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ORIGINAL RESEARCH

hsa_circRNA_101237: A Novel Diagnostic and Prognostic Biomarker and Potential Therapeutic Target for Multiple Myeloma

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Background: It has been demonstrated that circular RNA (circRNA) plays a crucial role in the occurrence and development of tumors, but the diagnostic and predictive value of most circRNAs in tumor patients remains unclear, especially for multiple myeloma (MM).

Methods: High-throughput circRNA microarray-based sequencing was used to identify the differentially expressed circRNAs in MM. qRT-PCR was then employed to detect hsa_circRNA_101237 expression levels in the bone marrow tissues from 143 MM patients (65 first-episode treatment-naive patients and 78 patients with recurrent/refractory disease), MM cells and bortezomib-resistant MM cell lines. Whether hsa_circRNA_101237 can be used as a potential biomarker and therapeutic target for MM was investigated.

Results: The average expressions of hsa_circRNA_101237 in the bone marrow tissues from MM patients (especially those with recurrent/refractory disease), MM cells and bortezomib-resistant MM cell lines were increased significantly ($P<0.01$). hsa_circRNA_101237 was overexpressed in patients positive for 13q14 deletion, 1q21 amplification, P53 deletion, and t(4,14) and t(14,16). hsa_circRNA_101237 was closely related to prognosis of the patients, and its high expression was associated with shorter OS and PFS. In addition, those over-expressing hsa_circRNA_101237 were less responsive to bortezomib treatment. Bioinformatic analysis indicated that hsa_circRNA_101237 interacted with 11 miRNAs and 10 candidate mRNAs. This finding may shed new light on the subsequent studies on the working mechanism and functions.

Conclusion: It was first reported that hsa_circRNA_101237 was significantly upregulated in MM. It was indicated that hsa_circRNA_101237 may be a novel biomarker for MM, and it plays a significant role in the occurrence and development of MM.

Keywords: circRNAs, MM, biomarker, diagnosis, prognosis

Background

Multiple myeloma (MM) is a plasma cell malignancy in which monoclonal plasma cells proliferate in bone marrow, resulting in an overabundance of monoclonal paraprotein (M protein), destruction of bone, and displacement of other hematopoietic cell lines.[1] It is one of the most common hematological malignancies in clinic, accounting for about 10% of all hematological malignancies. The occurrence and development of MM are closely related to genetic variations, including chromosome translocation, gene mutations and cytogenetic abnormalities.[2] It has been reported that abnormalities of important chromosomes and genes may lead to the progression of MM and independently predict the prognosis of patients with MM.[3] According to one study,

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t (4;14), t (14; 16) and t(14:20) chromosomal abnormalities along with *KRAS, NRAS, FAM46C, DIS3, BRAF, TRAF3 and TP53* gene mutations are associated with poor prognosis of MM patients.[4,5] In spite of advance in MM treatment, about 20% of the patients relapse or die within 2 years after the diagnosis.[6] Therefore, early diagnosis and effective treatment are highly important for improving the prognosis of MM patients. Individualized treatment modalities can be adopted based on the levels of specific biomarkers in MM patients, so as to optimize the efficacy and reduce the toxic and side effects.[7] Identifying new biomarkers for MM facilitates the diagnosis and treatment of MM and also improves prognosis of the patients.

Circular RNA (circRNA), an emerging class of non-coding RNA, has a covalently closed loop structure in which the 3′ and 5′ ends are linked in a non-collinear way by a process termed "back-splicing".[8] After its first discovery in 1976 in RNA viruses,[9] circRNA was later reported in eukaryotes in 1979.[10] Due to the recent progress in high-throughput sequencing technology, circRNAs are found in large quantities in eukaryotes,[11] especially in the brains of mammals.[12] According to some reports, circRNAs are considered relevant to human neurodegenerative disease.[13] During pre-RNA splicing, the 5′ and 3′ ends of exon(s) can be covalently ligated to form circRNAs through back-splicing. circRNAs are highly expressed in human cells and not easily degraded by the exonuclease, and therefore, they exist more stably in humans compared with linear RNAs, with a highly conserved sequence. circRNAs are featured by specificity to tissue/cell types and high stability, which make them suitable as biomarkers.[14] Since circRNAs are stably and specifically expressed in different diseases and tissues, such as breast cancer, lung cancer, colorectal cancer, liver cancer, gastric cancer and non-small cell lung cancer, they can be used as novel biomarkers and therapeutic targets for cancers.[15,16] In the present study, circRNA expressions were detected in the bone marrow tissues from patients with MM and iron deficiency anemia (IDA), and the clinical value of the candidate circRNAs was analyzed. It was found that hsa_circRNA_101237 may be a potential diagnostic biomarker for MM.

Materials and Methods

Clinical Data

From January 2012 to January 2019, 143 MM patients (65 first-episode treatment-naive patients and 78 patients with recurrent/refractory disease) and 23 IDA patients treated at the Hematology Department of the Third Xiangya Hospital of Central South University were recruited. They were divided into the experimental group (i.e., MM group) and control group, respectively. Bone marrow samples (cryopreserved at −80°C) were collected from both groups. The clinical data (including age, gender, MM classification, staging and cytogenetic abnormalities) and laboratory indicators were also collected from the experimental group.

