Long non-coding RNA DANCR represses the viability, migration and invasion of multiple myeloma cells by sponging miR-135b-5p to target KLF9

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Abstract. Multiple myeloma (MM) is a malignancy of plasma cells that leads to marrow failure and bone lesions. Numerous studies have verified the link between long non-coding RNAs (lncRNAs) and MM. The present study aimed to examine the role and underlying mechanism of differentiation antagonizing non-protein coding RNA (DANCR) in MM cells. The relative expression levels of DANCR, microRNA (miR)-135b-5p and Krüppel-like factor 9 (KLF9) were examined using reverse transcription-quantitative PCR. Cell viability was assessed using the MTT assay, while relative cell migration and invasion were evaluated using Transwell assays. Moreover, the dual-luciferase reporter assay was used to examine the interplay between DANCR, miR-135b-5p and KLF9. Western blotting was performed to determine the expression level of KLF9. It was found that lncRNA DANCR and KLF9 were downregulated, while miR-135b-5p was upregulated in the serum of patients with MM and in MM cells compared with the controls. Overexpressing DANCR or knocking down miR-135b-5p reduced the viability of the MM cells, as well as restrained MM cells from migrating and invading. Furthermore, DANCR directly targeted miR-135b-5p and was negatively correlated with miR-135b-5p. It was also found that KLF9 was targeted by miR-135b-5p and was inversely correlated with miR-135b-5p expression. The impact of lncRNA DANCR-mediated suppression on cell viability, invasion and migration was partially abolished by short hairpin RNA KLF9 or miR-135b-5p mimics transfection in MM cells. Thus, it was suggested that lncRNA DANCR repressed the viability, migration and invasion of MM cells by sponging miR-135b-5p to target KLF9.

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Introduction

Multiple myeloma (MM), a hematopoietic malignancy, is caused by the malignant expansion of plasma cells in the bone marrow (1,2). It is the second most diagnosed hematological malignancy in China with an incidence rate of ~1.1/100,000, which accounted for 2.1% of all new cancer cases in 2017 (2). Clinical manifestations of MM include bone fractures, hypercalcemia, renal impairment and anemia (3,4). MM involves numerous mechanisms associated with molecules and cells, such as chromosomal abnormality, epigenetic alteration, mutations and the imbalance of stromal cells in the microenvironment of the bone marrow (5). According to statistics, the survival time of patients with MM ranges from several months to >10 years (6). Despite the fact that significant progress has been achieved with conventional chemotherapy, nanomedicine and stem cell transplantation (7,8), the survival rate of patients with MM remains poor (3,9). Therefore, it is imperative to identify the underlying molecular mechanisms of MM and develop innovative approaches for MM therapy.

Long non-coding (lnc)RNAs, consisting of >200 base pairs, are a set of RNA transcripts that do not encode proteins (10,11). Previous studies have revealed that lncRNAs exert pivotal roles in the progression of MM (12,13). Over the past few years, 176 lncRNAs have been identified as biomarkers for MM prognosis (14,15). For example, Liu *et al* (12) reported that silencing lncRNA metastasis associated lung adenocarcinoma transcript 1 repressed cell viability and invasion in MM cells. Moreover, Wang *et al* (13) observed that the upregulation of lncRNA OIP5-antisense RNA (AS) 1 suppressed the viability, migration and invasion of MM cells, as well as inhibited MM tumorigenesis *in vivo*. As an anti-differentiation ncRNA, differentiation antagonizing non-protein coding RNA (DANCR) has been implicated in the development of multiple cancer

types (16,17). For example, silencing of lncRNA DANCR has been shown to suppress the proliferative, migratory and invasive abilities of cells in cervical cancer (16). Furthermore, knockdown of DANCR represses the proliferative, migratory and invasive abilities of osteosarcoma cells (17). Importantly, a previous study has reported the downregulation of DANCR expression in blood samples of patients with MM compared with blood samples from healthy controls (18). Nevertheless, the precise function and mechanism of lncRNA DANCR it yet to be elucidated in MM.

Previous studies have shown that lncRNAs regulate the translation of mRNAs by sponging microRNAs (miRNAs/miRs) (19). miRNAs are a genre of single-stranded RNA molecules with a length of 20-23 nucleotides that affect the stability and translation of their target mRNAs (20,21). It has been revealed that miRNAs participate in MM development (22). For example, Liu *et al* (23) reported that the inhibitory effect of circular RNA SMARCA5 on cell activity was partially abolished by miR-767-5p in MM. Kong *et al* (24) also observed that decreasing miR-17-5p inhibited the proliferation and colony formation of MM cells, and repressed tumor growth in mouse models of MM. Moreover, miR-135b is upregulated and associated with MM (25,26). Hao *et al* (25) revealed that miR-135b was upregulated in the serum of patients with MM and was essential for the prediction of MM prognosis. Furthermore, Xu *et al* (26) reported that miR-135b was notably upregulated in human mesenchymal stem cells from patients with MM (MM-hMSCs), and repression of miR-135b facilitated osteogenic differentiation in MM-hMSCs. However, the regulatory mechanism between lncRNA DANCR and miR-135b-5p in MM remains largely unknown.

Krüppel-like factor 9 (KLF9) is a basic transcription element-binding protein (27,28). An increasing number of studies have shown that KLF9 exerts regulatory roles in the pathogenesis of several cancer types (29,30). Zhong *et al* (29) found that KLF9 decreased the proliferative, migratory and invasive abilities of tumor cells in pancreatic cancer. Moreover, Kong *et al* (30) demonstrated that KLF9 partially reversed the promoting effect of miR-141 on cell invasion and proliferation in non-small cell lung cancer (NSCLC). Notably, Mannava *et al* (31) revealed that KLF9 was a crucial regulator of drug-induced apoptosis in MM cells. However, to the best of our knowledge, the regulatory impact of miR-135b-5p on KLF9 has not been examined in MM.

Therefore, the current study focused on investigating the expression levels and functions of lncRNA DANCR and miR-135b-5p in MM cells, and the relationship between lncRNA DANCR, miR-135b-5p and KLF9 in MM cells was further examined. Thus, the findings from this study may lay a foundation for the molecular therapy of MM.

Materials and methods

Collection of clinical samples. Serum samples (10 ml) were obtained from patients with MM (n=55; age range, 49-86 years old; 29 female patients and 26 male patients) and healthy donors (n=40) at the Anhui No. 2 Provincial People's Hospital (Hefei, China) between March 2017 and January 2020. The inclusion criteria were as follows: i) The patients were diagnosed with MM by pathological examinations, which were in accordance with the updated 2014 International Myeloma Working Group criteria for MM diagnosis (32); and ii) the clinical data for MM were complete. The exclusion criteria included patients who had received chemotherapy before the operation. The patients were selected according to the aforementioned conditions with an eventual sample size of 55. In addition, all MM patients were divided into a low group (n=27) and high group (n=28), according to the median expression level of lncRNA DANCR (0.491), as shown in Table I. This study was approved by the Ethics Committee of Anhui No. 2 Provincial People's Hospital (approval no. 2020-16-27), and adhered to the guidelines of the Declaration of Helsinki. Written informed consent was obtained from all participants.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from serum samples and cells using TRIzol® reagent (Beyotime Institute of Biotechnology). Total RNA was quantified at 260 nm with a spectrophotometer, and the purity was evaluated using the ratio of readings at 260 and 280 nm. A PrimeScript RT Reagent kit (Takara Biotechnology Co., Ltd.) was used to synthesize cDNA at 42˚C for 45 min. RT-qPCR was conducted using a SYBR ExScript qRT-PCR kit (Takara Biotechnology Co., Ltd.). The reaction procedures were as follows: Initial denaturation at 95˚C for 10 min; followed by 38 cycles of 95˚C for 30 sec, annealing at 58˚C for 30 sec, elongation at 72˚C for 1 min; and a final extension at 72˚C for 5 min. Primer sequences acquired from Sangon Biotech Co., Ltd., are listed in Table II. The relative expression levels of DANCR, miR-135b-5p and KLF9 were quantified according to the $2^{-\Delta\Delta Cq}$ calculation (33) and normalized to GAPDH (for DANCR and KLF9) or U6 (for miR-135b-5p).

