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CircKCNQ5 controls proliferation, migration, invasion, apoptosis, and glycolysis of multiple myeloma cells by modulating miR-335-5p/BRD4 axis

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Running title: The functional roles of circKCNQ5 in multiple myeloma
Abstract

Background: Circular RNAs (circRNAs) are key players in tumorigenesis progression. However, the role and molecular mechanisms of circKCNQ5 in multiple myeloma (MM) progression remain unclear.

Methods: The quantitative real-time polymerase chain reaction was used for examining circKCNQ5, miR-335-5p, and Bromodomain-containing protein 4 (BRD4) levels. The proliferation ability of MM cells was determined by Cell Counting Kit-8 and colony-forming assays. The migration and invasion were analyzed by transwell assay. Flow cytometry was used to assess cell apoptosis. The lactate production, glucose consumption, and ATP/ADP ratios were determined by commercialized kits. The protein levels were quantified by western blot analysis. The interactions between circKCNQ5 and miR-335-5p, along with miR-335-5p and BRD4 were analyzed by dual-luciferase reporter and RNA immunoprecipitation assays.

Results: The overexpression of circKCNQ5 was confirmed in MM tissues and cells. Importantly, knockdown of circKCNQ5 suppressed proliferation, migration, invasion, and glycolysis while it increased apoptosis of MM cells in vitro. Interestingly, the downregulation of miR-335-5p was able to rescue the circKCNQ5 inhibition-induced effects on MM cells. MiR-335-5p interacted with circKCNQ5, and was able to target BRD4 in MM cells. MiR-335-5p upregulation inhibited malignant phenotypes of MM cells depending on BRD4.

Conclusion: CircKCNQ5 was found to stimulate MM progression through competitively sponging to miR-335-5p.

Keywords: circKCNQ5, miR-335-5p, BRD4, multiple myeloma
Introduction

Multiple myeloma (MM) is a malignant hematological disease with no cure, characterized by malignant expansion and gathering of malignant plasma cells (Kazandjian, 2016; Bringle and Rogers, 2017). The clinical prognosis of MM patients remains unsatisfactory due to shortage of effective therapeutic regimens (Zhang et al., 2020). Thus, it is urgently needed to identify appropriate diagnostic markers and develop effective treatments.

Circular RNAs (circRNAs) are a class of non-coding RNAs with a covalently closed loop structure, which are produced by backsplicing of pre-mRNAs (Patop et al., 2019; Su et al., 2019). High-throughput sequencing analysis revealed that circRNAs are widely expressed in human tissues and exhibit cell type-specific and tissue-specific expression patterns. In contrast to linear RNAs, the lack of 5’-3’ polarity and the polyadenylated tail make circRNAs more resistant to exonuclease degradation (Su et al., 2019; Vo et al., 2019). Therefore, circRNAs might be used as reliable biomarkers or therapeutic targets for cancers. For instance, circRNA_101237 was obviously upregulated in MM tissues and had the potential application value for diagnosis and therapeutics (Liu et al., 2020). CircKCNQ5 (hsa_circ_0007165) is coded by potassium channel gene (KCNQ5) and is located on chr6 (73713630-73830300). A previous report has revealed that circKCNQ5 was upregulated in MM tissues, but its biologic function is unknown (Zhou et al., 2020).

MicroRNAs (miRNAs) are acknowledged as non-coding RNAs with 19-25 nucleotides and no ability to encode proteins (Saliminejad et al., 2019). Recent evidence supported that miRNAs functioned as gene regulators at the post-transcriptional level by binding with 3’untranslated regions (UTR) of mRNAs (Bartel, 2009). MiR-335-5p is a well-explored miRNA, which acted as a cancer-inhibitory miRNA to implicate in cell function in different cancers, including thyroid cancer (Luo et al., 2018), colorectal cancer (Zhang and Yang, 2019), and lung adenocarcinoma (Wang et al., 2020a). Importantly, increased expression of miR-335-3p suppressed invasion of MM cells depending on type 1 IGF receptor, which could contribute to predicting both the prognosis and progression of MM.
patients (Qi et al., 2019). Herein, through bioinformatics analysis, miR-335-3p was predicted to be the target of circKCNQ5 in MM. Therefore, we further investigated the clinical significance and exact role of miR-335-5p in MM development.