Cell Culture

All cell lines, including THP-1 human monocytic cell line, MM cell lines MM.1S and H929, were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA). The bortezomib concentration was gradually increased to obtain the bortezomib-resistant MM cell lines MM.1S/BTZ and H929/BTZ. All cells were cultured in the 1640 medium (HyClone, Logan, UT, USA) and incubated in a 5% CO_2 incubator at 37°C. The log-phase cells were harvested.

RNA Extraction

Plasma cells in the bone marrow samples from MM and IDA patients were enriched by magnetic-activated cell sorting (MACS) using CD138 magnetic l' bead. Total RNA was extracted from the enriched plasma cells using the TRIzol reagent kit (Invitrogen, USA) and preserved at −80°C. The RNA concentration and purity were detected by using a Nanodrop ND-1000 Spectrophotometer and agarose gel electrophoresis, respectively.

circRNA Microarray-Based Sequencing

Linear RNAs in the total extracted RNA were degraded using RNase R (EPicentre, USA). The residual circRNA was amplified and fluorescent cRNA was obtained by transcription. The labeled circRNA were hybridized to the Arraystar Human circRNA Array V2 (8x15K, Arraystar). The microarray was scanned with Agilent Scanner G2505C. Image analysis was conducted using Agilent Feature Extraction software (version 11.0.1.1). circRNA data were analyzed using the GeneSPring 13.0 software (Agilent). Differentially expressed circRNAs were defined as those with fold change>2.0 and $P<0.05$.

Verification by Quantitative RT-PCR

RNA was reversely transcribed into cDHA by SuPerScriPt Reverse TranscriPtase (Invitrogen, USA). Then circRNA

Table 1 The Primer of three circRNAs Choose to Validate by qRT-PCR

CircRNA	Primer
hsa_circRNA_105034	F:5′ CGGACATCTCACCAACACTG 3′ R:5′ TCATCATAGTTTCCAAGCGTG 3′
hsa_circRNA_101237	F:5′ CCTCAGGTGGACTTATCATGAC 3′ R:5′ CGTCTGATGTAGGAAGTGGG 3′
hsa_circRNA_102158	F:5′ CCGACCTCACAATAGAAAAAAT 3′ R:5′ TGTTGCCAGATCCCGTAGA 3′
β-actin (Human)	F:5′ GTGGCCGAGGACTTTGATTG3′ R:5′CCTGTAACAACGCATCTCATATT3′

expressions were detected by real-time qRT-PCR (Arraystar) on the ViiA 7 Real-time PCR System (APPlied Biosystems). Each reaction system was 10μL, consisting of 5u 2×MasterMix, 1μL/PCR specific primers F/R, and 2μL cDNA. The reaction system was added to the well-plate for PCR reaction. β-actin was used as internal control for qPCR, and the relative expression level of circRNA was reflected by the ΔCt method. All primer sequences in this paper were designed by primer 5 and synthesized by Shanghai Biotech. The primer sequences are shown in Table 1.

Statistical Analysis

All statistical analyses were performed using SPSS 20.0 software. Data were expressed as means±standard deviation. Two groups' means were compared by *t*-test. ANOVA was performed to compare the means between multiple groups. The reduction rate of M protein was compared by chi-square test. Receiver operating characteristic curve (ROC) was drawn to determine the diagnostic value of circRNA. When AUC was 0.5, it was considered that circRNA had no diagnostic value. The Kaplan-Meier (K-M) survival curve was plotted and Log rank test was used to analyze whether there was significant difference in the survival rate between patients with high and low circRNA expressions. $P<0.05$ indicated significant difference.

Results

Selection and Validation of circRNAs

According to the results of high-throughput circRNA microarray-based sequencing in 3 MM patients and 3 IDA patients, 147 circRNAs were differentially expressed (fold change>2). Figure 1A shows the differentially expressed circRNAs, including 40 upregulated and 10 downregulated circRNAs. From them 3 most differentially expressed circRNAs were chosen (Table 2) for qRT-PCR (20 samples for MM and IDA patients, respectively). The results are shown in Figure 1B. It can be seen that hsa_circRNA_101237 was upregulated most significantly and its expression was significantly higher than that in the control group.

hsa_circRNA_101237 Overexpression in MM Patients and MM Cell Lines

In order to further verify the results of qRT-PCR, hsa_circRNA_101237 expression levels were determined in the bone marrow tissues of 120 MM patients (Figure 2A). The results were consistent with our previous results of qRT-PCR verification, and the hsa_circRNA_101237 expression was significantly higher than that in the control group. This indicated that hsa_circRNA_101237 was stably overexpressed in MM patients and might serve as a biomarker for MM. In the next step, 143 MM patients were divided into first-episode treatment-naive group (n=65) and recurrent/refractory group (n=78). The hsa_circRNA_101237 expressions were further detected in the bone marrow tissues of the two groups (Figure 2B). The results showed that the hsa_circRNA_101237 expression in the recurrent/refractory patients was significantly upregulated compared with the first-episode treatment-naive patients, indicating that hsa_circRNA_101237 may be closely associated with the development of MM. The hsa_circRNA_101237 expression levels were also determined in the THP-1 cells and MM.1S, H929, MM.1S/BTZ and H929/BTZ cells (Figure 2C). It was found that the hsa_circRNA_101237 expression was the highest in the resistant cell lines MM.1S/BTZ and H929/BTZ, followed by the MM cell lines MM.1S and H929, and it was the lowest in the THP-1 cells, with significant differences. The above facts suggested that the hsa_circRNA_101237 upregulation may be one mechanism of bortezomib resistance in MM patients.

