ORIGINAL RESEARCH

# Effect of the Up-Regulation of Circular RNA Hsa_circ_0069767 Derived from C-KIT on the Biological Behavior of Multiple Myeloma Cells

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**Purpose:** Multiple myeloma (MM) is an incurable disease. This study focused on the expression of circular RNA *circ_0069767* in MM and its influence on prognosis, in order to provide a potential target.

**Patients and Methods:** Totally 66 MM patients participated in this research. Using RT-PCR method to determine the expression level of *circ_0069767* in 66 sorted samples from multiple myeloma patients and 21 normal control bone marrow samples, Kaplan–Meier was applied for survival analysis. We constructed stable over-expressing *circ_0069767* and silenced circ_0069767 cell lines and used MTS experiment to detect cell viability, transwell experiment to detect cell migration and invasion ability and flow cytometry to detect cell apoptosis. Dual luciferase experiment, qRT-PCR experiment and Western blot were used to explore miRNA and downstream genes.

**Results:** The expression of *circ_0069767* in MM was significantly higher than that of the normal control group. Patients with high expression of *circ_0069767* had longer PFS and OS. Cell function experiments showed that overexpression of *circ_0069767* in MM cells led to decreased proliferation, migration and invasion, but increased apoptosis; meanwhile, knockdown of *circ_0069767* caused opposite biological behaviors. *Circ_0069767* by sponging *miR-636* in MM cells regulates the expression of *K-RAS* while the *K-RAS* gene remained unmutated.

**Conclusion:** *Circ_0069767* plays an antitumor role and its expression can be used as a reliable prognostic indicator for MM patients.

**Keywords:** circular RNA, multiple myeloma, prognostic factor

## Introduction

Multiple myeloma (MM) is a clonal plasma cell proliferative disorder as the second most common hematological malignant tumor, accounting for about 15% of all hematological malignances. The disease is considered incurable,[1] but due to the introduction of autologous stem cell transplant (ASCT), the availability of new drugs, and the application of novel immunotherapies, the therapeutic effect, prognosis of MM, and the 5-year overall survival (OS) has been significantly improved; nonetheless, most patients eventually suffer from relapse.[2] Therefore, identifying factors that affect the prognosis of MM remains clinically important.

Circular RNA (circRNA) is a covalently closed endogenous non-coding RNA that is gradually recognized following miRNA and long non-coding RNA (lncRNA). CircRNA has neither the polarity of the 3′ and 5′ ends nor a polyA

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tail and is characterized by a stable structure (uneasily degraded by endonucleases)[3–6] and tissue and disease-specific expression patterns. Moreover, circRNA plays multiple regulatory roles in carcinogenesis, and therefore has potential diagnostic and prognostic values in cancer evaluation.

With the development of sequencing technique, a large number of circRNAs have been discovered. Researches on this new type of non-coding RNA revealed that it is highly associated with molecular biology and molecular oncology. To date, a number of human circRNAs have been identified.[7–13] With the in-depth study of structure and function of circRNAs, it was found to be involved in the occurrence and development of multiple diseases and is considered to be a key regulator in a wide range of biological processes.[14–20] However, it is rarely reported in MM.

In our previous studies, MM patients with high *C-KIT* (*CD117*) expression were observed of better prognosis compared with patients with low *C-KIT* (*CD117*) expression.[21] According to the circBase database, there are 12 circular RNAs derived from the *C-KIT* gene, and *circ_0069767* was found in the K562 cells, which belong to the same blood tumor as multiple myeloma. So I tried qRT-PCR detection of *circ_0069767* first, and the pre-experiment results showed that there was a difference in expression of MM compared with the normal control group. So we chose *circ_0069767* as the target gene for follow-up research. To further explore the effect of *circ_0069767* on tumor cells, we built stable overexpressed *circ_0069767* and silenced *circ_0069767* cell lines based on MM cell KM3 and explored its function through in-vitro experiments which demonstrated that KM3 cells altered biological behaviors including decreased cell viability, migration and invasion capacities, and increased cell apoptosis.

## Patients and Methods
### Patients and Clinical Samples
A total of 66 bone marrow samples from MM patients and 21 healthy control samples (healthy donors for bone marrow transplants) were collected from patients treated at Shengjing Hospital of China Medical University between January 2014 and December 2018. All patients were diagnosed and newly treated by the updated International Myeloma Working Group (IMWG) criteria and evaluated for staging based on the International Staging System (ISS) and Durie-Salmon (DS). All patients enrolled in this study did not receive any treatment before sampling and were treated with bortezomib-based combination chemotherapy after admission. The study protocol was reviewed and approved by the Ethics Committee of Shengjing Hospital of China Medical University, and all participants provided informed consents.

### Magnetic-Activated Cell Sorting
All bone marrow samples were residual samples from routine clinical laboratory tests, from which MM mononuclear cells (MNCs) were extracted using magnetic-activated cell sorting (Miltenyi, Germany) following the manufacturer's instruction. The extracted bone marrow MNCs were added to Trizol reagent (Takara, Japan) and cryopreserved at −80°C for future testing.

### Total RNA Extraction and qRT-PCR
The Trizol method was used to extract total RNAs from the sorted MM cells following the manufacturer's instructions. The extracted total RNA was reverse transcribed using PrimeScript™RT reagent Kits, and real-time PCR was performed using TAKARA SYBR ® Premix Ex Taq ™ II kits (TaKaRa, Dalian, China) on the ABI 7500HT. The process of the thermal cycler was as follows: 95°C5min→(95°C5s, 60°C34s)×40cycles→95°C15s →60°C1min→ 95°C15s.