Bromodomain-containing protein 4 (BRD4) is a member of the bromodomain and extra-terminal domain (BET) family that can interact with DNA sequences and then recruit transcription factors to enhance gene expression through exerting the N-acetylation function in lysine residues on histone tail (Taniguchi, 2016; Lambert et al., 2019). The upregulation of BRD4 has important impacts on the progression of MM (Handa et al., 2020; Zheng et al., 2020), and BRD4 inhibitor was shown to be a promising target for MM therapy (Li et al., 2020).

Therefore, we investigated the abundance of circKCNQ5, miR-335-5p, and BRD4 in MM. The result of functional experiments identified that circKCNQ5 downregulation decreased cell growth, mobility, and glycolysis while it increased apoptosis of MM cells through the miR-335-5p/BRD4 axis, providing new biomarkers for MM.

Materials and methods
Patient specimens
The bone marrow tissue samples from MM patients (N=43) and 43 healthy samples from donors were collected at Tianjin Fourth Central Hospital following support and supervision by the Ethics Committee of Tianjin Fourth Central Hospital. All patients and donors provided written informed consent before surgery. The removed tissues were maintained at -80°C. The clinical characteristics of MM patients are shown in Table 1. All patients were continuously followed up by telephone or clinical visit. The progression-free survival (PFS) was defined as the duration from initial treatment to disease progression or death. The overall survival (OS) was calculated from the date of treatment initiation to the date of death.
Cell lines

Human normal plasma cells (nPCs) were obtained from the bone marrow of healthy donors. MM cells (MM1S, RPMI-8226, H929, U266, and OPM2) were obtained from the Nanjing Key Gen Biotech (Nanjing, China) and then grown in RPMI 1640 medium (HyClone, Logan, UT, USA) supplemented with 10% FBS (HyClone) under the conditions (5% CO$_2$, 37°C).

RNA extraction and quantitative real-time polymerase chain reaction (RT-qPCR)

TRIzol (Vazyme, Nanjing, China) was used for RNA extraction from tissue specimens. For measurement of circRNAs and mRNAs, cDNA was generated from total RNA with Universal cDNA synthesis kit II (Exiqon, Woburn, MA, USA) and then RT-qPCR was conducted by SYBR Green Master Mix (Vazyme) under Thermal Cycler CFX6 System (Bio-Rad, Hercules, CA, USA), with β-actin as housekeeping gene. The level of miR-335-5p expression was determined by Mir-X miR First-Strand Synthesis Kit (Takara, Dalian, China) using stem-loop primer: 5’-CTCAACTGGTGTCGGAGTGGCAATTCAGTTGAGACATTGATTTT-3’, and then quantified by RT-qPCR, with U6 as control. The relative level was evaluated using comparative threshold cycle (Ct) method. Furthermore, total RNA was incubated with or without 3U/mg RNase R (Geneseed, Guangzhou, China) for 15 min at 37°C to analyze RNA stability. The primer sequences were: circKCNQ5, (sense) 5’-TAACCTTGGCTGGAAGATTG-3’ and (antisense) 5’-AGAGGCAACTTGGAGGCGAAC-3’; KCNQ5, (sense) 5’-TGTTGTCGATATAGAGGATGGCA-3’ and (antisense) 5’-GAGTGCAGACGTGGCAAAAAT-3’; miR-335-5p, (sense) 5’-GCCGAGTCAAGAGCAATAA-3’ and (antisense) 5’-CTGGTGTCGGAGTGGGCA-3’; BRD4, (sense) 5’-GAGCTACCCACAGAAGAAACC-3’ and (antisense) 5’-GAGTCGATGCTTGGAGTTGTT-3’; U6, (sense) 5’-GTGCTCGCTTCCGGCAGCACA-3’ and (antisense) 5’-GTGCTCGCTTCCGGCAGCACA-3’. 
5'-GGAACGCTTCACGAATTTG-3';  \( \beta \)-actin (sense)

5'-ATTGCCGACAGGATGCAGAA-3';  (antisense)

5'-CGCTCAGGAGGAGCAATGAT-3'.