Cell culture. Human normal plasma cells (nPCs) were purchased from Hunan Fenghui Biotechnology Co., Ltd., and MM cells (RPMI-8226, H929, U266 and MM1S cells) were purchased from the American Type Culture Collection. All cells were cultured in RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and penicillin-streptomycin mixed solution (1%). Cultures were incubated at 37˚C in a humidified atmosphere with 5% CO_2.

Cell transfection. The overexpression vectors of DANCR (pcDNA-DANCR), pcDNA-KLF9 and pcDNA-negative control (NC) were provided by Hanbio Biotechnology Co., Ltd. Short hairpin RNA (sh)-KLF9 and sh-NC were purchased from Guangzhou RiboBio Co., Ltd. miR-135b-5p inhibitor (5'-UCACAUAGGAAUGAAAAGCCAUA-3'), inhibitor NC (5'-UUCAUCGUGUUAUUAGCGUUCCU-3'), miR-135b-5p mimics (5'-UAUGGCUUUUCAUUCCUAUGUGA-3') and miR-NC (5'-UAUAUCGUGUUAUUAGCGUUCCU-3') were purchased from Shanghai GenePharma Co., Ltd. The transcripts (20 nM) were transfected into MM cells using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). Transfection was performed for 48 h at 37˚C. Subsequently, 48 h after transfection, the transfected cells were harvested to perform further experiments.

Cell proliferation assay. Cell viability was measured using the MTT assay. Briefly, RPMI-8226 and H929 cells were seeded in

Table I. Clinical parameters of the patients with multiple myeloma who were included in this study.

		DANCR expression		
Variable	Total	Low (n=27)	High (n=28)	P-value
Age, years				0.882
<60	30	15	15	
≥60	25	12	13	
Sex				0.504
Male	26	14	12	
Female	29	13	26	
WBC, x10^9/l				0.139
<5.40	27	16	11	
≥5.40	28	11	17	
ISS stage				0.002[a]
I	13	1	12	
II	20	11	9	
III	22	15	7	

[a]P<0.01. WBC, white blood cell; ISS, International Staging System; DANCR, differentiation antagonizing non-protein coding RNA.

Table II. Primer sequences for reverse transcription-quantitative PCR.

Gene	Forward	Reverse
DANCR	5'-GCCACAGGAGCTAGAGCAGT-3'	5'-GCAGAGTATTCAGGGTAAGGGT-3'
miR-135b-5p	5'-GGTATGGCTTTTCATTCCT-3'	5'-CAGTGCGTGTCGTGGAGT-3'
KLF9	5'-TGGCTGTGGGAAAGTCTA TGG-3'	5'-CTCGTCTGAGCGGGAG-3'
GAPDH	5'-GCATCCTGGGCTACACTG-3'	5'-TGGTCGTTGAGGGCAAT-3'
U6	5'-GCTTCGGCAGCACATATACTAAAAT-3'	5'-CGCTTCACGAATTTGCGTGTCAT-3'

DANCR, differentiation antagonizing non-protein coding RNA; miR, microRNA; KLF9, Krüppel-like factor 9.

96-well plates (5x10^3 cells/well). Following incubation for 24, 48, 72 and 96 h at 37˚C, MTT (20 μl; Sigma-Aldrich; Merck KGaA) was pipetted into each well and the reaction mixture was incubated at 37˚C for 4 h. Thereafter, 100 μl dimethyl sulfoxide (200 μl/well; Sigma-Aldrich; Merck KGaA) was added to dissolve the formazan. To determine the cell viability, the optical density value was monitored at 490 nm using a microplate reader (Bio-Rad Laboratories, Inc.).

The 5-ethynyl-2'-deoxyuridine (EdU) assay kit (cat. no. C10310-1; Guangzhou RiboBio Co., Ltd.) was used to measure the number of aforementioned cells undergoing DNA replication, as the thymidine analogue EdU is incorporated into DNA during DNA replication. Briefly, cells were first cultured with 50 μM EdU for 2 h at 37˚C, followed by fixing with 4% formaldehyde for 20 min at room temperature, permeabilization with 0.5% Triton X-100 for 20 min and incubation with 1X Apollo reaction cocktail for 30 min at room temperature. Following which, cell nuclei were stained with DAPI (1 μg/ml; 10 min; Sigma-Aldrich; Merck KGaA) at room temperature for 2 h. EdU-positive cells were determined using fluorescence microscopy (magnification, x400).

Cell migration and invasion assays. The cell invasive ability was assessed using Transwell chambers precoated with Matrigel (8-μm pore size; Corning, Inc.) overnight at 37˚C. Briefly, cells (1x10^5) resuspended in serum-free medium were inoculated into the upper chamber, accompanied by the addition of RPMI-1640 medium containing 10% FBS to the lower chamber. After incubation for 48 h at 37˚C, non-invaded cells were wiped off. Invaded cells were fixed with 4% paraformaldehyde and stained with 0.5% crystal violet for 15 min at 37˚C. Finally, the stained cells were analyzed using an inverted light microscope (Olympus Corporation; magnification, x400).

For the assessment of cell migration, the operation was similar to the cell invasion assay, but the Transwell chambers were not pre-coated with Matrigel.

Target prediction. The miRNA targets of DANCR were predicted using starBase software version 2.0 (http://starbase.sysu.edu.cn/), and 53 targets were predicted. Among these miRNA targets, miR-135b-5p was selected for the following assays due to its important role in MM (25,26) and its unknown regulatory relationship. In addition, the mRNA targets of

miR-135b-5p were also predicted using starBase software. In total, 3,791 targets were predicted. KLF9 was selected for the following assays due to its important role in MM (31) and its unknown relationship with miR-135b-5p in MM.

Dual-luciferase reporter (DLR) assay. The 3'-untranslated region (UTR) fragment of DANCR or KLF9, including the assumed binding sites for miR-135b-5p, was introduced into psiCHECK2 (Promega Corporation), and named as DANCR wild-type (wt) or KLF9 wt. Similarly, the 3'-UTR portion of DANCR or KLF9 harboring the mutated complementary sites for the miR-135b-5p seed region were inserted into psiCHECK2 (Promega Corporation), and named DANCR mutant (mut) or KLF9 mut. Subsequently, the aforementioned vectors (80 ng), along with the miR-135b-5p mimics or miR-NC (50 nM), were incubated with RPMI-8226 and H929 cells using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.) for 48 h at 37˚C. The luciferase activity of cell lysates was examined using a DLR Assay system (Promega Corporation). Relative luciferase activity was defined as the ratio of firefly luciferase activity/*Renilla* luciferase activity.

