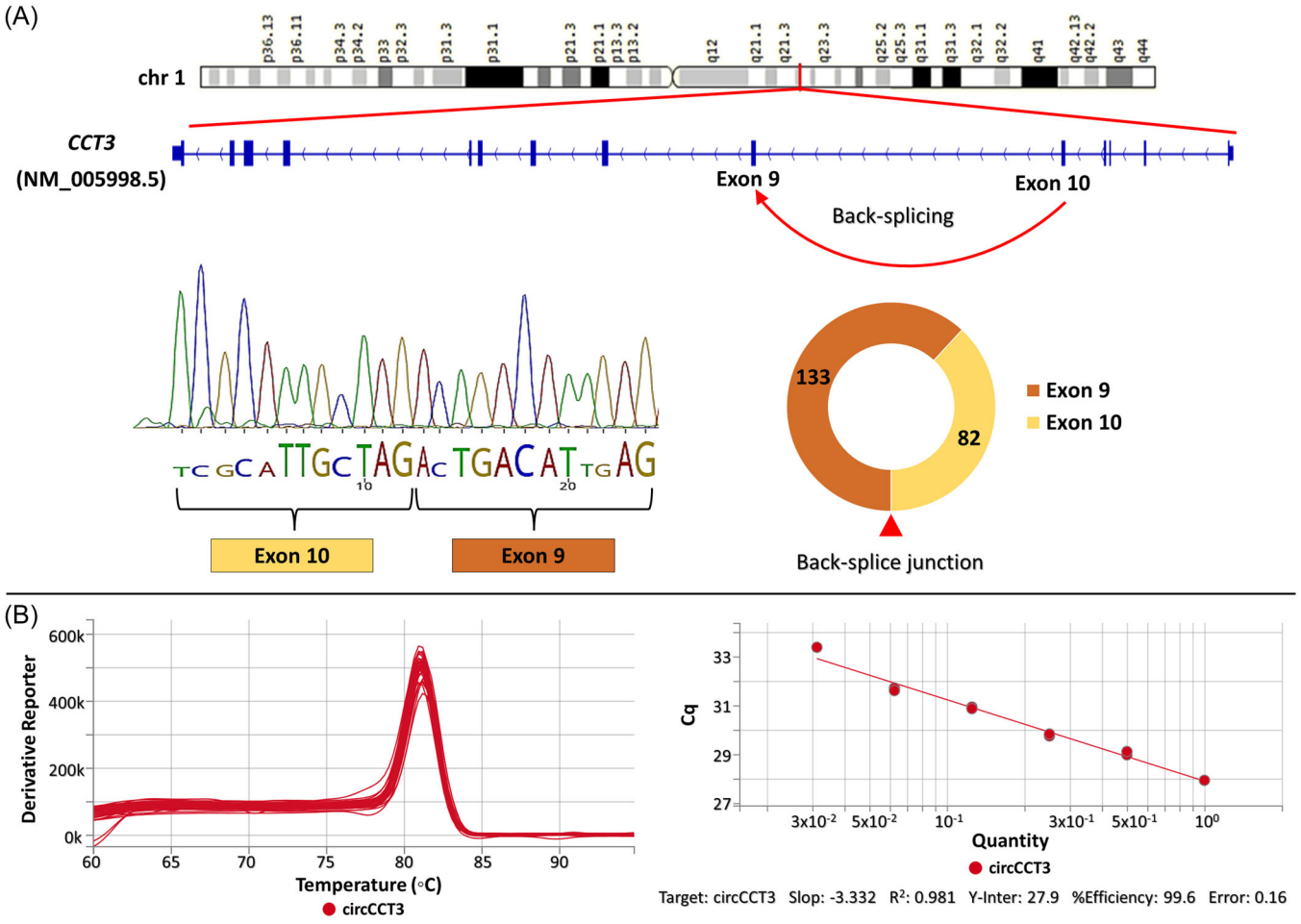

FIGURE 1 Design and optimization of the nested real-time qPCR assay for the accurate relative quantification of circCCT3. (A) Confirmation of the back-splice junction of circCCT3 between the 3′ end of the *CCT3* exon 10 (donor site) and the 5′ end of the *CCT3* exon 9 (acceptor site), based on Sanger sequencing results (illustrated using Geneious Prime). (B) The melt curve plot for the circCCT3 amplicon that was produced by nested real-time qPCR, and the standard curve plot for the amplification of circCCT3. The developed and optimized qPCR assay exhibited a 99.6% efficiency of circCCT3 amplification. qPCR, quantitative polymerase chain reaction.