**Cell transfection**

The oligonucleotides were synthesized from RiboBio (Shanghai, China): short interfering RNA (siRNA) targeting circKCNQ5 (si-circKCNQ5#1 and si-circKCNQ5#2), siRNA scrambled control (si-NC), the mimics of miR-335-5p and negative control (miR-335-5p and miR-NC), and inhibitors of miR-335-5p and negative control (in-miR-335-5p and in-NC). BRD4-upregulated vector (BRD4), and scrambled group (pcDNA) were provided by HanBio (Shanghai, China). The oligonucleotides/vectors-lipid mixtures using Lipofectamine 3000 reagent (Promega, Madison, WI, USA) were incubated with MM cells for 6 h at 37°C when cells reached 60%-70% confluence. The cells were cultured with fresh medium.

**Cell Counting Kit-8 (CCK-8) and colony-forming assays**

U266 and OPM2 cells at logarithmic growth phase were planted into 96 wells plates (3000 cells/well). After that, cells were incubated with 10 µL of CCK-8 (Vazyme) and further cultured for 2 h. A microplate reader (Bio-Rad) was used to measure the absorbance at 450 nm. For colony-forming, U266 and OPM2 cells were introduced into 6-well plates and then cultured at the conditions (5% CO\(_2\), 37°C). At the end of 14 d, the cells were fixed with fixation solution for 5 min and stained by 0.2% crystal violet (GE Healthcare, Piscataway, NJ, USA). Colonies (>50 cells) were counted under a microscope (Leica Microsystems GmbH, Wetzlar, Germany).

**Migration and invasion assay**

Transwell chambers (Corning, Franklin Lakes, NJ, USA) were used to determine the migratory capacity of U266 and OPM2 cells. Transfected U266 and OPM2 cells were suspended in 200 µL of serum free medium (2 \( \times \) 10\(^4\) per chamber) and then added into the top chamber, while the medium containing 10% FBS was used as
nutrient in the lower chamber. Following treatment with 0.2% crystal violet (GE Healthcare), the migrated cells were measured under a microscope (Leica Microsystems GmbH) in five random fields of view at 100× amplification. Transwell chambers pro-adhered with Matrigel (Corning) were used for invasion assay.

**Cell apoptosis assay**

To assess cell apoptosis, U266 and OPM2 cells after transfection were collected by trypsin and then washed with phosphate buffer saline. Afterwards, cells were exposed to saline buffer solution from Annexin V-FITC Apoptosis Detection Kit (TransGen Biotech, Beijing, China). After reaction for 15 min without light, apoptotic cells were assessed under FACSARia flow cytometer (Becton Dickinson, San Jose, CA, USA).

**Glucose consumption, lactate production, and intracellular ATP assays**

U266 and OPM2 cells were seeded into 12-well plates (1×10^5 cells/well) and then incubated for 24 h. The amount of glucose present in cell culture medium was analyzed by Glucose Assay Kit (Invitrogen, Carlsbad, CA, USA) referring to the user’s guidebook. Similarly, lactate production in the medium was assessed using a lactic acid assay kit (Invitrogen). ATP/ADP ratio was determined with ApoSSENSOR™ ADP/ATP Ratio Bioluminescence Assay Kit (Biovision, San Francisco, CA, USA).

**Western blot assay**

Following the quantitation of protein concentration, protein samples (the same amount in each sample) were electrophoresed on 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis. The isolated proteins were transferred to the nitrocellulose membranes (Roche, Basel, Switzerland) by electrophoretic transfer method. The nitrocellulose membranes were blocked by 5% skim milk solution in tris buffered saline tween (TBST) buffer and then interacted with antibodies for 12 h at
4 °C. Next, HRP-linked secondary antibodies (# 7074S; 1:2000 dilution; Cell Signaling Technology, Danvers, MA, USA) were added into membranes. Bands on the membrane were visualized using ECL-PLUS/Kit (GE Healthcare). The primary antibodies were bought from Cell Signaling Technology, such as β-actin (# 4970S; 1:1500 dilution), BRD4 (# 13440S; 1:1500 dilution), E-cadherin (# 3195S; 1:1500 dilution), N-cadherin (# 13116S; 1:1500 dilution), and Vimentin (# 5741S; 1:1500 dilution).