Western blotting. RIPA buffer (Beyotime Institute of Biotechnology) was used to extract the protein from RPMI-8226 and H929 cells. The BCA Protein Assay kit (Pierce; Thermo Fisher Scientific, Inc.) was used to measure the protein concentration. Protein samples (30 μg) were separated via 10% SDS-PAGE, and then transferred onto PVDF membranes. After blocking the membranes with 5% skimmed milk for 2 h at room temperature, the membranes were incubated at 4˚C overnight with primary antibodies, including anti-KLF9 (1:1,000; cat. no. sc-12996; Santa Cruz Biotechnology, Inc.) and anti-GAPDH (1:1,000; cat. no. ab8245; Abcam). Then, the membranes were washed with TBS containing 0.1% Tween-20. The secondary antibody (1:2,000; cat. no. ab205723; Abcam) was then added and the membranes were incubated with the protein samples at 37˚C for 1 h. The immunoreactive signals were visualized using an ECL system (BD Biosciences), and the relative protein expression level of KLF9 was analyzed using Alpha Innotech imaging software version 3.1.2 (ProteinSimple).

Statistical analysis. All experiments were conducted in triplicate in at least three independent experiments. Experimental data were analyzed using SPSS 22.0 software (IBM Corp.) and are presented as the mean ± SD. Differences between two groups were compared using an unpaired Student's t-test. Comparisons between multiple groups were assessed by one-way ANOVA, which was then followed by the Tukey's post hoc test. A χ^2 test was used for analysis the data in Table I. Correlations among DANCR, miR-135b-5p and KLF9 were assessed using Pearson correlation analysis. P<0.05 was considered to indicate a statistically significant difference.

Results

lncRNA DANCR is downregulated in the serum of patients with MM and MM cells. The expression level of lncRNA DANCR was determined using RT-qPCR. The relative expression level of DANCR was lower in serum samples from patients with MM compared with that in serum samples from healthy donors (P<0.01; Fig. 1A). Moreover, DANCR expression was consistently decreased in the MM cell lines (RPMI-8226, U266, MM1S and H929 cells) compared with that in the nPCs (all P<0.01; Fig. 1B), especially in RPMI-8226 and H929 cells. Therefore, RPMI-8226 and H929 cells were chosen for further experiments.

The association between DANCR and the clinicopathological characteristics of MM cases was also studied, and it was found that DANCR expression was associated with the International Staging System (ISS) stage (P=0.002; Table I).

Overexpression of lncRNA DANCR suppresses the viability, migration and invasion of RPMI-8226 and H929 cells. To evaluate the role of lncRNA DANCR in MM cells, DANCR was initially overexpressed by transfection with pcDNA-DANCR. The addition of pcDNA-DANCR led to a significant increase in the expression level of DANCR compared with the addition of pcDNA-NC in RPMI-8226 and H929 cells (all P<0.01; Fig. 2A). Functional experiments were also performed. The results of the MTT assay indicated that overexpression of DANCR significantly reduced cell viability at 96 h of incubation compared with the pcDNA-NC group in RPMI-8226 and H929 cells (all P<0.01; Fig. 2B). Moreover, the number of RPMI-8226 or H929 cells incorporating EdU in the pcDNA-DANCR group was significantly lower compared with that in the pcDNA-NC group (all P<0.01; Fig. 2C). The results of the Transwell assay also demonstrated that the relative migration and invasion of RPMI-8226 and H929 cells were decreased by overexpression of DANCR (all P<0.01; Fig. 2D and E).

lncRNA DANCR directly binds to miR-135b-5p. To examine the downstream mechanism of lncRNA DANCR in RPMI-8226 and H929 cells, bioinformatics analysis was performed using starBase and it was shown that DANCR had combinative sites for miR-135b-5p (Fig. 3A), indicating that miR-135b-5p was the target of DANCR. Accordingly, the expression level of miR-135b-5p was determined and the association between DANCR and miR-135b-5p in MM cells was investigated. It was identified that miR-135b-5p was upregulated in the MM cell lines compared with that in nPCs (all P<0.01; Fig. 3B). Moreover, miR-135b-5p expression was decreased by the overexpression of DANCR in RPMI-8226 and H929 cells (all P<0.01; Fig. 3C). Subsequently, a DLR assay was applied to further confirm the cooperation of DANCR and miR-135b-5p, and it was found that the relative luciferase activity of RPMI-8226 and H929 cells co-transfected with DANCR wt and miR-135b-5p mimics was lower compared with that of RPMI-8226 and H929 cells co-transfected with DANCR wt and miR-NC (all P<0.01; Fig. 3D).

It was demonstrated that miR-135b-5p was highly expressed in serum samples of patients with MM as opposed to serum samples from healthy donors (P<0.01; Fig. 3E). There was also a moderate negative correlation between miR-135b-5p expression and DANCR expression in serum samples from patients with MM (P<0.01; Fig. 3F).

Knockdown of miR-135b-5p represses viability, migration and invasion in RPMI-8226 and H929 cells. The expression of miR-135b-5p was knocked down by the

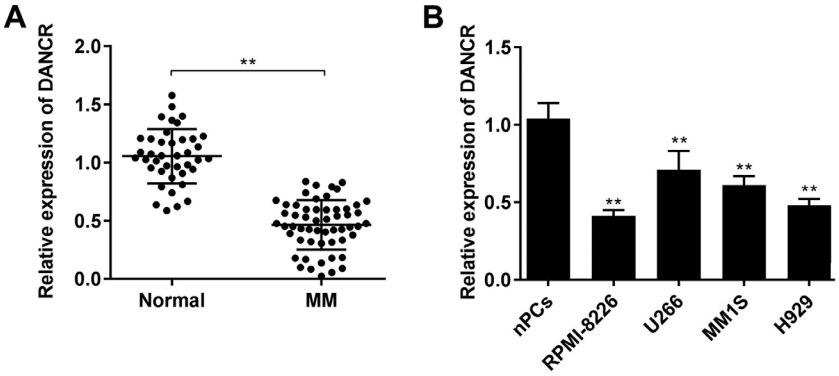

Figure 1. DANCR is downregulated in serum of patients with MM and MM cells. (A) Relative expression level of DANCR was determined via RT-qPCR in serum from patients with MM and healthy donors. **P<0.01. (B) Relative expression level of DANCR was determined via RT-qPCR in RPMI-8226, U266, MM1S, H929 cells and nPCs. **P<0.01 vs. nPCs. RT-qPCR, reverse transcription-quantitative PCR; DANCR, differentiation antagonizing non-protein coding RNA; nPCs, normal plasma cells; MM, multiple myeloma.