High circCCT3 expression constitutes a favorable prognostic factor in MM, independent of R-ISS staging and patient age

The univariate Cox regression analysis uncovered the favorable prognostic value of circCCT3 overexpression in MM (Table 4), revealing a significantly lower risk for progression and MM-related death of patients with high circCCT3 expression levels (for progression-free [PFS]: HR = 0.48, 95% CI = 0.26–0.87, $p = 0.015$; for overall survival [OS]: HR = 0.26, 95% CI = 0.093–0.72, $p = 0.010$). Additionally, multivariate Cox regression analysis showed that the prognostic value of circCCT3 expression is independent of the R-ISS stage and age of MM patients (Table 4), with regard to both PFS (HR = 0.43, 95% CI = 0.23–0.78, $p = 0.005$) and OS (HR = 0.24, 95% CI = 0.090–0.66, $p = 0.010$). Bootstrapping based on 1000 samples strengthened the validity of these results.

Furthermore, we found that circCCT3 expression could be exploited as a surrogate molecular biomarker of prognosis, particularly in MM patients of R-ISS stage II, as it can further stratify patients of this prognostic group in two distinct subgroups with substantially different prognoses. More specifically, the subgroup of R-ISS II patients with high intracellular circCCT3 expression was shown to have better PFS and OS cumulative probabilities than R-ISS II patients exhibiting low expression of this circRNA in their bone marrow $CD138^+$ plasma cells ($p = 0.002$ and $p = 0.010$, respectively) (Figure 3A,B). Interestingly, we observed that the PFS and OS of the MM patients' subgroup with R-ISS II disease and concomitant high expression levels of circCCT3 were represented by survival curves that were very similar to those of R-ISS I patients (particularly with regard to OS), whereas the MM patients' subgroup with R-ISS II disease and low intracellular circCCT3 expression exhibited survival curves very similar to those of R-ISS III patients (Figure 3C,D).

DISCUSSION

Noncoding RNAs, such as miRNAs, long noncoding RNAs, and circRNAs, have gained increased attention in recent years due to their function as key regulators of gene expression.[1,28,29] In particular, circRNAs are considered crucial regulators of major biological processes, like cell proliferation and differentiation, apoptosis, and cell