**Dual-luciferase reporter assay**

Circinteractome (https://circinteractome.nia.nih) was utilized to predict the binding miRNAs of circKCNQ5. The target gene of miR-335-5p was predicted by DianaTools-microT_CDS (http://diana.imis.athena-innovation.gr/DianaTools/index.php?r=microT_CDS/index).

A fragment of circKCNQ5 sequences comprising the putative binding sites on miR-335-5p was cloned into downstream of pGL3-promoter (Promega). A second construct without the putative binding sites fragment (circKCNQ5 MUT) was also created by Quik-Change II XL Site-Directed Mutagenesis Kit (Vazyme, Nanjing, China). U266 and OPM2 cells were transfected with a wild-type or a mutant reporter vector along with miR-335-5p mimic or control using Lipofectamine 3000 (Promega). Dual-Luciferase Assay Kit (Promega) was used to assess firefly luciferase activity, with Renilla luciferase activity as control. Similarly, the association between miR-335-5p and BRD4 was analyzed.

**RNA immunoprecipitation (RIP) assay**

For RIP assay, U266 and OPM2 cells were incubated with RIP lysis buffer from EZ-Magna RIP kit (Millipore, Billerica, MA, USA), and then collected cellular lysates were probed with magnetic beads conjugated Argonaute-2 (Ago2; Abcam, Cambridge, MA, USA) or IgG (Abcam) at 4°C for 4 h. After proteinase K treatment at 55°C for 15 min to digest the protein, immunoprecipitated RNA was subjected to RT-qPCR assay.
Statistical analysis

The data were statistically analyzed with SPSS 21.0 (IBM, Somers, NY, USA), and P-value less than 0.05 was considered as the threshold for statistical significance. Comparison between groups was measured by Student’s t-test or analysis of variance, as appropriate.

Results

CircKCNQ5 was upregulated in MM tissues and cells

In this study, we investigated the functional effects of circKCNQ5 on MM by assessing the expression of circKCNQ5 in human bone marrow tissue samples from MM patients and healthy controls. As presented in Figure 1A, circKCNQ5 was upregulated in MM tissues in contrast to control tissues. Besides, circKCNQ5 was also overexpressed in MM cells relative to control nPCs cells, and U266 and OPM2 cells were chosen in the next experiments (Fig. 1B). We also found that circKCNQ5 was more resistant to RNase R degradation than KCNQ5 mRNA (Fig. 1C-D). In addition, the correlation between circKCNQ5 expression and the clinical characteristics of MM patients was analyzed. As shown in Table 1, patients with high expression of circKCNQ5 had significantly shortened OS (P=0.0001) and PFS (P=0.0024) time, as well as advanced International staging system (ISS) stage (P=0.034). These data indicated the clinical significance of circKCNQ5 in evaluating prognosis in MM.

Depletion of circKCNQ5 impaired proliferation, migration, invasion, and glycolysis while it increased apoptosis of MM cells

The loss-of-function assay was performed using si-circKCNQ5-mediated circKCNQ5 knockdown in U266 and OPM2 cells. As compared to si-NC transfected U266 and OPM2 cells, circKCNQ5 was downregulated in si-circKCNQ5-transfected cells (Fig. 2A-B). Further, the results of CCK-8 assay showed that circKCNQ5 inhibition decreased proliferation of U266 and OPM2 cells (Fig. 2C-D). Consistent
with these findings, circKCNQ5 knockdown also inhibited the colony-forming ability of U266 and OPM2 cells (Fig. 2E). In addition, circKCNQ5-silencing U266 and OPM2 cells showed strong inhibitory effects on migration and invasion capacities compared with si-NC transfected cells (Fig. 2F-G). On the contrary, the downregulation of circKCNQ5 significantly enhanced cell apoptosis (Fig. 3A). Reduction in lactate production and glucose consumption in the culture medium were found in circKCNQ5-silencing U266 and OPM2 cells; besides, ATP/ADP ratios were decreased after knockdown of circKCNQ5, suggesting glycolysis inhibition in U266 and OPM2 cells (Fig. 3B-D). As confirmed by western blot analysis, E-cadherin was increased while N-cadherin and Vimentin were downregulated in circKCNQ5-silencing U266 and OPM2 cells (Fig. 3E-F). Hence, suppression of circKCNQ5 repressed malignant phenotypes of MM cells.