All reactions were performed in triplicate. A reverse primer was designed to amplify the head-to-tail splice junction of the *circ_0069767*, the sequences were F:5′-GTAATCGTAGCTGGCATGAT-3′, R:5′-GAATGCTTCATATTCAACAATC-3′, with the GAPDH as an internal reference gene, the primer sequences was F: 5′-TGTTCGTCATGGGTGTGAAC-3′, R: 5′-ATGGCATGGACTGTGGTCAT-3′. The relative expression of *circ_0069767* was calculated using the 2-ΔΔCT method. We used the median value of *circ_0069767* expression as a boundary, and divided 66 patients into high expression group and low expression group.

### Cell Culture and Construction of Stably Transfected Cell Lines
MM cell line KM3 was selected for subsequent experiments for positive expression of *CD117*. The expression of *CD117* in KM3 lines is about 82% ([Figure S1](#)). MM cell line KM3 and human embryonic kidney cell line HEK-293T were purchased from Cell Bank of the Chinese Academy of Sciences (Shanghai, China) and ATCC. Cells were kept in RPMI 1640 or Dulbecco's Modified

Eagle Medium (DMEM) containing 10% fetal bovine serum (HyClone, USA) in humidified 37°C incubators with 5% CO2.

The lentivirus vector mediating the overexpression of *circRNA_0069767* was provided by Liaoning Baihao Biotechnology Co., Ltd. (Benxi, China), which was constructed by cloning *circRNA_0069767* cDNA into PLCDH vector. A reverse indirect linker sequence of the silenced sh-circRNA specifically targeting *circRNA_0069767* was designed with an shRNA sequence of:

AAGTTTACAGATAAAGGATTCATTTTGGATCCA-AAATGAATCCTTTATCTGTAAC.

After lentivirus infection, antibiotics screenings were performed to select stably transfected cells which were overexpressing *circ_0069767* cells (circ_0069767), empty vector cells (vector), silenced *circ_0069767* cells (sh-circ_0069767), and silenced empty vector cells (sh- vector).

## Cell Viability Assay

The MTS assay (Promega) was used to test the viability of all cells. The four groups of cells were centrifuged, the supernatant was discarded, and approximately 3mL of RPMI 1640 was added then mixed. The cells were seeded in 96-well plates with $1 * 10^4$ cells per well. Cell colony formation was detected in 5 replicates for each group (24h, 48h, 72h, and 96h, respectively). MTS (20 µl) was added to each well and incubated for 2 hours before detection.

## Cell Migration and Invasion Assays

The cells were centrifuged, the supernatant was discarded, and approximately 5 mL of culture solution was added. The cells were seeded in cell chamber slides with $1 \times 10^5$ cells per chamber. A layer of matrigel was coated onto the chambers' polycarbonate membrane for the invasion assay. Culture solution that contained a semi-solid medium made from agarose (500 µl) was added to each chamber (3 duplicate chambers were set). After a cultivation period of 24h images were obtained under a microscope. The cells in the lower chamber were counted with a cell counter.

## Apoptosis Assay

The cells were centrifuged at 1000 rpm for 5 min, washed twice with PBS and then centrifuged at 1000 rpm for 5 min. Then, the cells were collected then added 100 µL binding buffer to prepare a single-cell suspension with a density of $2 \times 10^5$/mL. 5 µL Annexin V-APC and 5 µL 7-AAD (Becton Dickinson) were added per tube and mixed thoroughly. Each mixture was then incubated at room temperature for 10 min and then detected using flow cytometry. Empty cell controls were set and the above procedures were repeated.

## Immunophenotyping

Immunophenotypic analysis was performed using a FACS Calibur 4-color flow cytometer (Becton, Dickinson and Company, USA). Monoclonal mouse anti-human antibodies were labeled using fluoresceins including CD45-Percp (Peridinin-Chlorophyll-Protein), CD117-PE (phycoerythrin), and CD38-APC (allophycocyanin). The antibodies (Becton Dickinson), after binding to antigen, were kept in a dark place for 15 mins. BD FACS Lysing Solution was added for hemolysis for 10 min. Following antibody labeling, 50,000 cells were analyzed with CellQuest software on a flow cytometer.

## Luciferase Reporter Assay

We predicted the targeted miRNAs that may be bound to *circ_0069767* by three online tools (https://omictools.com/circnet-tool, http://regrna2.mbc.nctu.edu.tw/, https://circinteractome.nia.nih.gov/). The full length of *circ_0069767* and 3′-UTR of *K-RAS* containing wild-type or mutant *miR-636* binding site were synthesized and cloned into pmirGLO REPORT vector, followed by co-transfection with miRNAs mimics into HEK-293T cells using Lipofectamine™ 3000 (Invitrogen). The relative luciferase activity was detected by the Dual-Luciferase Reporter Assay Kit purchased from Promega company (Madison, WI, USA).

## Western Blot

Cells from each group were collected and protease and phosphatase inhibitors were added to extract total protein, which was then dyed and electrophoresed on a 10% SDS-PAGE gel. After electrophoresis, the total protein was transferred to polyvinylidene difluoride (PVDF) parts, and then blocked with 5% skimmed milk powder. Thereafter, the membrane was incubated with an anti-K-RAS (# 180772, Abcam) primary antibody at 4° C overnight and with an anti-rabbit secondary antibody for 1h at room temperature the next day. The membrane was developed using a chemiluminescent solution in a dark room.

## Next-Generation Sequencing

The next-generation sequencing technology used Illumina/Sol-exa GenomeAnalyzer (Illumina, USA) to complete K-RAS gene sequencing.