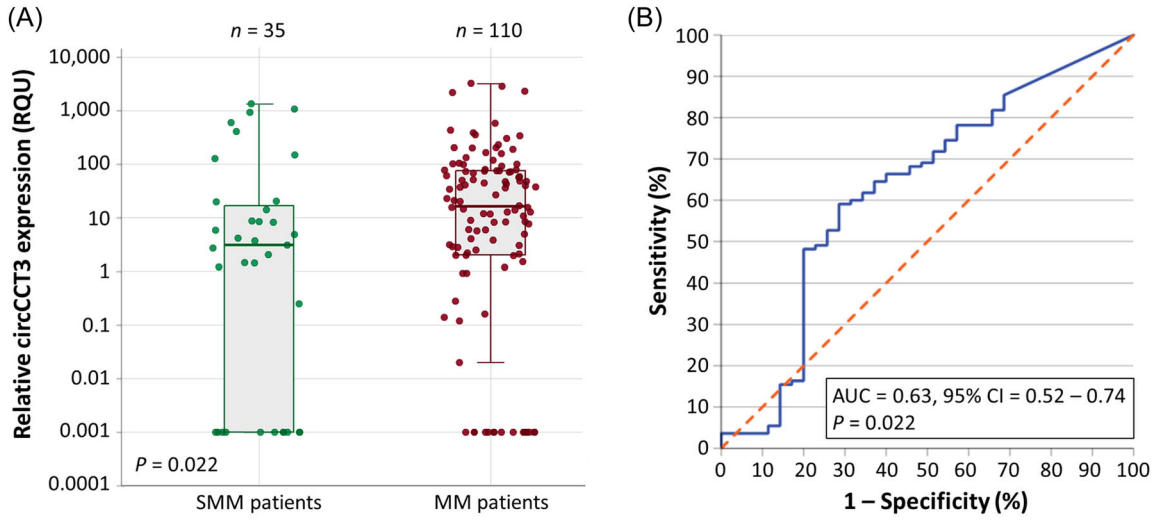

FIGURE 2 The differential expression of circCCT3 between MM and SMM. (A) Scattered boxplots of intracellular circCCT3 expression levels in CD138$^+$ plasma cells from patients with SMM and MM. Each data point represents an individual value; the line inside each box denotes the median value of each distribution, while the lower and upper box limits denote the 25th and 75th percentiles, respectively. The *y*-axis is on a $\log_{10}$ scale. The *p* value was calculated using the Mann–Whitney *U* test. (B) ROC curve illustrating the ability of circCCT3 expression to efficiently distinguish MM patients from SMM patients. AUC, area under the ROC curve; CI, confidence interval; MM, multiple myeloma; ROC, receiver operating characteristic; SMM, smoldering multiple myeloma.

TABLE 3 Distribution of circCCT3 expression in CD138$^+$ plasma cells of patients with MM or SMM

Patient diagnosis	circCCT3 expression[a] (mean ± SEM)	Range	Percentiles		
			25th	50th (median)	75th
MM (*n* = 110)	154.1 ± 48.19	0.001–3.261	1.99	16.24	76.25
SMM (*n* = 35)	135.8 ± 56.23	0.001–1.348	0.001	3.07	19.86

Abbreviation: SEM, standard error of the mean.

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

signaling.[6,30] Mainly due to their role as miRNA sponges, circRNAs are implicated in the regulation of the hallmarks of cancer, and their deregulation can affect the division rate, metastatic potential, and therapeutic sensitivity of cancer cells.[5,31,32] There have already been several studies that examine circRNAs as potential prognostic and diagnostic biomarkers in MM and investigate their mechanism of action[32,33]; circCCT3 is such an indicative example. The oncogenic role of circRNAs from the *CCT3* gene has also been demonstrated in other malignancies, such as non-small-cell lung cancer and pancreatic cancer.[34,35] Based on these, we aimed to investigate the expression levels of circCCT3 in plasma cell dyscrasias and shed light on its potential as a molecular biomarker in MM.

According to our findings, circCCT3 is significantly down-regulated in CD138$^+$ plasma cells of SMM patients, compared to samples from MM patients. Moreover, based on ROC analysis, the expression of circCCT3 can successfully distinguish between cases of SMM and MM. These findings are both intriguing and important, showcasing that there is a gradual increase in circCCT3 levels during the multistep process of myelomagenesis. Moreover, as circCCT3 may be a noninvasive and easily measured biomarker for the early diagnosis of MM, these results emphasize the potential of circRNAs as prognostic indicators for MM. Considering that circRNAs are abundant in bodily fluids and extracellular vesicles,[36,37] the detection of circCCT3 via liquid biopsy could easily provide insights regarding an MM patient. The early detection of MM is essential, since it is frequently asymptomatic or manifests with vague symptoms in its early stages, which can delay detection and result in more advanced disease at the time of diagnosis.[17,38] The likelihood of a successful treatment plan may be decreased and the patient's quality of life may be negatively impacted by a delayed diagnosis. Therefore, monitoring circCCT3 levels of SMM, especially high-risk SMM patients, could aid in the early diagnosis of MM. In this way, early treatment onset could be decided, thus raising the likelihood of full remission, decreasing the risk of complications, and improving the patient's OS. Additionally, early detection of MM allows for better tracking of the disease development and response to therapy, allowing for timely alterations in therapy to improve treatment results.

Moreover, the Kaplan–Meier survival analysis showed that MM patients with high circCCT3 expression had higher OS and PFS probabilities, indicating that circCCT3 expression may be a valuable prognostic factor for MM. These findings further highlight the clinical significance of circCCT3 as a biomarker for MM, since the stratification of MM patients according to the ISS and R-ISS stages showed that those with low expression levels of circCCT3 and ISS II or R-ISS II stages had significantly shorter PFS time intervals than those with high expression levels of circCCT3. This result could be particularly valuable for clinicians since it could guide treatment choices and help identify individuals who would benefit from more aggressive therapy interventions. More specifically, by enabling more precise risk assessment and individualized treatment strategies, the ability to distinguish between patients with high and low circCCT3 expression may contribute to an overall improvement in the management of