MiR-335-5p was a direct target of circKCNQ5

To clarify the regulatory mechanism of circKCNQ5 in MM, the target miRNAs of circKCNQ5 were predicted by Circinteractome. Several miRNAs, including miR-127-5p, miR-599, miR-595, miR-622 and miR-335-5p, were predicted to contain the complementary binding sites of circKCNQ5. Among these miRNAs, miR-335-5p was the most downregulated in MM cells (Fig. 4), and has been reported to have a prognostic role in MM (Qi et al., 2019). Thus, miR-335-5p was chosen for subsequent research. The complementary binding sites between circKCNQ5 and miR-335-5p were shown in Figure 5A, and a schematic illustration showed the construct of luciferase reporter vectors. We found that transfection with miR-335-5p mimic increased, while transfection with of in-miR-335-5p decreased miR-335-5p expression in U266 and OPM2 cells (Fig. 5B). The ectopic expression of miR-335-5p decreased luciferase reporter activity of circKCNQ5 WT group, but there was no observed difference in circKCNQ5 MUT group (Fig. 5C-D). RIP assay revealed that miR-335-5p and circKCNQ5 were enriched by AGO2 but not IgG in U266 and OPM2 cells (Fig. 5E-F). Furthermore, miR-335-5p was obviously decreased in MM tissues and cells in contrast to matched controls (Figure 5G-H). Interestingly,
knockdown of circKCNQ5 increased the expression of miR-335-5p in U266 and OPM2 cells (Fig. 5I). MiR-335-5p expression was regulated by circKCNQ5 in MM cells.

Depletion of circKCNQ5 suppressed proliferation, migration, invasion, and glycolysis while it increased apoptosis of MM cells depending on miR-335-5p

To assess the undetermined biological relevance between circKCNQ5 and miR-335-5p, rescue experiments were performed. As shown in Figure 6A-B, si-circKCNQ5#1 was used to increase the expression of miR-335-5p, which was abolished by transfection with in-miR-335-5p. The suppressive effects on proliferation and clone formation of U266 and OPM2 cells induced by circKCNQ5 depletion were rescued by miR-335-5p inhibition (Fig. 6C-E). CircKCNQ5 knockdown also repressed migration and invasion while it promoted apoptosis of U266 and OPM2 cells, and these effects were neutralized by miR-335-5p inhibition (Fig. 6F-H). Importantly, the downregulation of miR-335-5p was able to partially rescue the glycolysis inhibition in si-circKCNQ5#1-transfected cells (Fig. 6I-K). The increased expression of E-cadherin and decrease of N-cadherin and Vimentin in U266 and OPM2 cells induced by circKCNQ5 inhibition were reversed by transfection with in-miR-335-5p (Fig. 6L-M). These results suggested that circKCNQ5 regulated proliferation, migration, invasion, apoptosis, and glycolysis of MM cells through miR-335-5p.

BRD4 was a downstream target of miR-335-5p

As shown in Figure 7A, BRD4 was predicted to contain the complementary binding sites to miR-335-5p. The reduced luciferase reporter activity was found in U266 and OPM2 cells co-transfected with miR-335-5p and BRD4 3’UTR WT (Fig. 7B-C). What’s more, BRD4 level was upregulated in MM tissues and cells (Fig. 7D-F). In addition, BRD4 expression was lower in U266 and OPM2 cells after overexpression of miR-335-5p compared with control (Fig. 7G). BRD4 was obviously downregulated after knockdown of circKCNQ5, which was rescued by miR-335-5p
inhibition (Fig. 7H). In summary, BRD4 was a functional target of miR-335-5p.