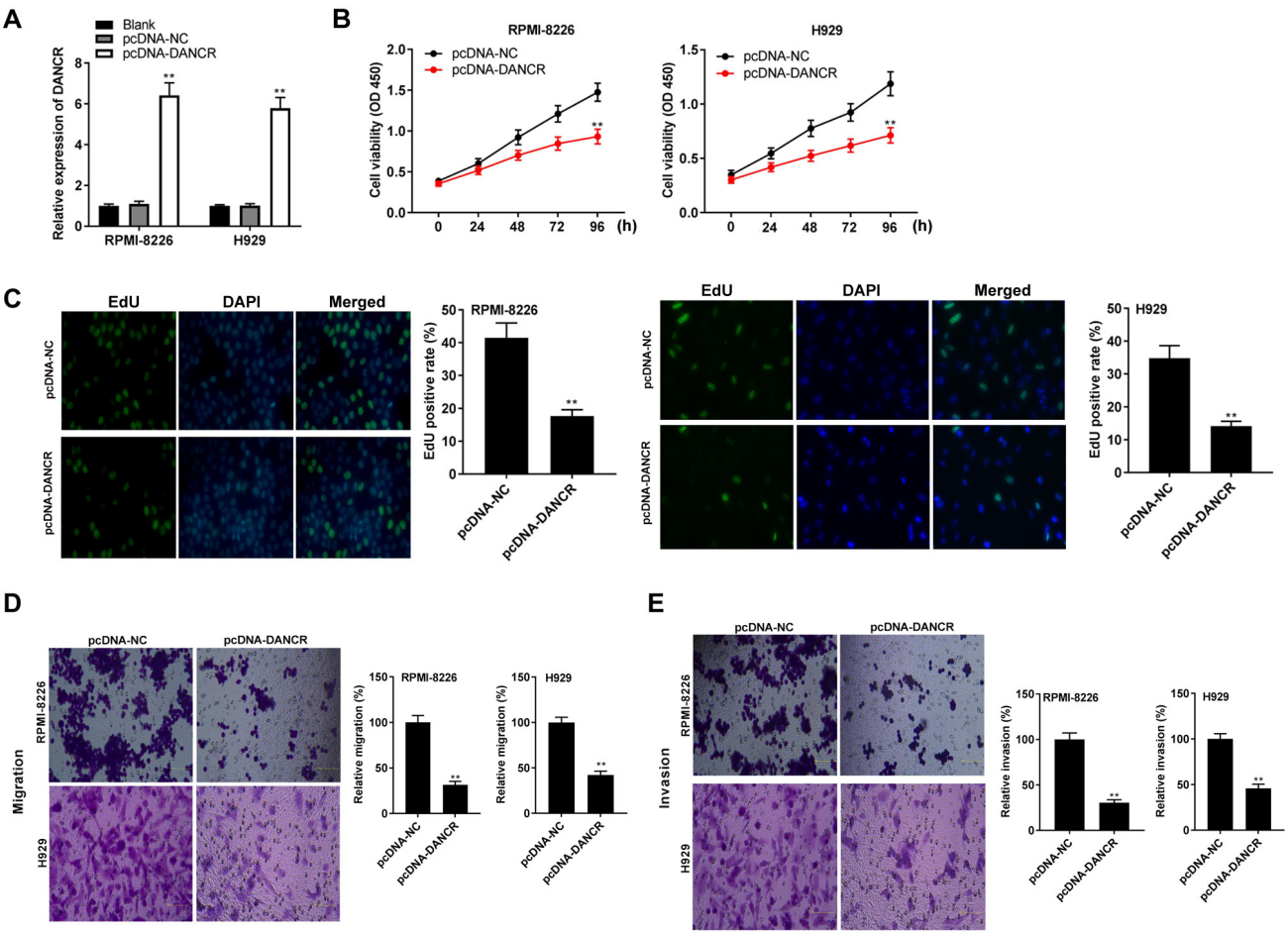

Figure 2. Overexpression of DANCR suppresses the viability, migration and invasion of RPMI-8226 and H929 cells. (A) After transfection of pcDNA-DANCR and pcDNA-NC, the relative expression level of DANCR was detected via reverse transcription-quantitative PCR in RPMI-8226 and H929 cells. (B) Cell viability was detected using an MTT assay in RPMI-8226 and H929 cells. (C) Cell proliferation in RPMI-8226 and H929 cells was determined using an EdU assay (magnification, x400). (D) Relative migration of RPMI-8226 and H929 cells was detected using a Transwell assay (magnification, x400). (E) Relative invasion of RPMI-8226 and H929 cells was detected using a Transwell assay (magnification, x400). **P<0.01 vs. pcDNA-NC. NC, negative control; DANCR, differentiation antagonizing non-protein coding RNA; EdU, 5-ethynyl-2'-deoxyuridine.

addition of miR-135b-5p inhibitor. The expression level of miR-135b-5p was significantly decreased after the addition of miR-135b-5p inhibitor in RPMI-8226 and H929 cells (all P<0.01; Fig. 4A). The specific effects of miR-135b-5p on RPMI-8226 and H929 cells were then studied. It was found that the knockdown of miR-135b-5p caused a significant decrease in the viability of RPMI-8226 and H929 cells (all P<0.01; Fig. 4B), as well as reduced the migratory and invasive abilities of RPMI-8226 and H929 cells (all P<0.01; Fig. 4C and D).