Correlation Between the hsa_circRNA_101237 Expression and Clinical Symptoms of MM Patients

In order to better understand the clinical value of hsa_circRNA_101237, we analyzed the correlations between the expression level of hsa_circRNA_ 101237 and the pathological features and laboratory indicators of MM patients, as shown in Table 3. It can be seen that the hsa_circRNA_101237 expression in IgG and IgA MM

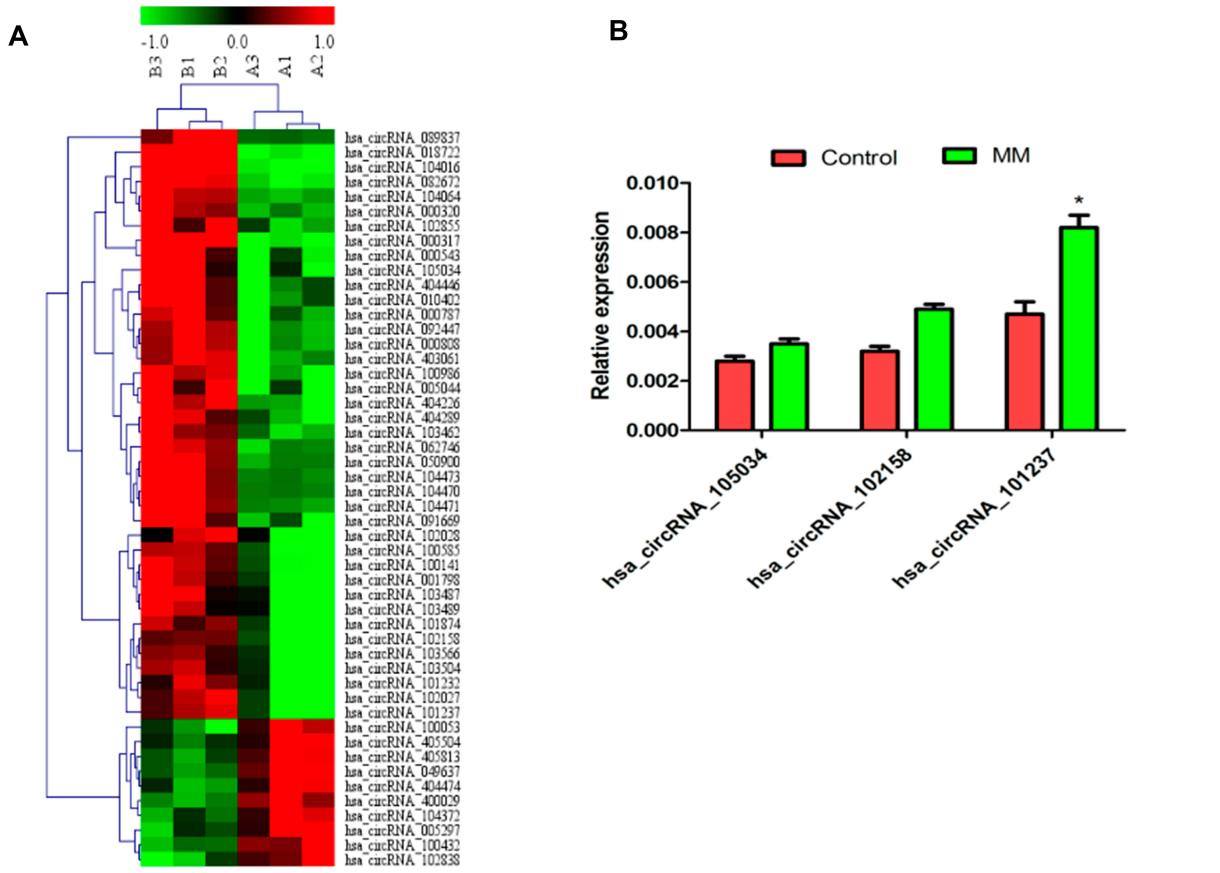

Figure 1 Selection and validation of differentially expressed circRNAs. Figure 1A shows the 50 differentially expressed circRNAs. Red indicates relative upregulation, and green downregulation. A1, A2 and A3, the control group; B1, B2 and B3, the MM group. The first 40 circRNAs were upregulated, and the last 10 circRNAs were downregulated. Figure 1B shows the qRT-PCR verification results of 3 circRNAs. *P<0.05, compared with the control group. All experiments were performed in triplicate.

increased (P<0.001). In MM patients with cytogenetic abnormalities and bone destruction which were of R-ISS stage III, hsa_circRNA_101237 was overexpressed (P<0.05). In addition, hsa_circRNA_101237 was significantly upregulated in MM patients with overexpression of β2-microglobulin and lactate dehydrogenase (P<0.05).

hsa_circRNA_101237 Could Be Used as a Diagnostic and Prognostic Indicator for MM

In order to determine the diagnostic value of hsa_circRNA_101237 for MM patients, the ROC curve was drawn for MM. The results are shown in Figure 3A. hsa_circRNA_101237 had a high diagnostic accuracy for MM, and its AUC was 0.92 (P<0.0001). This indicated that hsa_circRNA_101237 may be a specific and sensitive diagnostic biomarker for MM. The prognosis of MM patients can be influenced by cytogenetic abnormalities. The expressions of hsa_circRNA_101237 in MM patients harboring different chromosomal or genetic variations were analyzed, and the results are shown in Figure 3B. It was found that the hsa_circRNA_101237 expression was significantly increased in patients positive for 13q14 deletion, 1q21 amplification, P53 deletion and t(4,14) and t(14,16) gene mutations (P<0.05), but it was decreased significantly in those positive for t(11,14) gene mutations (P<0.05). The correlation between hsa_circRNA_101237 expressions and