Overexpression of miR-335-5p regulated proliferation, migration, invasion, apoptosis, and glycolysis of MM cells through BRD4

To characterize the cellular effects of miR-335-5p/BRD4 axis, we assessed functional experiments in U266 and OPM2 cells. The downregulation of BRD4 in miR-335-5p-transfected cells was overturned by transfection with BRD4 (Fig. 8A-B). The overexpression of BRD4 blocked miR-335-5p-dependent proliferation inhibition in U266 and OPM2 cells (Fig. 8C-E). We found that miR-335-5p upregulation suppressed migration and invasion while it increased cell apoptosis in U266 and OPM2 cells, which was rescued by overexpression of BRD4 (Fig. 8F-H). Decreased glucose consumption, lactate production, and ATP/ADP ratios were observed in the miR-335-5p-treated group, and the co-transfection of miR-335-5p and BRD4 significantly counteracted these effects (Fig. 8I-K). The increased expression of miR-335-5p increased E-cadherin and decreased N-cadherin and Vimentin expression in U266 and OPM2 cells, which was reversed by BRD4 upregulation (Fig. 8L-M). The miR-335-5p/BRD4 axis played important roles in proliferation, migration, invasion, apoptosis, and glycolysis of MM cells.

Discussion

Currently, our results displayed that knockdown of circKCNQ5 repressed malignant phenotypes of MM cells. Importantly, the regulatory mechanism of circKCNQ5 was dependent on miR-335-5 promotion and suppression of BRD4 expression, underlining the significance of circKCNQ5 in MM progression.

CircRNAs have been considered as potential diagnostic and prognostic targets for multiple diseases, including MM (Zhou et al., 2020; Sun et al., 2021). Through circRNA microarray analysis, Zhou et al. uncovered that circ-PTK2, circ-RNF217 and circ-AFF2 expression are closely related to the treatment response and survival of MM patients, which could be potential prognostic biomarkers for MM (Zhou et al., 2020). In the present research, we disclosed the abnormally elevated circKCNQ5 in
MM tissues and cells, and found that circKCNQ5 knockdown constrained the progression of MM cells by regulating cell proliferation, migration, invasion, apoptosis and glycolysis.

In previous reports, circRNAs have been reported to act miRNA sponges through competing with miRNA response elements, thereby mediating the expression of downstream genes targeted by miRNAs at the post-transcriptional level (Hansen et al., 2013). Although circKCNQ5 was aberrantly expressed in human MM (Zhou et al., 2020), the functional roles of circKCNQ5 were explored. We identified circKCNQ5 as a key regulator of MM progression by serving as a miR-335-5p sponge. It has been confirmed that several circRNAs can sponge miR-335-5p in cancers. For instance, miR-335-5p was sponged by circ_0009910 and participated in the regulation of hepatocellular carcinoma cell proliferation and metastasis (Li and Liu, 2020). Circular RNA 0007255 acted as a sponge for miR-335-5p to regulate the progression of breast cancer (Jia et al., 2020). In addition, miR-335-5p was sponged by circZMYM2 and regulated pancreatic cancer development (An et al., 2018). Conclusively, circRNA/miRNA-based regulatory mechanism plays significant roles in MM progression.

Considering that miRNAs are reported to control gene expression by targeting 3’ UTR of its downstream targeting mRNA, it was no surprise that miRNAs were demonstrated to play multifunctional roles in tumorigenesis (Bartel, 2009). Previous studies have identified many target mRNAs of miR-335-5p, and miR-335-5p was a dysregulated miRNA involved in occurrence and development of human cancers (Luo et al., 2018; Zhang and Yang, 2019; Wang et al., 2020a). Here, the tumor-suppressive functions of miR-335-5p were also revealed in MM through regulating BRD4 expression.

Additionally, the abnormal activation BRD4 signaling was necessary and sufficient to promote development of hematologic cancers, including MM (Stubbs et al., 2019). As a key epigenetic protein, BRD4 was shown to play a significant role in multiple cell behaviors (White et al., 2019). For instance, a report discovered that BRD4 inhibition blocked MYC expression and induced degradation, thereby leading
cancer cell growth and metastasis in cancer cells (Andrews et al., 2017). Zou et al. found that BRD4 could bind to acetylated RelA and then regulate the transcriptional activity of NF-κB to stimulate lung carcinoma progression (Zou et al., 2014); supporting a role for BRD4 in MM, Guo et al. revealed that I-BET151 decreased excessive osteoclast formation and inflammatory cytokine secretion by targeting BRD4-mediated RANKL-NF-κB signal pathway (Guo et al., 2019). Besides, BRD4 also was reported to regulate programmed death 1 ligand (PD-L1) expression in immune cells, while PD-L1 played a critical role of T cell immune checkpoint activation (Zhu et al., 2016; Jing et al., 2020). Consistent with previous conclusions (Wang et al., 2020b), BRD4 was upregulated in MM tissues and cells, and we elucidated that the upregulation of miR-335-5p regulated MM cells function through BRD4. Thus, BRD4 was confirmed to exert functions as a tumor promoter, suggesting attractive therapeutic targets for patients with MM.