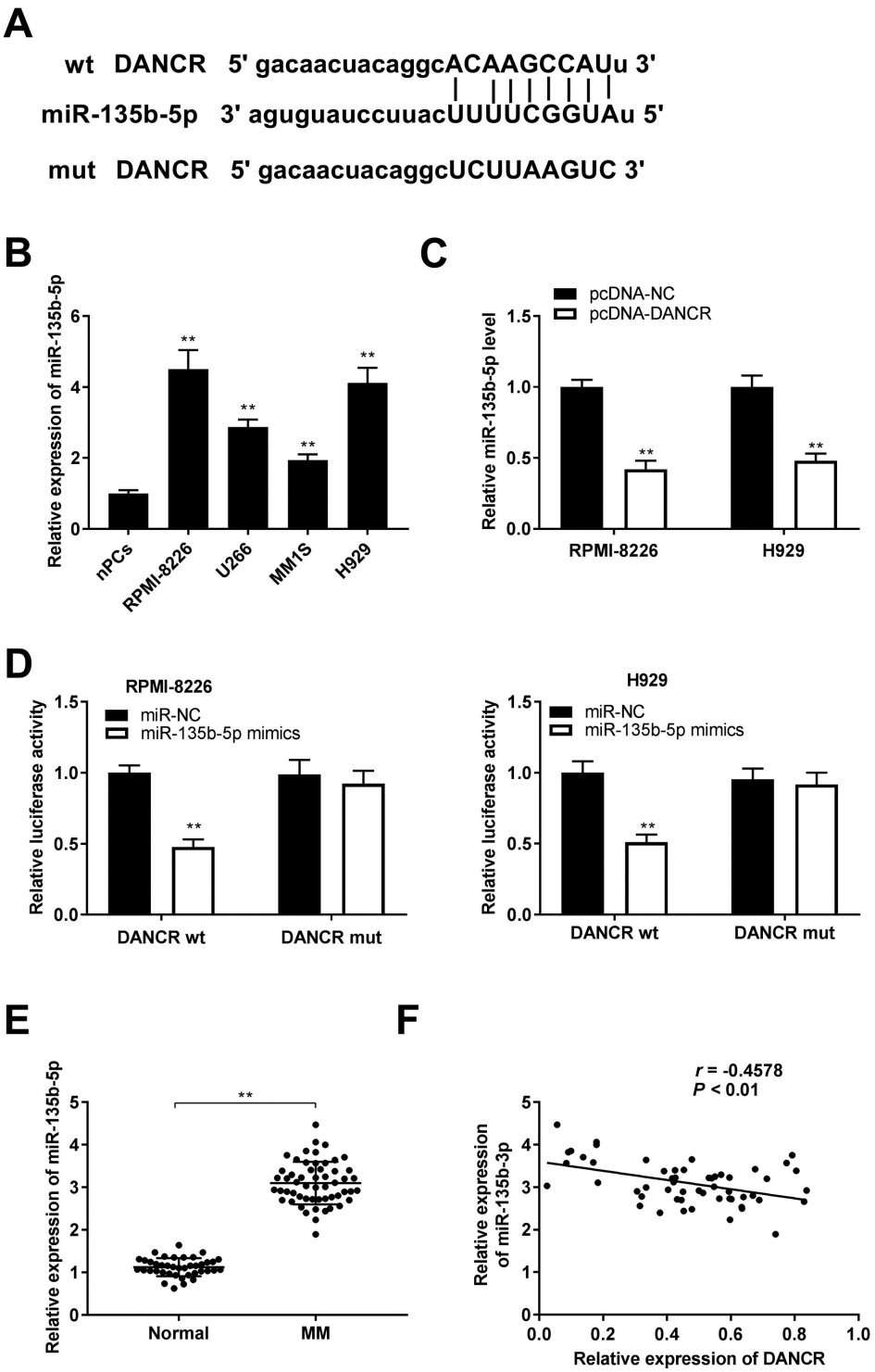

Figure 3. DANCR directly binds to miR-135b-5p. (A) Binding sequence between DANCR and miR-135b-5p was predicted using starBase. (B) Relative expression level of miR-135b-5p was detected via RT-qPCR in RPMI-8226, U266, MM1S, H929 cells and nPCs. **P<0.01 vs. nPCs. (C) After transfection of pcDNA-DANCR and pcDNA-NC, the relative expression level of miR-135b-5p was detected via RT-qPCR in RPMI-8226 and H929 cells. **P<0.01 vs. pcDNA-NC. (D) The interaction between DANCR and miR-135b-5p was confirmed using a dual luciferase reporter assay in RPMI-8226 and H929 cells. **P<0.01 vs. miR-NC. (E) Relative expression level of miR-135b-5p was detected via RT-qPCR in serum from patients with MM and healthy donors. **P<0.01 vs. Normal. (F) Correlation between DANCR expression and miR-135b-5p expression in serum of patients with MM was analyzed via Pearson's correlation analysis. NC, negative control; DANCR, differentiation antagonizing non-protein coding RNA; miR, microRNA; wt, wild-type; mut, mutant; nPCs, normal plasma cells; MM, multiple myeloma; RT-qPCR, reverse transcription-quantitative PCR.

KLF9 is targeted by miR-135b-5p. To elucidate the regulatory mechanism of miR-135b-5p in MM cells, the targets of miR-135b-5p were predicted using starBase and it was found that there were base pairing sites between miR-135b-5p and KLF9 (Fig. 5A). A DLR assay was also performed to corroborate the association between miR-135b-5p and KLF9. It was demonstrated that the addition of miR-135b-5p mimics significantly decreased the relative luciferase activity of the KLF9 wt

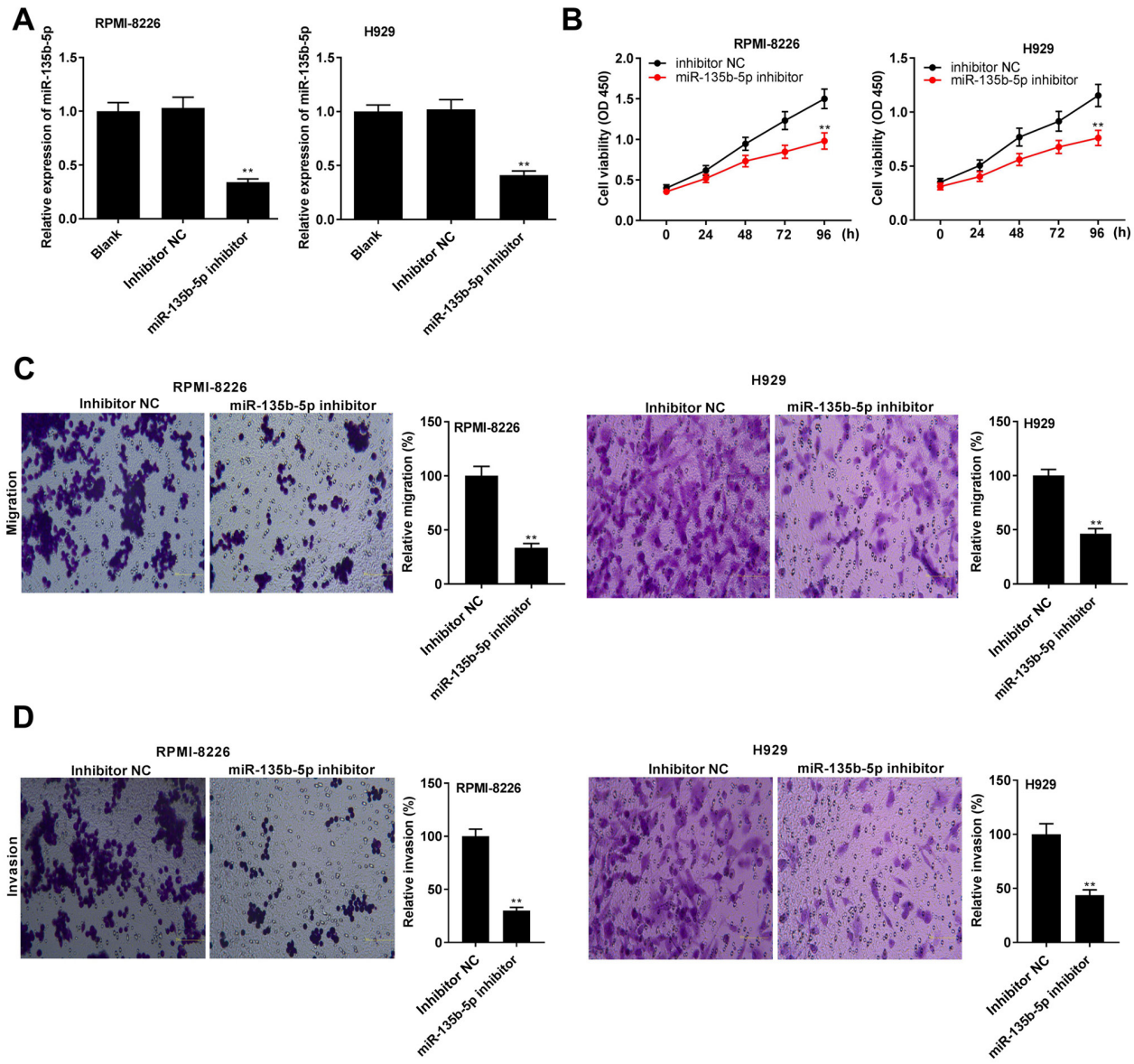

Figure 4. Knockdown of miR-135b-5p represses the viability, migration and invasion of RPMI-8226 and H929 cells. (A) After transfection of inhibitor NC and miR-135b-5p inhibitor, the relative expression level of miR-135b-5p was detected via reverse transcription-quantitative PCR in RPMI-8226 and H929 cells. (B) Cell viability was determined using an MTT assay in RPMI-8226 and H929 cells. (C) Relative migration of RPMI-8226 and H929 cells was determined using a Transwell assay (magnification, x400). (D) Relative invasion of RPMI-8226 and H929 cells was detected using a Transwell assay (magnification, x400). **P<0.01 vs. inhibitor NC. NC, negative control; miR, microRNA; OD, optical density.

compared with the addition of miR-NC (all P<0.01; Fig. 5B), but showed no significant effect on the luciferase activity of the KLF9 mut in RPMI-8226 and H929 cells (Fig. 5B). Simultaneously, it was observed that KLF9 expression was significantly downregulated in the MM group compared with the normal group (P<0.01; Fig. 5C). It was found that KLF9 expression was weakly, negatively correlated with miR-135b-5p expression in serum samples from patients with MM (P<0.01; Fig. 5D). In addition, the regulatory association between KLF9 and miR-135b-5p was verified via western blotting, and it was found that the relative protein expression level of KLF9 was downregulated by miR-135b-5p overexpression in RPMI-8226 and H929 cells (all P<0.01; Fig. 5E).