Table 2 The Basic Information of three circRNAs Choose to Validate by qRT-PCR

CircRNA	Alias	Chrom	CircRNA Type	FC	Regulation	Gene Symbol	P value
hsa_circRNA_105034	hsa_circ_0001947	chrX	Exonic	4.86	Up	AFF2	0.03
hsa_circRNA_101237	hsa_circ_0003489	chr13	Exonic	4.57	Up	CDK8	0.04
hsa_circRNA_102158	hsa_circ_0008438	chr17	Exonic	3.39	Up	TLK2	0.03

Abbreviation: FC, Fold change.

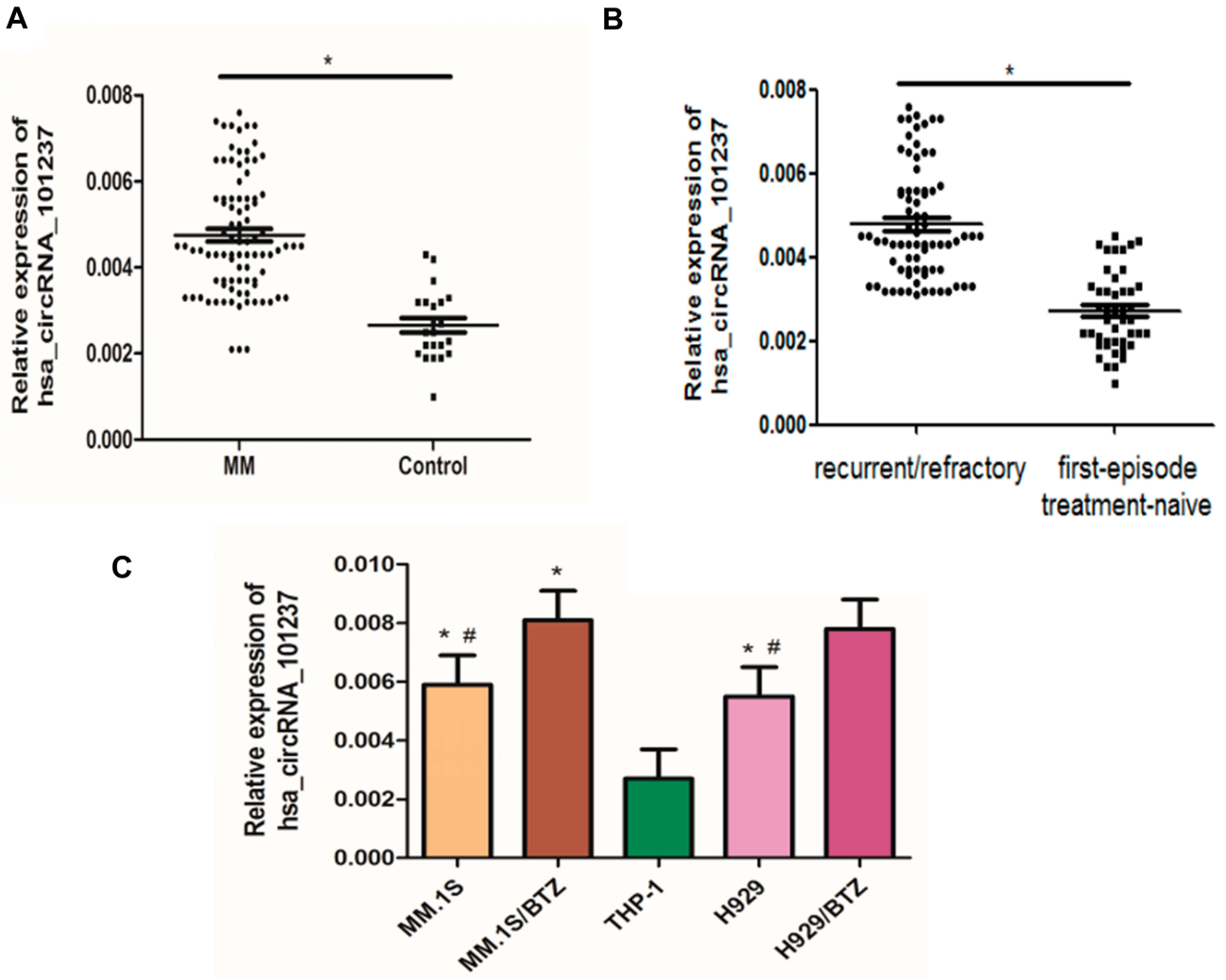

Figure 2 Expressions of hsa_circRNA_101237 in MM patients (**A** and **B**) and cell lines (**C**). *P<0.05, compared with the control group (**A**), first-episode treatment-naive group (**B**) and TH-1 cells (**C**), respectively; #P<0.05, compared with the resistant cell lines (P<0.05). All experiments were performed in triplicate.

prognosis of MM patients was further analyzed through the survival curve. Survival analysis indicated that the overall survival (OS) and progression-free survival (PFS) were shortened significantly in those with upregulated hsa_circRNA_101237 (Figure 3C and D, P<0.05).