## Statistical Analyses

All statistical analyses were performed using SPSS 19.0 software (IBM, USA), and the data reported as mean ± SD from at least three independent experiments. Differences between groups were analyzed by Student's *t*-test and Kruskal–Wallis test. *P* value less than 0.05 was considered statistically significant. *$p<0.05$, **$p<0.01$, ***$p<0.001$.

## Results
### Increased Circ_0069767 Expression in MM

QRT-PCR analysis was performed on the 66 sorted MM bone marrow samples with purity of 90.76% (Figure S2) and 21 normal control bone marrow samples which confirmed that *circ_0069767* expressed higher in MM samples than in normal control samples (Figure 1A). By the median expression of *circ_0069767* in MM patients (median = 8.81), patients were divided into "high" *circ_0069767* and "low" *circ_0069767* groups. The results showed that low *circ_0069767* was closely correlated with advanced Durie-Salmon (DS) stage (Table 1). Our present study results indicated low expression *circ_0069767* patients had more frequent p53 deletion than high expression *circ_0069767* patients (Table 2). QRT-PCR analysis showed that the higher expression of mRNA of *CD117* was related to higher expression of *circ_0069767* (Figure 1C), also there was a strong positive correlation between *circ_0069767* and mRNA expression of *CD117* in MM tissues (Figure 1D). And the average fluorescence intensity of *CD117* protein encoded by the *KIT* gene showed that patients with stronger *CD117* fluorescence intensity had higher expression of *circ_0069767* (Figure 1B). At the same time, we used qRT-PCR to test some rechecked patient samples, including 6 CR patients, 7 VGPR
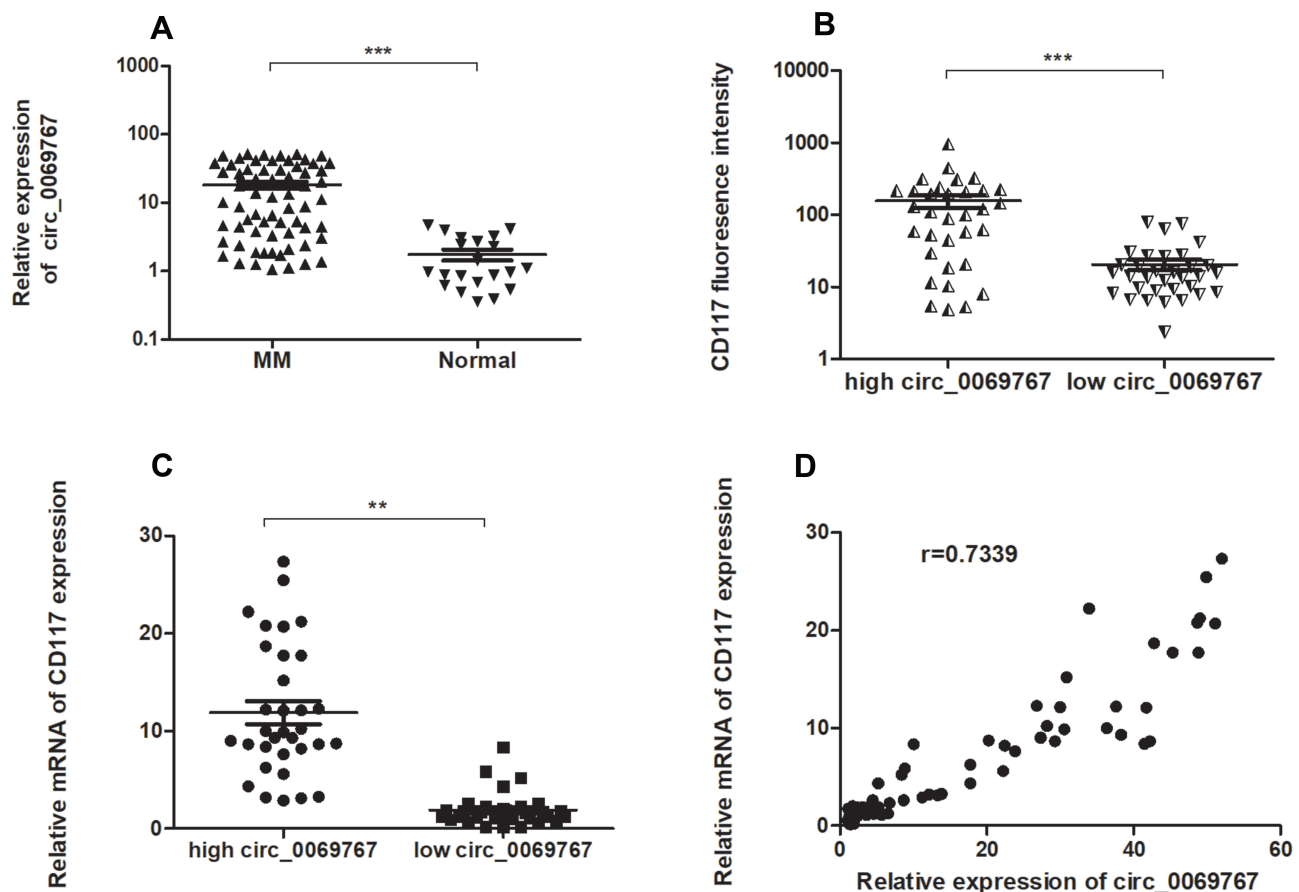


**Figure 1** Circ_0069767 is high-regulated in MM. By the median expression of *circ_0069767* in MM patients (median = 8.81), patients were divided into "high" *circ_0069767* and "low" *circ_0069767* groups. (**A**) RT-PCR analysis of *circ_0069767* in human MM and normal healthy control tissues. MM vs Normal, *p* < 0.001. (**B**) *CD117* mean fluorescence intensity (channel) analysis of high or low expression of *circ_00669767* in MM patients. (**C**) RT-PCR analysis of *CD117* mRNA of high or low expression of *circ_00669767* groups in MM patients. (**D**) The correlation between *CD117* mRNA and *circ_0069767* expression in MM tissues. (**D**) The more of expression *CD117* mRNA, the higher expression of *circ_0069767*, *p*<0.001. **p<0.01, ***p<0.001..