Overexpression of KLF9 inhibits the viability, migration and invasion in RPMI-8226 cells. The expression level of KLF9 in MM cell lines was determined. As presented in Fig. 6A, it was found that KLF9 expression was significantly downregulated in MM cell lines compared with that in nPCs (P<0.01). Next, pcDNA-KLF9 or NC was transfected into RPMI-8226 cells to determine transfection efficiency. The results demonstrated that KLF9 expression was significantly increased after pcDNA-KLF9 transfection (P<0.01; Fig. 6B). The effects of KLF9 overexpression on cell viability, migration and invasion were also studied. As shown in Fig. 6C-E, the overexpression of KLF9 significantly repressed the viability, migration and invasion of RPMI-8226 cells (P<0.01).

lncRNA DANCR represses cell viability, migration and invasion by sponging miR-135b-5p to target KLF9 in RPMI-8226 cells. To determine the association between lncRNA DANCR, KLF9 and miR-135b-5p, KLF9 was

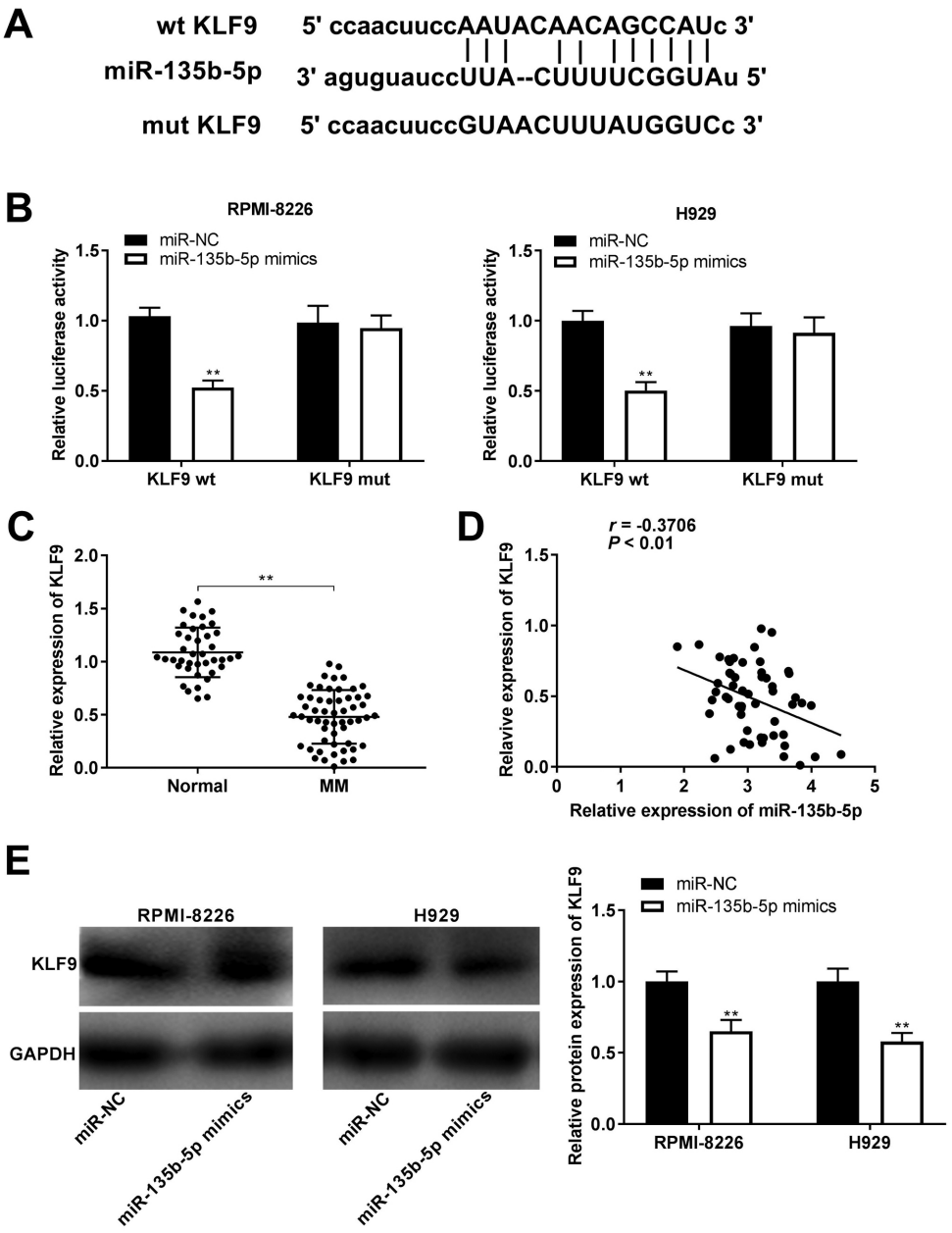

Figure 5. KLF9 is targeted by miR-135b-5p. (A) The binding sites between miR-135b-5p and KLF9 were predicted using starBase. (B) A dual luciferase reporter assay was used to determine the targeting relationship between miR-135b-5p and KLF9 in RPMI-8226 and H929 cells. **P<0.01 vs. miR-NC. (C) Relative expression level of KLF9 was detected via reverse transcription-quantitative PCR in serum from patients with MM and healthy donors. **P<0.01 vs. Normal. (D) Correlation between miR-135b-5p expression and KLF9 expression in serum of patients with MM was analyzed using Pearson's correlation analysis. (E) Relative protein expression level of KLF9 in RPMI-8226 and H929 cells was detected via western blotting after transfection of miR-135b-5p mimics and miR-NC. **P<0.01 vs. miR-NC. NC, negative control; wt, wild-type; mut, mutant; MM, multiple myeloma; KLF9, Krüppel-like factor 9; miR, microRNA.

silenced and miR-135b-5p was overexpressed in the RPMI-8226 cells. As presented in Fig. 7A and B, the introduction of sh-KLF9 significantly decreased the relative expression level of KLF9, and the introduction of miR-135b-5p mimics elevated the relative expression level of miR-135b-5p in RPMI-8226 cells (all P<0.01). Rescue experiments were then performed. It was demonstrated that the viability of RPMI-8226 cells was reduced in the pcDNA-DANCR group compared with the pcDNA-NC group, whereas this reduction in cell viability mediated by DANCR was partially abrogated by the overexpression of miR-135b-5p or the knockdown of KLF9 in RPMI-8226 cells (all P<0.01; Fig. 7C). Additionally, the relative migration and invasion of RPMI-8226 cells were repressed in the pcDNA-DANCR group compared with the pcDNA-NC group, which was partially abrogated by the overexpression of miR-135b-5p or the knockdown of KLF9 in RPMI-8226 cells (all P<0.01; Fig. 7D and E).