miRNA and mRNA Target Prediction of hsa_circRNA_101237

Competitive inhibition of miRNA by the sponging effect is the most important working mechanism of circRNA. In order to explore the possible working mechanism of hsa_circRNA_101237, TargetScan and miRanda were used to predict the miRNA and mRNA targets that might interact with hsa_circRNA_101237. As shown in Figure 4, 11 differentially expressed miRNA and 10 candidate mRNAs interacted with hsa_circRNA_101237. Later, GO (Gene ontology) enrichment analysis was performed to investigate the possible roles of hsa_circRNA_101237 in the cellular activities, including biological processes (Figure 5A), cell components (Figure 5B) and cell functions (Figure 5C). Figure 5D shows the possible signal pathways related to hsa_circRNA_101237 revealed by the KEGG (Kyoto Encyclopedia of Genes and Genomes) analysis, including the PI3K-Akt signaling pathway, chemokine signaling pathway, cell cycle-related pathway, and the signaling pathway related to the interactions between cytokines and their receptors.

hsa_circRNA_101237 Overexpression Influenced the Treatment Response of MM Patients

One hundred forty-three patients were enrolled for efficacy evaluation, including 79 patients in the bortezomib-containing treatment group (BD group) and 64 patients in

Table 3 A Correlation Analysis Between the Clinical Data of 143 MM Patients and hsa_circRNA_101237 Expressions

Clinical Data	Cases	hsa_circRNA_101237 Expression	P value
Age(years)			0.535
≥60	78	9.934±4.617	
<60	65	9.545±4.228	
Gender			0.298
Male	75	11.218±4.725	
Female	68	9.435±4.228	
Type			0.001*
IgG	65	10.298±4.871	
IgA	38	12.977±4.147	
Light-chain	23	8.174±2.831	
Unclassified	17	7.344±1.840	
Cytogenetic abnormalities			0.002*
Yes	82	12.448±4.425	
No	61	8.396±1.440	
Plasma cells (%)			0.767
≥40	60	9.851±4.260	
<40	83	10.332±3.980	
Bone destruction			0.018*
Yes	89	10.443±4.316	
No	54	6.882±1.455	
β2-microglobulin			0.037*
< 5.5 mg/L	63	8.277±3.428	
≥5.5 mg/L	80	11.228±4.295	
Lactate dehydrogenase			0.039*
<220 U/L	74	7.547±1.634	
≥220 U/L	69	12.764±2.864	
CRP			0.376
>10mg/L	65	10.836±3.896	
≤10mg/L	78	8.835±1.973	
Serum calcium			0.523
>2.65mmol/L	82	10.753±2.859	
≤2.65mmol/L	61	7.826±1.974	
Creatinine			0.642
<177 μmol/L	79	9.244±2.752	
≥177 μmol/L	64	12.865±3.874	
ISS staging			0.023*
Stage I+II	89	5.459±1.537	
Stage III	54	8.478±3.627	
DS staging			0.224
Stage I+II	79	8.737±2.847	
Stage III	64	10.197±3.221	

Note: *Indicates that the comparison between the two groups is statistically significant, P <0.05.

the non-bortezomib-containing treatment group (TT group). After 4 cycles of bortezomib-containing treatment, patients with a low expression of hsa_circRNA_101237 showed a higher M protein reduction rate compared to those receiving non-bortezomib-containing treatment (78.1% vs. 32.5%, p<0.001); for patients with an overexpression of hsa_circRNA_101237, after four cycles of bortezomib-containing treatment, the M protein reduction rate was lower compared to those receiving non-bortezomib-containing treatment (38.3% vs. 61.4%, p<0.05) (Figure 6). It was indicated that the hsa_circRNA_101237 expression may influence the responsiveness of MM patients to bortezomib treatment.

Discussion

MM is a hematological malignancy featured by abnormal proliferation of plasma cells in the bone marrow,[17] which may lead to a variety of symptoms, including hypercalcemia, anemia, renal failure, lytic bone lesions, infection, neurologic symptoms and amyloidosis. MM is more common in the middle-aged and elderly males,[18] and its incidence has been increasing gradually as China enters the aging society. MM is a highly heterogeneous disease, and the patients' survival varies considerably with clinical manifestations, chromosomal and genetic variations. So far, there are no treatment modalities to cure such a high malignant disease as MM. Developing novel early diagnostic, prognostic and therapeutic targets is crucial for MM treatment.

circRNA is a special class of endogenous noncoding RNAs widely present in the organisms and mainly composed of exons and (or) introns. circRNAs regulate the expressions of target genes primarily through the sponging effect of miRNA,[19] and play an important role in disease development and progression, especially cancers. The use of second-generation sequencing technology and microarray analysis has resulted in the identification of more and more differentially expressed circRNAs, which are stably expressed in many tumor cells and can be used as a novel marker for tumors. For example, Chen et al[20] reported the use of hsa_circ_0000190 as a biomarker for gastric cancer, the sensitivity and specificity of which even surpassed those of carcinoembryonic antigen (CEA) and CA19-9. Yao et al[21] proposed that circ RNA_100876 was closely associated with non-small cell lung cancer and that its overexpression predicted shorter survival. Gao et al[22] and Tian et al[23] identified circRNA1656 and hsa_circ_0004585 as the biomarkers for ovarian cancer and colorectal cancer, respectively, by using