**Table 1** Difference in the *Hsa_circ_0069767* Expression in Multiple Myeloma Patients Grouped by Clinicopathological Characteristics

| Clinicopathological Characteristics | Total (n=66) | Hsa_circ_0069767 Expression | | P value |
|---|---|---|---|---|
| | | Low (n=33) | High (n=33) | |
| Age (years) | | | | 0.438 |
| Male | 43 | 20 | 23 | |
| Female | 23 | 13 | 10 | |
| Gender | | | | 0.618 |
| ≤60 | 26 | 14 | 12 | |
| >60 | 40 | 19 | 21 | |
| M protein | | | | 0.166 |
| IgG | 27 | 15 | 12 | |
| IgA | 13 | 8 | 5 | |
| Light chain | 18 | 5 | 13 | |
| No secretion | 8 | 5 | 3 | |
| ISS stage | | | | 0.831 |
| I | 5 | 2 | 3 | |
| II | 11 | 5 | 6 | |
| III | 50 | 26 | 24 | |
| DS stage | | | | 0.037 |
| I | 10 | 4 | 6 | |
| II | 11 | 3 | 8 | |
| III | 45 | 26 | 19 | |
| Hypercalcemia | | | | 0.648 |
| With | 10 | 6 | 4 | |
| Without | 48 | 25 | 23 | |
| Renal insufficiency | | | | 0.716 |
| With | 24 | 11 | 13 | |
| Without | 37 | 21 | 16 | |
| Albumin | | | | 0.447 |
| ≥30 g/L | 25 | 11 | 14 | |
| <30 g/L | 41 | 22 | 19 | |
| Anemia | | | | 0.625 |
| With | 54 | 27 | 27 | |
| Without | 12 | 6 | 6 | |
| Cytogenetic abnormality | | | | 0.950 |
| With | 26 | 10 | 16 | |
| Without | 16 | 6 | 10 | |
| CD117 | | | | 0.026 |
| Positive | 29 | 10 | 19 | |
| Negative | 37 | 23 | 14 | |

**Abbreviations:** ISS, International Staging System; DS, Durie-Salmon.

patients, 8 PR patients and 13 PD patients. The results showed that the expression of *circ_0069767* in PD patients was the lowest (Figure S3).

## Kaplan–Meier Survival Analysis Results Indicated That High Circ_0069767 Led to a Longer PFS and OS

All 66 patients were treated with bortezomib-based chemotherapy. Kaplan-Meier survival analyses of the high and low *circ_0069767* groups indicated that the high *circ_0069767* group had a longer progression-free survival (median, 15 vs 10 months; *p*<0.05) and overall survival (median, 35 vs 21 months; *p*<0.001; Figure 2).

## Circ_0069767 Overexpression Led to Reduced Cell Viability

To clarify the biological function of *circ_0069767* in MM, we built stable *circ_0069767* overexpressed and silenced cell lines (Figure 3A) and performed a series of functional assays. Multiple myeloma cell lines were used for viability assays. MTS assay results suggest that the cells with overexpressed *circ_0069767* had decreased viability, while sh-circ cells had restored viability (Figure 3B).

## The Cells Overexpressing Circ_0069767 Had Decreased Migration and Invasion Capacities

We performed transwell assays to detect the invasion and migration capacities of the four cell lines (*circ_0069767*, vector, sh-circ_0069767, sh-vector). The stable cell line overexpressing *circ_0069767* showed decreased invasion and migration capacities (Figure 4A and B).

## The Cells Overexpressing Circ_0069767 Showed Increased Apoptosis

Flow cytometry was used to test apoptosis of *circ_0069767*, vector, sh-circ_0069767, and sh-vector cell lines. Originally there were few apoptotic cells in each group, but after treated with bortezomib (IC50 values 100 ng/mL, 24h), the apoptosis of cells overexpressing *circ_0069767* was significantly increased compared to other groups (Figure 5).

## Circ_0069767acts as a Sponge of miR-636 in MM Cells

We predicted the targeted miRNAs capable of binding to *circ_0069767* using different online tools, the overlapped results that met the criteria were *miR-29*, *miR-370*, *miR-491*, *miR-636* and *miR-1264*. The full length of *circ_0069767* wild-type was synthesized and cloned into pmirGLO REPORT vector, followed by co-transfection

**Table 2** The *Hsa_circ_0069767* Expression as Grouped by FISH

| Chromosomal Aberrancy, n = 35 | P53 Deletion | IgH Rearrangement | 13q14.3 Deletion | 1q21 Gain | RB1 Deletion |
|---|---|---|---|---|---|
| Low circ_0069767, n = 16 | 6 (35.7%) | 5 (31.3%) | 6 (35.7%) | 6 (35.7%) | 6 (35.7%) |
| High circ_0069767, n = 19 | 1 (5.26%) | 8 (42.1%) | 12 (63.2%) | 11(57.9%) | 12 (63.2%) |
| *P* value | 0.046* | 0.353 | 0.097 | 0.159 | 0.097 |
| * *p*< 0.05 | | | | | |