In summary, all findings confirmed that circKCNQ5 contributed to tumorigenesis and development of MM, representing the first functional characterization of circKCNQ5 and identifying it as a key driver of MM progression, which was dependent on its interaction with the miR-335-5p/BRD4 axis.

Conclusion

In summary, circKCNQ5 promoted MM progression by acting as miR-335-5p sponges to increase BRD4 expression. These results shed light on the mechanisms of MM and improve the understanding of circKCNQ5 in MM progression.

Acknowledgment

None.

Disclosure of interest

The authors declare that they have no financial conflicts of interest.

Funding
None.

**Availability of data and materials**

Please contact the correspondence author for the data request.

**Ethics approval and consent participate**

Written informed consent was obtained from patients with approval by the Institutional Review Board in Tianjin Fourth Central Hospital.

**Consent for publication**

Not applicable.

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Table 1 Correlation between circKCNQ5 expression and clinical characteristics of MM patients.

Figure legends

Figure 1 The expression level of circKCNQ5 in multiple myeloma tissues and cells. (A) RT-qPCR was used for measuring circKCNQ5 level in multiple myeloma tissues (n=43) and healthy control tissues (n=43). (B) The expression level of circKCNQ5 was assessed by RT-qPCR in multiple myeloma cells and control nPCs cells. (C-D) The relative levels of circKCNQ5 and KCNQ5 mRNA were determined by RT-qPCR after treating with RNase R. *P < 0.05.

Figure 2 Effects of circKCNQ5 knockdown on cell proliferation, migration and invasion in multiple myeloma cells. (A-M) U266 and OPM2 cells were transfected with si-NC, si-circKCNQ5#1, or si-circKCNQ5#2. (A-B) The expression level of circKCNQ5 was analyzed by RT-qPCR. (C-E) The proliferation of U266 and OPM2 cells were determined by CCK-8 and colony-forming assays. (F-G) Enumeration of migration and invasion was analyzed by transwell assay. *P < 0.05.
Figure 3 Effects of circKCNQ5 knockdown on cell apoptosis, glycolysis and EMT in multiple myeloma cells. (A-F) U266 and OPM2 cells were transfected with si-NC, si-circKCNQ5#1, or si-circKCNQ5#2. (A) The apoptosis rate was measured by flow cytometry. (B-D) The lactate production, glucose consumption, and ATP/ADP ratios were determined by commercialized kits. (E-F) The protein levels of E-cadherin, N-cadherin, and Vimentin were assessed by western blot analysis. *P < 0.05.

Figure 4 MiR-335-5p was a target of circKCNQ5. (A) Schematic illustration showing the luciferase reporters containing circKCNQ5 binding sites on miR-335-5p, and mutant form of circKCNQ5. (B) The expression level of miR-335-5p was examined by RT-qPCR in U266 and OPM2 cells transfected with miR-NC, miR-335-5p, in-miR-NC, or in-miR-335-5p. (C-F) Dual-luciferase reporter and RIP assays were used to confirm the association between miR-335-5p and circKCNQ5. (G-H) The expression level of miR-335-5p was assessed by RT-qPCR in multiple myeloma tissues and cells. (I) RT-qPCR assay was performed to test miR-335-5p in U266 and OPM2 cells were transfected with si-NC, or si-circKCNQ5#1. *P < 0.05.