TABLE 4 Cox regression analyses of MM patients' progression-free (PFS) and overall survival (OS)

Covariate	Univariate analysis					Multivariate analysis				
	HR	95% CI	*p* value	BCa bootstrap 95% CI	Bootstrap *p* value	HR	95% CI	*p* value	BCa bootstrap 95% CI	Bootstrap *p* value
PFS										
circCCT3 expression										
Low	1.00					1.00				
High	0.48	0.26–0.87	*0.015*	0.25–0.90	*0.017*	0.43	0.23–0.78	*0.005*	0.21–0.78	*0.013*
ISS stage			*0.005*							
I	1.00									
II	0.82	0.31–2.12		0.24–2.22	0.68					
III	2.47	1.16–5.23		1.23–5.84	*0.010*					
R-ISS stage			0.14					0.090		
I	1.00					1.00				
II	1.93	0.79–4.72		0.89–6.84	0.11	2.18	0.88–5.41		1.01–7.23	0.061
III	2.64	1.01–6.88		1.11–10.20	*0.027*	2.96	1.12–7.82		1.25–11.32	*0.019*
Age	1.01	0.99–1.04	0.31	0.99–1.05	0.33	1.30	0.67–2.54	0.44	0.61–2.72	0.46
OS										
circCCT3 expression										
Low	1.00					1.00				
High	0.26	0.093–0.72	*0.010*	0.056–0.69	*0.006*	0.24	0.085–0.66	*0.006*	0.048–0.57	*0.004*
ISS stage			0.29							
I	1.00									
II	0.98	0.25–3.94		0.15–6.62	0.98					
III	2.05	0.65–6.45		0.68–12.07	0.19					
R-ISS stage			0.19					0.11		
I	1.00					1.00				
II	2.23	0.49–10.19		$0.70–9.55 \times 10^4$	0.18	2.76	0.60–12.80		$0.76–1.22 \times 10^5$	0.099
III	4.01	0.83–19.32		$1.00–1.35 \times 10^5$	*0.037*	5.10	1.04–25.05		$1.20–1.94 \times 10^5$	*0.031*
Age	1.02	0.98–1.07	0.30	0.98–1.07	0.25	0.80	0.31–2.05	0.64	0.30–2.36	0.63

Note: Italics indicate a statistically significant *p* value ($p < 0.050$).

Abbreviations: BCa, bias-corrected and accelerated; CI, confidence interval; HR, hazard ratio; ISS, International Staging System; R-ISS, revised International Staging System.