with *miR-29*, *miR-370*, *miR-491*, *miR-636* and *miR-1264* mimics into HEK-293T cells using Lipofectamine™ 3000. The relative luciferase activity was detected by the Dual-Luciferase Reporter Assay Kit. The results showed that overexpression of *miR-636* was able to dramatically reduce the luciferase activity of wild-type *circ_0069767* reporter, whereas other miRNAs had no effects on the wild-type *circ_0069767* reporter. Then the full length of *circ_0069767* mutant-type of *miR-636* binding site and *miR-636* mimics were co-transfected into HEK-293T cells, and the results showed that there was no interaction between them (Figure 6A and B). Then, we used the online miRWalk database to identify the downstream target of miR-636, and found that *K-RAS*, the well-known oncogene, may be bound
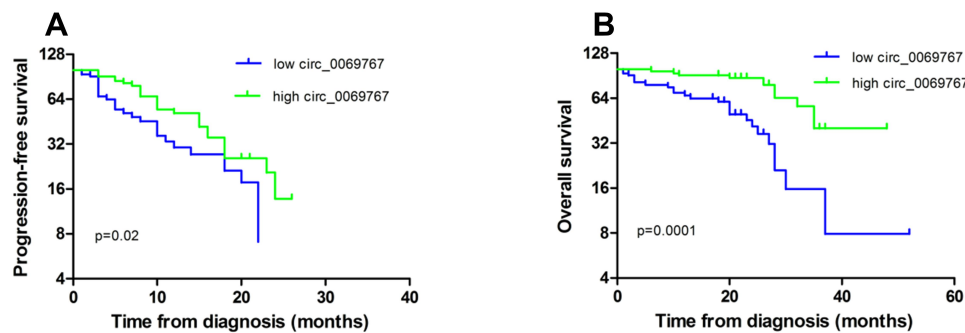


**Figure 2** The patients with high expression of *circ_0069767* demonstrate improved PFS and OS. (**A**) Progression-free curve analysis of high or low expression of *circ_0069767* in MM patients. (**B**) Overall survival curve analysis of high or low expression of *circ_0069767* in MM patients.
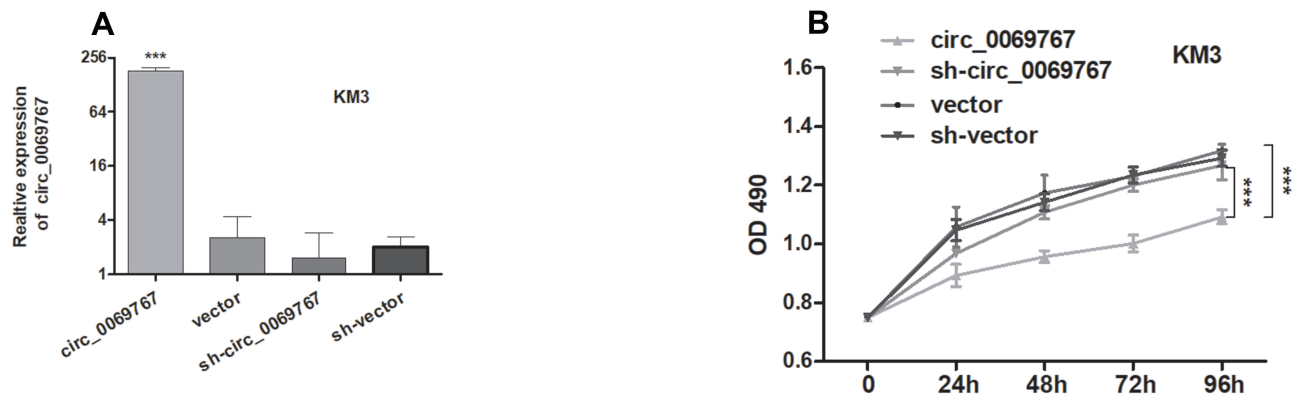