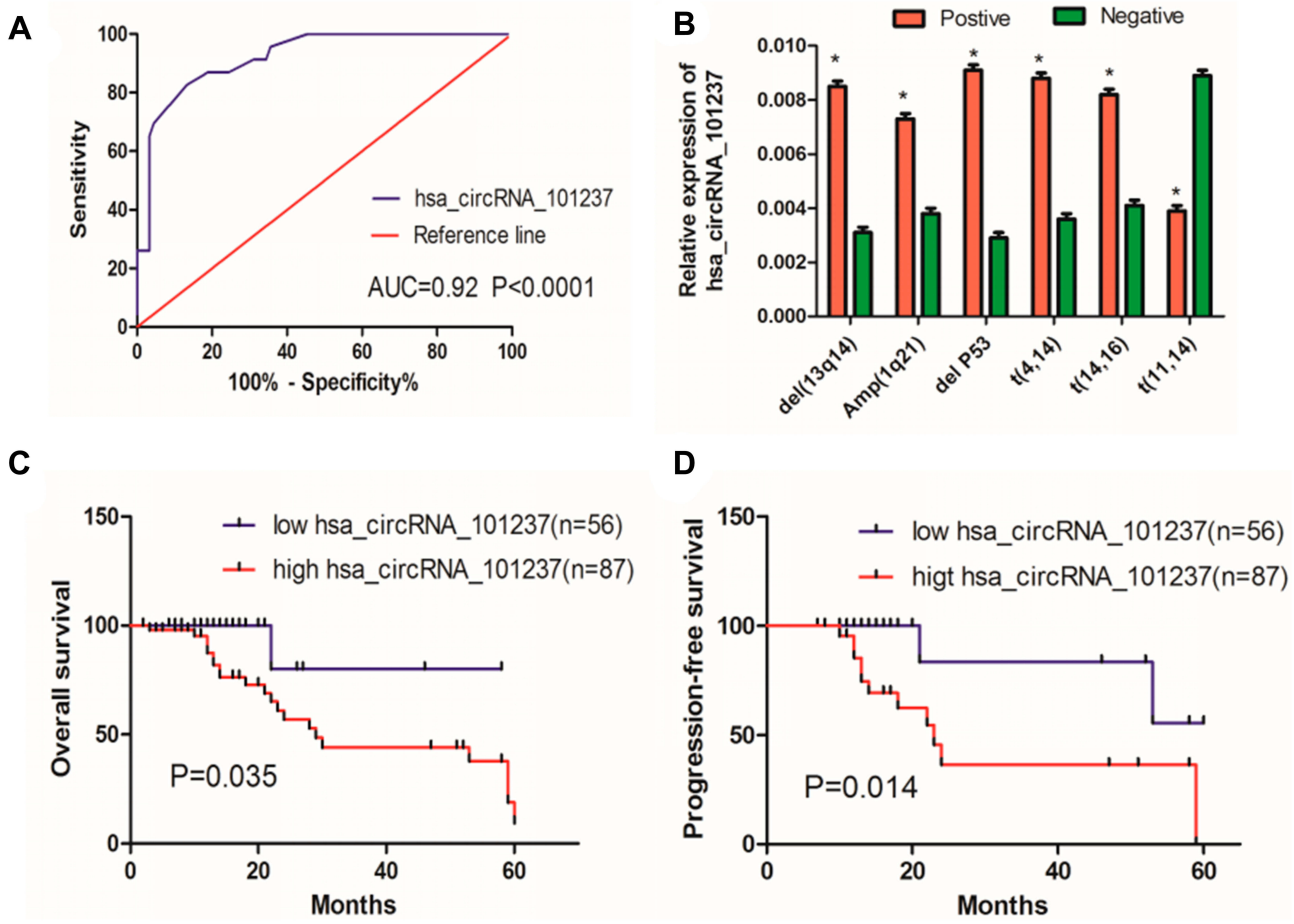

Figure 3 Analysis of the diagnostic and prognostic value of hsa_circRNA_101237 in MM patients. Figure 3A shows the ROC analysis of hsa_circRNA_101237 in MM patients. Figure 3B indicates the expression level of hsa_circRNA_101237 in MM patients with chromosomal or genetic variations; Figure 3C and D indicate OS and PFS of MM patients, respectively. *P<0.05, compared with the negative control group.

microarray analysis. Besides, other studies have indicated that tumor cells contain thousands of circRNA transcripts, which account for the majority of all transcripts. This fact implies the potential of circRNAs as novel diagnostic biomarkers and therapeutic targets for cancers.[24] The present study showed that hsa_circRNA_101237 was overexpressed in the bone marrow tissues of MM patients and might serve as a novel diagnostic and prognostic biomarker.

By high-throughput circRNA microarray-based sequencing, the differentially expressed circRNAs in the bone marrow of MM patients were identified, from which the three most significantly differentially expressed circRNAs were selected for qRT-PCR and verification. The results showed that hsa_circRNA_101237 was significantly upregulated. Our study was the first attempt to demonstrate that hsa_circRNA_101237 was upregulated in MM patients, and the diagnostic and prognostic value of hsa_circRNA_101237 in MM patients was investigated. Combining with the clinical data, it was found that the hsa_circRNA_101237 expression was closely associated with MM typing, bone destruction, chromosomal and genetic variations. It can be inferred that hsa_circRNA_101237 was clinically valuable. Chromosomal or genetic variations may affect the prognosis of MM patients. In order to investigate whether hsa_circRNA_101237 influences the prognosis of MM patients, the hsa_circRNA_101237 expressions in patients with different chromosomal or genetic variations were further analyzed. The results showed that hsa_circRNA_101237 was upregulated in patients positive for 13q14 deletion, 1q21 amplification, P53 deletion, and t(4,14) and t(14,16) gene mutations, while it was contrary for t(11,14) gene mutations. It has been long confirmed that the genes mentioned above are closely associated with the prognosis of MM patients. For example, Jung et al[25] showed that del (13q) was the only important genetic factor influencing OS of the first-episode MM patient. Grzasko et al[26] believed that compared with 13q14 deletion alone, 13q14 deletion with 1q21 amplification could significantly decrease OS of the patients. Studies by Oh et al[27] and Shin et al[28] confirmed that the poor