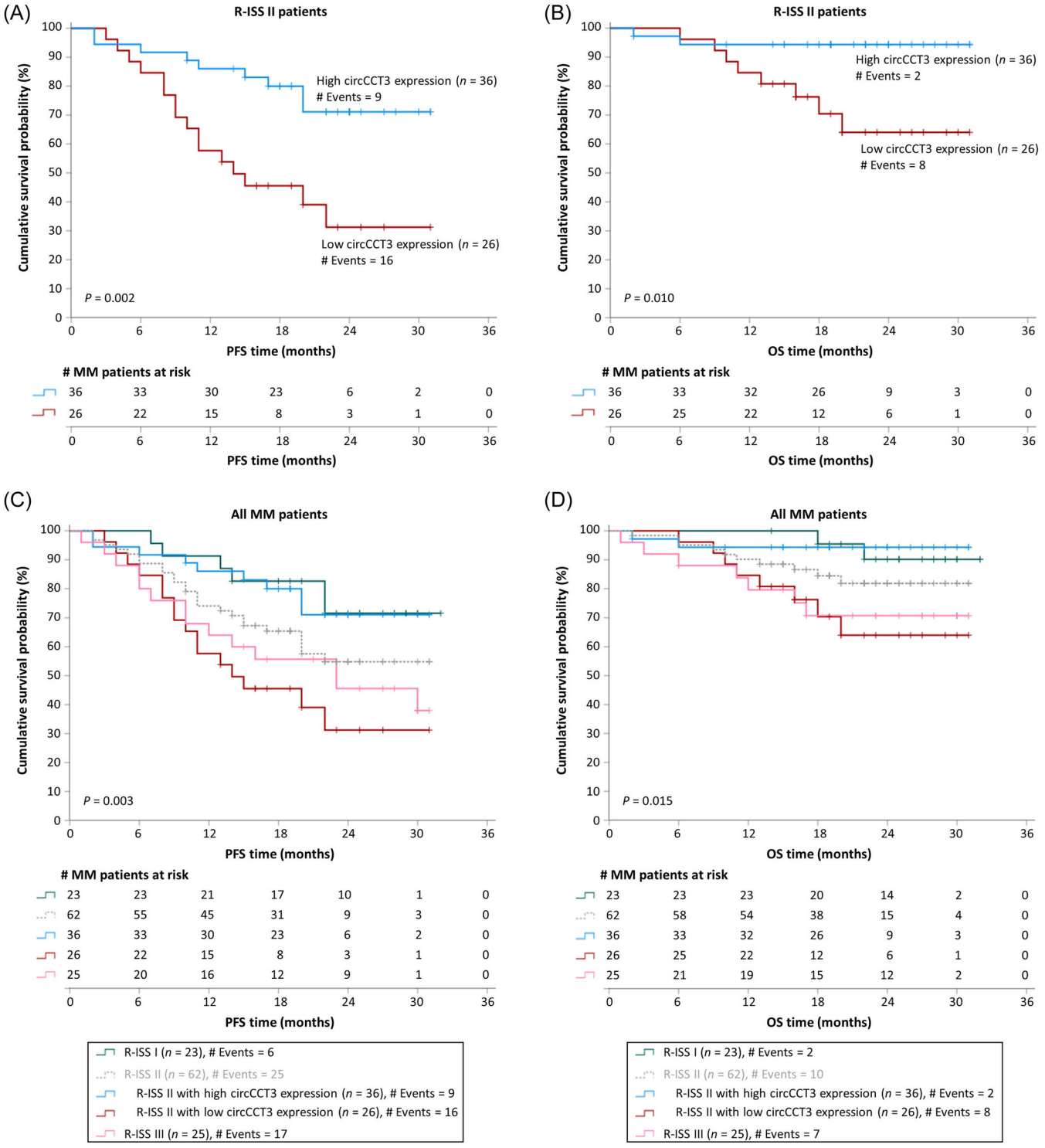

FIGURE 3 Kaplan–Meier survival curves for the PFS and OS of MM patients, stratified based on the R-ISS stage. circCCT3 expression may effectively separate the group of R-ISS II patients into two subgroups with substantially different prognoses, with regard to both PFS (A) and OS (B). In particular, the PFS (C) and OS (D) of R-ISS II patients with circCCT3 overexpression in bone marrow CD138$^+$ plasma cells were similar to those of R-ISS I patients, respectively; on the other hand, R-ISS II patients with low intracellular circCCT3 expression had cumulative survival probabilities similar to those of R-ISS III patients. ISS, International Staging System; MM, multiple myeloma; OS, overall survival; PFS, progression-free survival; R-ISS, revised International Staging System.

MM. For instance, patients with MM and low circCCT3 expression might benefit from more aggressive therapy or tighter monitoring, whereas individuals with MM and high circCCT3 expression might be able to benefit from less intensive therapies with similar results. It should be highlighted that we found no association between cytogenetic abnormalities or the existence of bone disease and circCCT3 expression, indicating that circCCT3 might not be a general biomarker for all aspects of MM etiology.

In addition, the importance of adding circCCT3 expression levels into current predictive tools is underlined by the intriguing finding that MM patients with R-ISS II disease who highly express circCCT3 in plasma cells had survival probabilities comparable to those of R-ISS I MM patients, while low expression of circCCT3 in MM patients with R-ISS II MM correlated with survival rates similar to those of R-ISS III MM. To distinguish MM patients who are more likely to have disease progression, R-ISS staging can be combined with circCCT3 expression as a prognostic biomarker. Furthermore, this finding provides more evidence that circCCT3 has a potential clinical significance in MM treatment. Since R-ISS III patients are considered to have the poorest prognosis,[39] circCCT3 expression may help to identify high-risk R-ISS II MM patients who require more aggressive treatment. Thus, the use of circCCT3 expression levels in clinical decision-making could help to improve patient outcomes by allowing clinicians to tailor treatment to individual patient risk levels. This could be achieved by integrating the quantification of circCCT3 expression levels into existing gene expression panels. Specifically, our circCCT3 assay could be incorporated into existing qPCR assays that are widely used in clinical laboratories; this integration would allow for the simultaneous assessment of circCCT3 alongside other relevant biomarkers routinely used for MM patients' prognosis and risk stratification.