**Figure 3** The cells overexpressing *circ_0069767* had reduced viability. (**A**) Transfection efficiency of *circ_0069767* overexpression and knocking down were determined by qRT-PCR. *circ_0069767* vs vector, sh-circ_0069767 and and sh-vector, *p*< 0.01. (**B**) The MTS assay detected the effect of circ_0069767 on cell viability of MM cells. *circ_0069767* vs vector, sh-circ_0069767 and and sh-vector, *p*< 0.01.
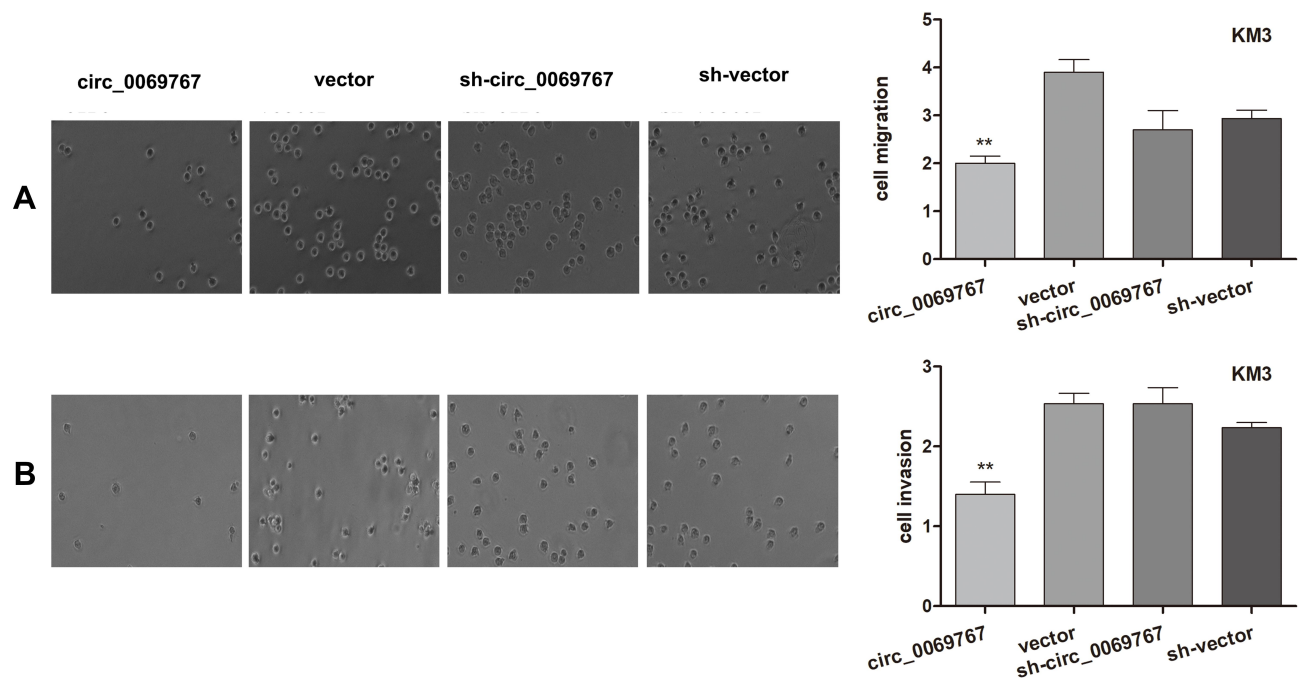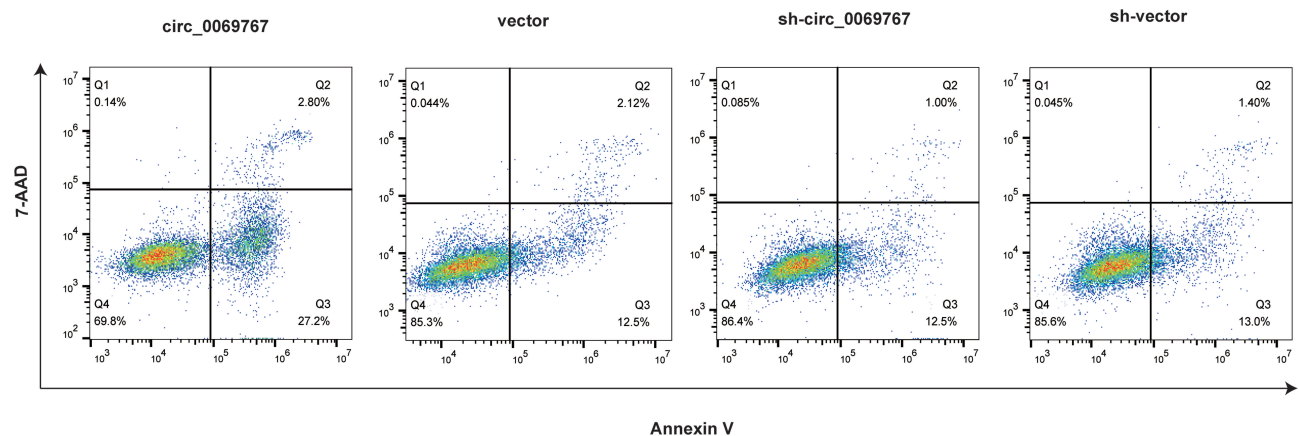
**Figure 4** Transwell assay detects cell migration and invasion. (**A**) The transwell assay detected the effect of *circ_0069767* on cell migration of MM cells. *circ_0069767* vs vector, sh-circ_0069767 and and sh-vector, *p*< 0.01. (**B**) The transwell assay detected the effect of *circ_0069767* on cell invasion of MM cells. *circ_0069767* vs vector, sh-circ _0069767 and and sh-vector, *p*< 0.01. **\*\****p*<0.01.



**Figure 5** Flow cytometry apoptosis method detected the effect of *circ_0069767*, sh-circ_0069767, vector and sh-vector on cell apoptosis. With bortezomib effecting on the cells (IC50 values 100 ng/mL) after 24h of treatment, the apoptosis of cells overexpressing *circ_0069767* was significantly increased than that of other groups. circ_0069767 vs vector, sh-circ_0069767 and and sh-vector, *p*< 0.01.

by *miR-636*. To confirm such prediction, we conducted a luciferase reporter assay in HEK-293T cells. As shown in Figure 6C, *miR-636* overexpression significantly decreased the luciferase activity of wild-type *K-RAS* 3′-UTR reporter, while this effect was not observed in the mutant reporter. We then performed qRT-PCR assay in KM3 cells, and the results displayed that *miR-636* expression was significantly decreased after *circ_0069767* overexpression (Figure 6D).