Discussion

MM is the second most common hematological cancer type, with high incidence and mortality (3,34). Given that the molecular mechanism of MM is largely unknown, the treatment of MM remains a great challenge (35). Currently, it has been revealed that lncRNA DANCR was abnormally expressed in

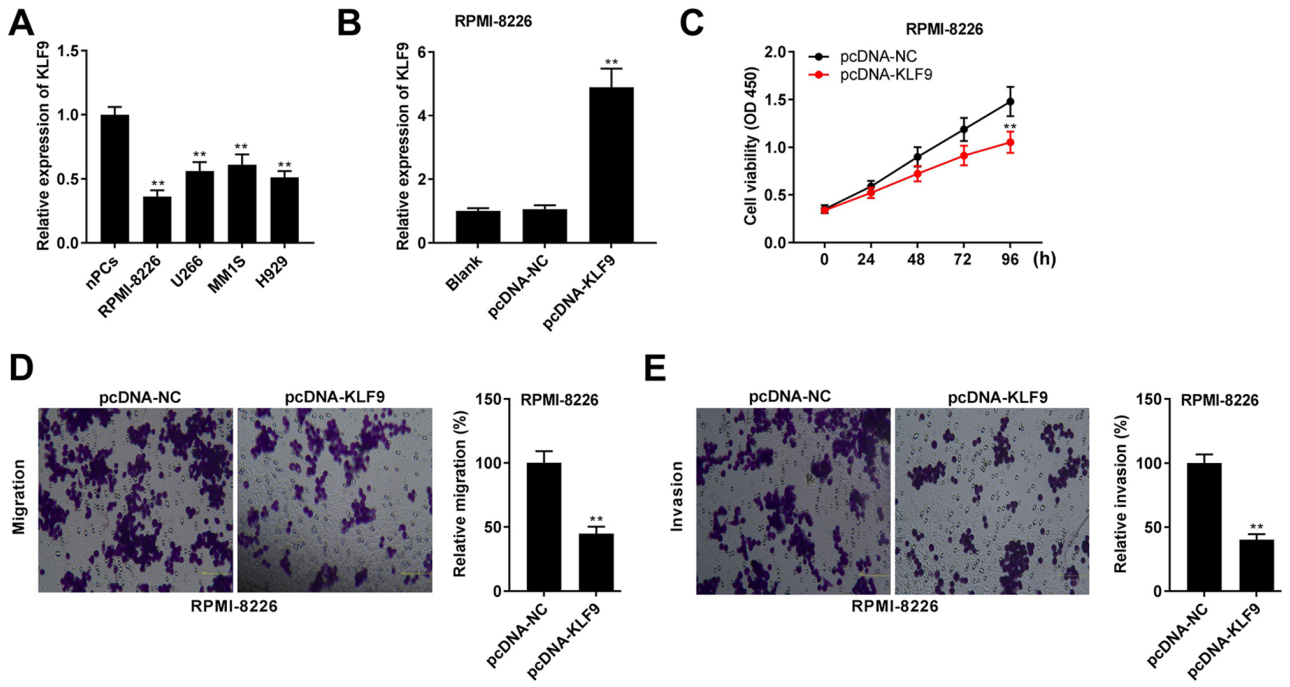

Figure 6. Overexpression of KLF9 inhibits the viability, migration and invasion of RPMI-8226 cells. (A) Relative expression level of KLF9 was detected via RT-qPCR in RPMI-8226, U266, MM1S, H929 cells and nPCs. **P<0.01 vs. nPCs. (B) After transfection of pcDNA-KLF9 and pcDNA-NC, relative expression level of KLF9 was detected via RT-qPCR in RPMI-8226 cells. (C) Cell viability was determined using an MTT assay in RPMI-8226 cells. (D) Relative migration of RPMI-8226 cells was determined using a Transwell assay (magnification, x400). (E) Relative invasion of RPMI-8226 cells was determined using a Transwell assay (magnification, x400). **P<0.01 vs. pcDNA-NC. NC, negative control; KLF9, Krüppel-like factor 9; nPCs, normal plasma cells; RT-qPCR, reverse transcription-quantitative PCR; OD, optical density.

various tumors and was considered as a prognostic biomarker for cancer (36,37). For instance, Chen *et al* (36) reported that DANCR expression was distinctly higher in bladder cancer tissues compared with in normal tissues, and its expression was associated with tumor stage, lymph node (LN) metastasis and histological grade. Moreover, Bai *et al* (37) indicated that DANCR expression was increased in NSCLC tissues compared with normal lung tissues, and its expression was correlated with advanced TNM stage, larger tumor size and LN metastasis. Importantly, a previous study by Allegra *et al* (18) identified the downregulation of DANCR in serum samples of patients with MM compared with serum samples from healthy controls. The present study also observed that DANCR was downregulated in the serum of patients with MM and MM cells as opposed to their controls, which was in agreement with the results of Allegra *et al* (18). Concurrently, it was discovered that DANCR was associated with ISS, suggesting the potential of DANCR as a molecular marker for MM diagnosis.

Several studies have suggested that lncRNA DANCR was a critical regulator in the development of different cancer types (38,39). For example, Jia *et al* (38) revealed that lncRNA DANCR facilitated the invasive and migratory abilities of cells in prostate cancer. Moreover, Lu *et al* (39) reported that silencing DANCR resulted in a decrease in cell proliferation, migration and invasion in lung adenocarcinoma *in vitro*. Similar to previous reports, in the present study, the overexpression of DANCR was shown to prevent RPMI-8226 and H929 cells from migrating and invading, as well as reduce the viability of RPMI-8226 and H929 cells. Based on the aforementioned findings, it was suggested that lncRNA DANCR may act as a tumor suppressor in MM.

Previous studies have shown that miR-135b was highly expressed in patients with MM compared with healthy controls, and miR-135b-5p has a crucial impact on the progression of several cancer types (25,26,40). For example, miR-135b expression is increased in the serum of patients with MM relative to that in the serum of healthy controls (25). miR-135b also shows distinct upregulation in MM-hMSC (26). It has been observed that miR-135b-5p enhances cell viability and facilitates the invasive and migratory abilities of gastric carcinoma cells (40). Moreover, miR-135b-5p has been shown to facilitate the invasive and migratory abilities of cells in pancreatic cancer (41). Similar to the aforementioned results, the present study identified that miR-135b-5p expression was elevated in the serum of patients with MM and MM cells as opposed to their controls, and knockdown of miR-135b-5p reduced cell viability and restrained RPMI-8226 and H929 cell migration and invasion. These findings suggest that decreasing miR-135b-5p expression repressed the progression of MM *in vitro*.

miR-135b-5p has been verified to be targeted by lncRNAs in several cancer types, including lncRNA GAS8-AS1 in papillary thyroid carcinoma (42) and lncRNA SMAD5-AS1 in diffuse large B cell lymphoma (43). The present study demonstrated that lncRNA DANCR could directly target miR-135b-5p, and there was a negative correlation between DANCR and miR-135b-5p in the serum samples of patients with MM. It was then investigated whether the impact of DANCR on MM cells was affected by miR-135b-5p, and it