To date, clinical considerations including age and comorbidities have been used to determine the best course of treatment for MM patients.[40] Owing to the broad development, validation, and clinical use of molecular technologies like next-generation sequencing, numerous prognostic biomarkers for PFS, OS, and therapy response have been discovered.[15,41] The results of this study demonstrate the potential prognostic significance of circCCT3 in MM, which was independent of the ISS, R-ISS, and age of MM patients, and its low expression levels were a strong indicator of poor OS and PFS. The R-ISS staging is presently the most effective method for MM survival prediction.[42] The largest and most diverse class, accounting for 62% of the entire MM population, is R-ISS stage II.[43] For this reason, molecular biomarkers that can further categorize patients with R-ISS stage II in terms of their prognosis are still needed, and circCCT3 is a potential predictive biomarker that might be able to determine how these individuals' prognoses would turn out. The incorporation of molecular biomarkers into the standard diagnostic workup of patients will enable the adoption of individualized, biologically based therapies, hence contributing to the ongoing improvement of myeloma patient outcomes. It is possible to imagine that in the next few years, the rapid developments in molecular biomarker detection will improve the current staging systems and circRNAs will be given greater weight when more data become available and our understanding of how these molecules reflect myeloma biology grows.

Regarding the future directions of this study, several steps need to be taken to assess the implementation of a circCCT3 assay into a standard clinical routine. The measurement of circCCT3 expression levels could be integrated into existing gene expression panels; however, to address the practicality of this suggestion, validation and optimization of the assay in larger cohorts and diverse populations are needed. This validation will be crucial for ensuring the reproducibility and accuracy of the assay in real-world clinical settings. In addition, conducting long-term follow-up studies will validate the prognostic significance of circCCT3 and its utility in guiding treatment decisions over extended periods. The comparison of a circCCT3 assay with other established molecular prognostic factors used in MM is also essential to evaluate the performance of circCCT3 as an independent prognostic factor and determine its potential as a complementary or superior marker to existing gene expression-based approaches. To achieve this, comprehensive comparative studies with well-established gene expression-based prognostic factors using well-characterized MM patient cohorts should be conducted. Examples of molecular prognostic factors that could be used are those derived from gene expression profiling or gene panels associated with MM prognosis,[44,45] as well as the GEP70 signature that utilizes the expression levels of 70 genes to categorize patients as high- or low-risk ones.[46]

In summary, the finding that circCCT3 expression significantly affects the prognostic impact of R-ISS staging highlights the importance of assessing circCCT3 expression levels in MM patients. Incorporating circCCT3 expression levels into existing prognostic tools may help to improve the accuracy of risk stratification and clinical decision-making for MM patients in the near future.

AUTHOR CONTRIBUTIONS

Christos K. Kontos and Evangelos Terpos: Conceptualization; **Christos K. Kontos and Diamantis C. Sideris:** Methodology. **Christos K. Kontos:** Software. **Maria Papatsirou:** Validation. **Maria Papatsirou and Christos K. Kontos:** Formal analysis. **Maria Papatsirou:** Investigation. **Ioannis Ntanasis-Stathopoulos, Panagiotis Malandrakis, Despina Fotiou, Christine-Ivy Liacos, Maria Gavriatopoulou, Efstathios Kastritis, Meletios A. Dimopoulos, and Evangelos Terpos:** Resources. **Christos K. Kontos:** Data curation. **Maria Papatsirou:** Writing—original draft preparation. **Christos K. Kontos:** Writing—review and editing. **Maria Papatsirou and Christos K. Kontos:** Visualization. **Christos K. Kontos:** Supervision. **Andreas Scorilas and Evangelos Terpos:** Project administration. **Andreas Scorilas and Evangelos Terpos:** Funding acquisition. All authors have read and agreed to the published version of the manuscript.

CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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SUPPORTING INFORMATION

Additional supporting information can be found in the online version of this article.

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