## The Cells Overexpressing Circ_0069767 Had Increased K-RAS Expression

RT-PCR analysis showed that relative expression of *K-RAS* was higher in cells overexpressing *circ_0069767* (Figure 7A). Western blot analysis was repeated triple times and the results demonstrated that *K-RAS* protein expression was increased in cells overexpressing *circ_0069767* (Figure 7B).
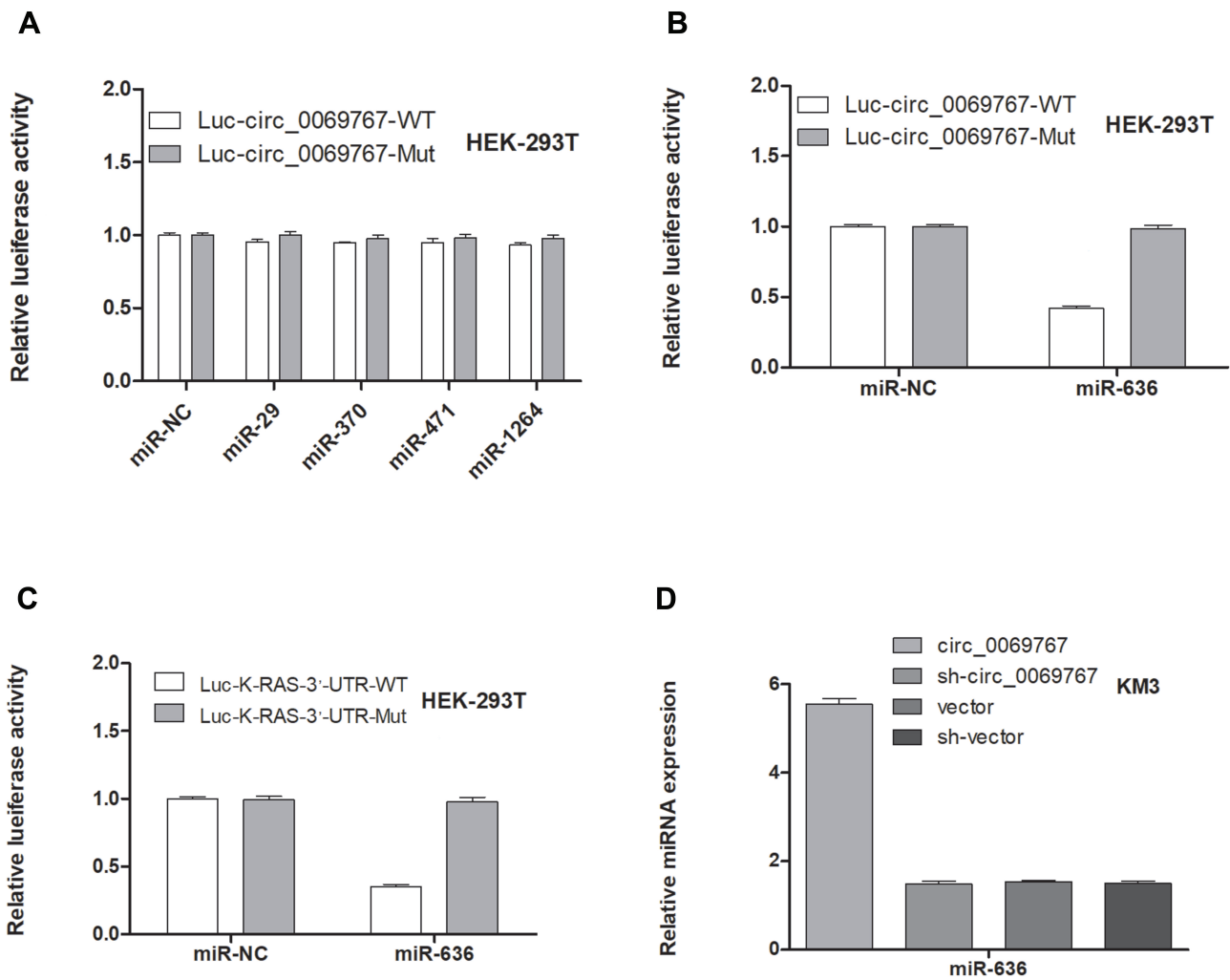
**Figure 6** *Circ_0069767* regulates *miR-636/K-RAS* axis in KM3 cells. (**A**) The luciferase activity of MM cells co-transfected with wild-type or mutant *circ_0069767* reporter and control or miRNAs mimics. (**B**) The luciferase activity of MM cells co-transfected with wild-type or mutant *circ_0069767* reporter and control or *miR-636* mimics. (**C**) The luciferase activity of MM cells co-transfected with wild-type or mutant *K-RAS* 3′-UTR reporter and control or *miR-636* mimics. (**D**) qRT-PCR analysis of *miR-636* expression in KM3 cells.
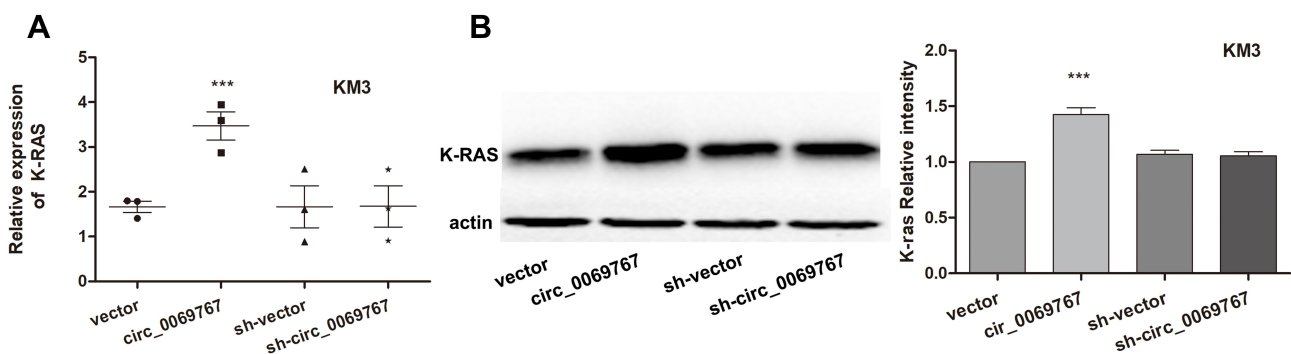


**Figure 7** Detection of K-RAS expression in cells overexpressing circ_0069767. (**A**) RT-PCR analysis of K-RAS in cells with overexpressing *circ_0069767*, vector, sh-circ _0069767 and sh-vector. *circ _0069767* vs vector, sh-circ_0069767 and sh-vector, $p < 0.001$. (**B**) Western blot analysis with repeated 3 times detected the effect of *circ_0069767*, sh-circ_0069767, vector and sh-vector on the expression of K-RAS. *circ_0069767* vs vector, sh-circ_0069767 and sh-vector, $p < 0.001$. ***$p < 0.001$.