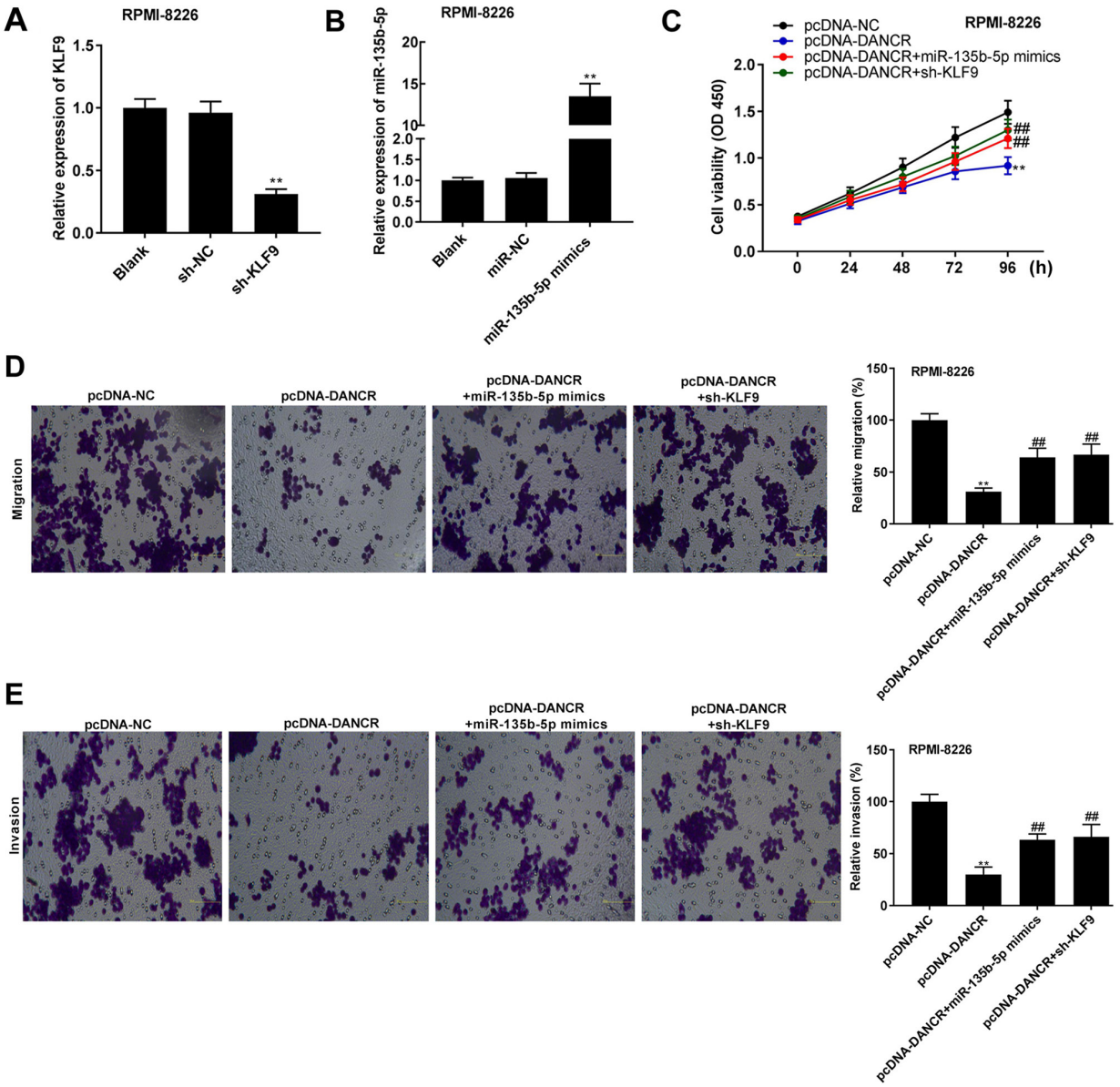

Figure 7. lncRNA DANCR represses the viability, migration and invasion of RPMI-8226 cells by sponging miR-135b-5p to target KLF9. (A) After transfection of sh-KLF9 and sh-NC, relative expression level of KLF9 was detected via RT-qPCR in RPMI-8226 cells. **P<0.01 vs. sh-NC. (B) After transfection of miR-135b-5p mimics and miR-NC, relative expression level of miR-135b-5p was detected via RT-qPCR in RPMI-8226 cells. **P<0.01 vs. miR-NC. (C) Cell viability was determined using an MTT assay in RPMI-8226 cells. (D) Relative migration of RPMI-8226 cells was determined using a Transwell assay (magnification, x400). (E) Relative invasion of RPMI-8226 cells was detected using a Transwell assay (magnification, x400). **P<0.01 vs. pcDNA-NC; $^{\#\#}$P<0.01 vs. pcDNA-DANCR. NC, negative control; KLF9, Krüppel-like factor 9; sh, short hairpin RNA; DANCR, differentiation antagonizing non-protein coding RNA; miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR.

was identified that the inhibitory effects of DANCR on the viability, invasion and migration of RPMI-8226 cells were partially reversed by miR-135b-5p. Based on these findings, it was concluded that DANCR served an anti-tumor role by interacting with miR-135b-5p in MM cells.

Over the past few years, KLF9 has gained increased attention as it is downregulated and involved in the pathological process of multiple cancer types (29,30). For instance, KLF9 is downregulated in pancreatic cancer tissues and cells, reduces cell viability and suppresses the migratory and invasive abilities of tumor cells (29). KLF9 expression was also shown to be decreased in NSCLC tissues and cells, and partially reversed the promoting effects of miR-141 on the proliferative and invasive abilities of NSCLC cells (30). It has been reported that the expression level of KLF9 was downregulated in the serum of patients with MM and MM cells compared with their controls, indicating that KLF9 may participate in the pathological processes of MM. In addition, KLF9 was shown to be a downstream target of miR-135b-5p in colorectal cancer (44). Consistently, the present study verified that KLF9 was a downstream target of miR-135b-5p and was inversely correlated with miR-135b-5p in serum samples of patients with MM, indicating that inhibition of miR-135b-5p suppressed MM tumorigenesis by targeting KLF9 *in vitro*. Moreover, the suppressive effects of lncRNA DANCR on cell viability, migration and invasion were partially abolished by sh-KLF9 in MM cells. Collectively, the present findings suggest that lncRNA DANCR represses

the development of MM via the miR-135b-5p/KLF9 axis *in vitro*.

In summary, the present study indicated that lncRNA DANCR was downregulated in MM tissues and cells compared with their controls, and it repressed the viability, invasion and migration of MM cells. Furthermore, DANCR directly targeted miR-135b-5p, which binds to KLF9. Thus, lncRNA DANCR repressed the malignant behavior of MM cells by sponging miR-135b-5p to target KLF9. The DANCR/miR-135b-5p/KLF9 axis offers a neoteric perspective for MM treatment. However, the current failed to verify the DANCR/miR-135b-5p/KLF9 axis in MM *in vivo*, and further investigation is required to confirm the current results.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

LW and LXi were involved in the conception and design, data analysis and were the major contributors in writing the manuscript. HJ contributed to the conception of the study and manuscript preparation. YH contributed significantly to analysis and manuscript preparation. LL performed the data analyses and wrote the manuscript. RX and LXu helped perform the analysis with constructive discussions. All the authors took part in the experiment, confirm the authenticity of all the raw data, and read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of Anhui No. 2 Provincial People's Hospital (Hefei, China). Written informed consent was obtained from all subjects.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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