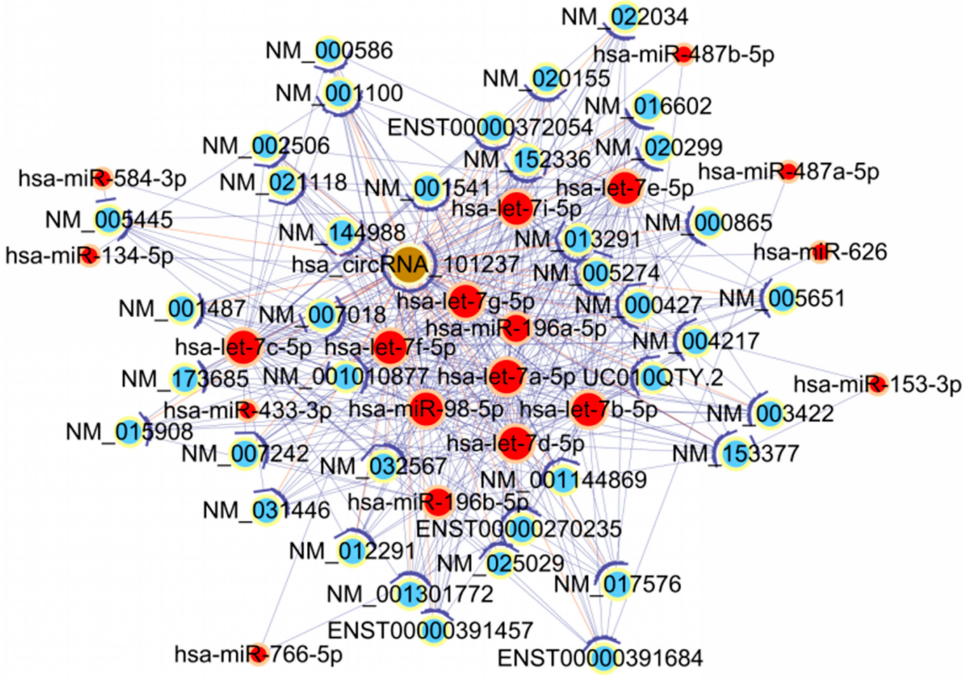

Figure 4 Functional analysis of circRNA. hsa_circRNA_101237-miRNA-mRNA coexpression network established using hsa_circcircRNA_101237.

prognosis of MM patients was associated with t(4,14) gene mutations. In addition, it was also found that the hsa_circRNA_101237 expression in recurrent/refractory MM patients was higher than that in the first-episode treatment-naive patients. It was indicated that hsa_circRNA_101237 was closely associated with the progression of MM.

Bortezomib is a novel inhibitor targeting protease, and its combination with conventional chemotherapeutic agents can increase the overall response of newly diagnosed MM (NDMM), improving the patients' life quality and prolonging OS.[29] Bortezomib has also achieved a good efficacy in refractory/recurrent MM (RRMM).[30] However, the long-term use of bortezomib may finally result in the resistance of MM cells. To better understand the resistance mechanism, we analyzed the correlation between hsa_circRNA_101237 and bortezomib resistance. Cell experiments showed that the hsa_circRNA_101237 was increased significantly in bortezomib-resistant cell lines and that hsa_circRNA_101237 overexpression was associated with a poor response to bortezomib-containing chemotherapy in MM patients. Moreover, the M protein reduction rate decreased after treatment. The results above suggested that hsa_circRNA_101237 was possibly involved in bortezomib resistance in MM patients.

Competitive inhibition of miRNA as the molecular sponge is the most important working mechanism of circRNA. It has been found that circRNAs adsorb specific miRNAs to influence the expression of mRNA, thereby performing their biological functions.[31,32] Our bioinformatic analysis identified 11 miRNAs and 10 candidate mRNAs which might interact with hsa_circRNA_101237. It was also found that hsa_circRNA_101237 might be involved in various cellular activities and signaling pathways, which further contributes to the development and occurrence of MM. These results provide a novel clue to further investigate the pathogenic and chemotherapeutic resistance mechanism of hsa_circRNA_101237 in MM.

In this study, hsa_circRNA_101237 overexpression in MM patients was found and verified by microarray-based sequencing, which was associated with development, prognosis and responsiveness of MM to bortezomib treatment. Thus hsa_circRNA_101237 may be used as a novel diagnostic and prognostic biomarker and potential therapeutic target for MM. However, more efforts should be made to develop novel biomarkers for MM and improve the diagnosis, treatment and prognosis of MM.

Ethics Approval and Informed Consent

This study was approved by the Ethics Committee of The Third Xiangya Hospital, Central South University. Informed consent was obtained from each subject in accordance with the Declaration of Helsinki.