## There Was No Mutation of K-RAS Gene

We performed second-generation gene sequencing on cells overexpressing *circ_0069767*, sh-circ_0069767, vector and sh-vector, and there was no mutation of *K-RAS* gene.

## Discussion

The development of sequencing technique has led to the identification of circRNAs that are considered to be widely expressed in human cells. By joining the 3′ and 5′ ends together, circRNA forms a covalently closed continuous loop that confers excellent stability. Therefore, circRNA is increasingly expected to be ideal indicator for tumor diagnosis.[22,23] An increasing number of studies have reported that circRNA is associated with the occurrence and development of tumors; for instance, the up-regulation or knockdown of circRNA_0020123 significantly affect the growth and metastasis of tumor cells in non-small cell lung cancer.[24] Reducing the expression of *hsa_-circ_0000064* has been shown to inhibit cell proliferation, promote apoptosis, and regulate the cell cycle in lung cancer cells. Similarly, in liver cancer cells, *circ_0000064* was revealed to promote the progression of the malignancy.[25,26] In diffuse large B-cell lymphoma (DLBCL), *circ-Foxo3* is closely related to cell cycle pro-gression—the intervention with *circ-Foxo3* accelerates the cell cycle and enhances proliferation capacity, while its overexpression has been shown to suppress cell cycle progression. Researchers also suggested that circ-Foxo3 inhibits cell cycle progression by forming a complex with cyclin-dependent kinase *CDK2* and cyclin-dependent kinase inhibitor *P21*.[27] These studies suggest that circRNAs are involved in the occurrence and development of tumors. In our study, based on information from the circBase database, *circ_0069767* is derived from the transcript of the *C-KIT* protein (*CD117*) gene, with a sequence length of 722bp and located on chromosome 4 chr4: 55573263–55593490. Our results showed that circRNA *circ_0069767* was differentially expressed in human MM cells and normal bone marrow cells and it was significantly correlated with the prognosis of MM patients. High expression of *circ_0069767* in MM patients leads to improved PFS and OS, suggesting that *circ_0069767* may be an eligible indicator to MM prog-nosis evaluation. To accurately assess the prognosis of MM and provide clinically reasonable and effective prog-nostic information, stable expression of biomarkers is particularly practical. For that circRNA has a closed-loop structure, it is very stable and easy to be detected, which makes it suitable as a biomarker for prognosis evaluation than linear RNA. In addition, MM cells overexpressing *circ_0069767* demonstrated decreased proliferation, migration, and invasion capacities, while the MM cells with *circ_0069767* knockdown demonstrated increased proliferation, migration, and invasion capacities. After bortezomib treatment, the apoptosis rate of each group was up-regulated, and the apoptosis of cells overexpres-sing *circ_0069767* was significantly increased than other groups. It may be that the pathway of apoptosis activated by *circ_0069767* and bortezomib was the same. And this is what our research group will study in the future. Cell function test results showed that *circ_0069767* played a role of tumor suppressor gene in MM, explained why the higher *circ_0069767* expression linked to better PFS and OS.

Our previous studies showed that patients with high expression of *C-KIT* protein (*CD117*) had better PFS and OS,[21] and *circ_0069767* was derived from the *C-KIT* gene, which may further explain why patients with positive *CD117* have improved PFS and OS. Some research showed that circRNA was produced by co-transcription and competed with conventional splicing during the pro-duction process. Therefore, the biogenesis of circRNA lead to a decrease in mRNA synthesis from the same locus.[28,29] But our research showed that *circ_0069767* is positively correlated with the mRNA expression of *CD117*, so there may be other regulatory pathways. However, it is not yet clear how *circ_0069767* affects the cellular biological behaviors, which will be the focus of subsequent studies.

We focused on the sponging function of circular RNA and bioinformatically predicted the targeted miRNAs and downstream mRNAs that *circ_0069767* may competitively bind with and confirmed through experiments, the results of which demonstrated that *circ_0060767* regulates the expression of *K-RAS* through *miR-636*. In other words, the *circ_0069767/miR-636/K-RAS* regulatory axis exists in the KM3 cells. We also noticed an interesting phenom-enon regarding an association between high *K-RAS* mRNA expression and increased protein expression ($p<0.001$) in circ_0069767 overexpressing cells. As it is known that only when the *K-RAS* gene is mutated can it activate downstream signaling pathways, promote cell growth and proliferation, and inhibit cell senescence and death, even-tually causing cell to become cancerous.[30,31] In our study,

the expression of *K-RAS* gene and protein increased after overexpression of *circ_0069767* while the *K-RAS* gene remain unmutated. This phenomenon further supported the hypothesis that the *K-RAS* gene does not cause disease and affect tumor cell behaviors unless mutated. Therefore, *circ_0069767* affects the biological behavior of MM cells through other pathways rather than the K-RAS pathway.

The mystery of circRNA is gradually being unveiled, but its structural specificity also foretells the diversity of its roles. As the current study suggests, *circ_0069767* plays an antitumor role and its expression can be used as a reliable prognostic indicator of MM patients.

## Author Contributions

All authors contributed to data analysis, drafting or revising the article, have agreed on the journal to which the article was submitted, 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 conflicts of interest for this work and declared no potential conflicts of interest with respect to the research, authorship, or publication of this article.

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