Figure 5 Knockdown of circKCNQ5 mediated effects on multiple myeloma cells depending on miR-335-5p. (A-M) U266 and OPM2 cells were transfected with si-NC, si-circKCNQ5#1, si-circKCNQ5#1+in-miR-NC, or si-circKCNQ5#1+in-miR-335-5p. (A-B) The relative level of miR-335-5p was examined by RT-qPCR. (C-E) CCK-8 and colony-forming assays were conducted to assess cell proliferation. (F-G) Transwell assay was performed to assess migration and invasion. (H) Cell apoptosis was presented by flow cytometry. (I-K) The glycolysis ability of U266 and OPM2 cells were checked by measuring lactate production, glucose consumption, and ATP/ADP ratios. (L-M) Western blot analysis was applied to test protein levels of E-cadherin, N-cadherin, and Vimentin. *P < 0.05.
Figure 6 MiR-335-5p targeted BRD4 in multiple myeloma cells. (A) Binding regions between miR-335-5p and BRD4, along with mutated nucleotides of BRD4 3’UTR were displayed. (B-C) Dual-luciferase reporter assay was conducted in U266 and OPM2 cells. (D-F) The relative levels of BRD4 were examined by RT-qPCR and western blot. (G) After transfecting with miR-335-5p or miR-NC, the expression of BRD4 was quantified by western blot. (H) The protein level of BRD4 was assessed by western blot in U266 and OPM2 cells transfected with si-NC, si-circKCNQ5#1, si-circKCNQ5#1+in-miR-NC, or si-circKCNQ5#1+in-miR-335-5p. *P < 0.05.

Figure 7 MiR-335-5p/BRD4 axis regulated proliferation, migration, invasion, apoptosis, and glycolysis of multiple myeloma cells. (A-M) U266 and OPM2 cells were transfected with miR-NC, miR-335-5p, miR-335-5p+pcDNA, or miR-335-5p+BRD4. (A-B) The protein level of BRD4 was calculated by western blot analysis. (C-D) CCK-8 assay was used to analyze cell viability. (E) Colony-forming assay was conducted in U266 and OPM2 cells. (F-G) Migration and invasion were estimated by transwell assay. (H) Cell apoptosis was assessed by flow cytometry. (I-K) The lactate production, glucose consumption, and ATP/ADP ratios were detected in U266 and OPM2 cells. (L-M) Western blot analysis was carried out to assess E-cadherin, N-cadherin, and Vimentin levels. *P < 0.05.

Supplementary files

Supplementary Figure 1 The expression levels of miRNAs in MM cells. The expression levels of miR-127-5p, miR-599, miR-595, miR-622 and miR-335-5p in MM cells. *P < 0.05.
Table 1 Correlation between circKCNQ5 expression and clinical characteristics of MM patients

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IgG: immunoglobulin G, IgA: immunoglobulin A, ISS: international staging system; OS: overall survival; PFS: Progression free survival; *P<0.05
HISTOLOGY AND HISTOPATHOLOGY

(A) Relative expression level of circKCNQ5

(B) Relative expression level of circKCNQ5 across different cell lines:
- nPCs
- MM1S
- RPMI-8266
- H929
- U266
- OPM2

(C) Relative expression of circKCNQ5 and KCNQ5 mRNA in U266

(D) Relative expression of circKCNQ5 and KCNQ5 mRNA in OPM2

* indicates statistical significance.
HISTOLOGY AND HISTOPATHOLOGY

![Bar chart showing relative expression of different miRNAs (miR-127-5p, miR-599, miR-595, miR-622, miR-335-5p) in normal and MM conditions. Bars are labeled with error bars and asterisks (*) indicating significant differences.](chart.png)
A circKCNQ5 WT
miR-335-5p
circKCNQ5 MUT

D OPM2
E U266
F OPM2

H

I

B

C

G

Normal
MM

Relative transcription level of miR-335-5p

si-NC
si-circKCNQ5#1
**A**

BRD4 3'UTR WT 5' ...AGGAGUCCUGGGC UGCUUUGA...3'  
miR-335-5p 3' 
UGUAAAAAGCAAU AA CGAGAACU 5'

BRD4 3'UTR MUT 5' ...AGGAGUCCUGGGC UG GAGAA GA...3'

**B**

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**C**

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**D**

Relative mRNA expression level of BRD4

**E**

Relative protein expression level of BRD4  
β-actin

**F**

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**H**

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