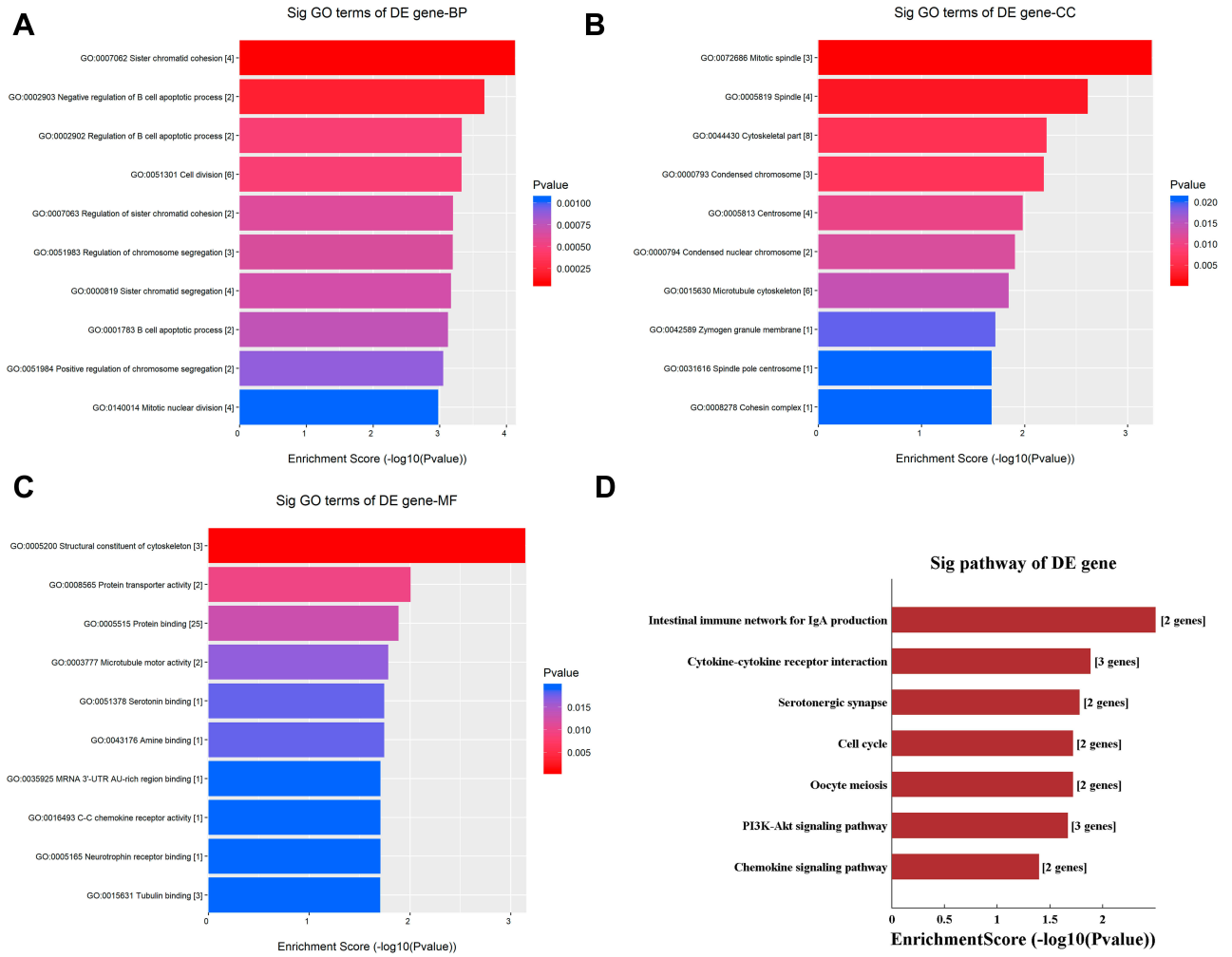

Figure 5 Functional analysis of circRNA. Figure **A**–**C** represent Gene ontology (GO) enrichment analysis for hsa_circRNA_101237 in terms of biological processes (BP), cellular component (CC) and molecular function (MF), respectively. Figure **D** represents KEGG pathway analysis for hsa_circRNA_101237.

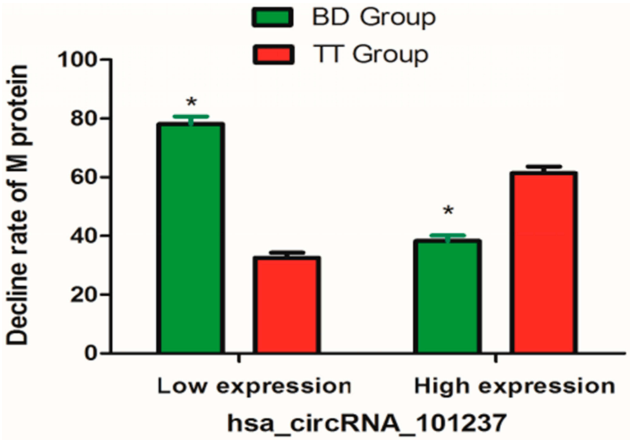

Figure 6 Influence of hsa_circRNA_101237 expressions on the responsiveness to bortezomib-containing treatment. *P<0.05, compared with the TT group.

Author Contributions

All authors contributed to data analysis, drafting and revising the article, gave final approval of the version to be published, and agree to be accountable for all aspects of the work.

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Disclosure

The authors report no declarations of interest in this work.

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