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ORIGINAL ARTICLE

MicroRNA-137 targets EZH2 to exert suppressive functions in uveal melanoma via regulation of Wnt/β-catenin signaling and epithelial-to-mesenchymal transition

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Summary

Purpose: *Uveal melanoma (UM) is one of the primary intraocular malignancies. Emerging studies have confirmed dysregulation of microRNA (miRNA/miR) in UM. The present study focused on the biofunctions of miR-137 in UM.*

Methods: *MiR-137 expressions in tissue samples were analyzed by qRT-PCR. MTT and transwell assays were applied to investigate the impacts of miR-137 on UM cell proliferation, invasion and migration. Luciferase assay was carried out to explore the downstream target of miR-137. Western blot was used to analyze the roles of miR-137 in UM cells, Wnt/β-catenin pathway and epithelial-mesenchymal transition (EMT).*

Results: *qRT-PCR showed that miR-137 expressions were lower in UM tissue samples than para-carcinoma tissues, whereas EZH2 was simultaneously upregulated. MiR-137 overexpression evidently suppressed UM cell proliferation, invasion and migration. The findings also indicated that miR-137 restoration could block Wnt/β-catenin pathway and EMT in UM cells thus resulting in downregulation of malignant behaviors. EZH2 was a downstream target of miR-137 as demonstrated by luciferase assay.*

Conclusion: *The present study indicated that EZH2 participated in the anti-UM functions of miR-137. Taken together, the data in our study established miR-137/EZH2 axis in regulating UM progression, suggesting that miR-137 may function as a novel therapeutic biomarker for UM patients.*

Key words: *EMT, EZH2, miR-137, uveal melanoma, Wnt/β-catenin*

Introduction

As one common primary intraocular malignant tumor, uveal melanoma (UM) is estimated to have an annual morbidity of approximately 5.1 per million [1]. UM has a high metastatic rate and hematogenously spreads to the liver [2,3]. Early metastasis frequently leads to high mortality of UM patients [4]. Despite the significant progress in UM diagnosis and treatment, the relative survival rates of UM patients, especially of metastatic patients, have not improved [5,6]. Currently, as the biology of UM aggressiveness is not elucidated, no effective therapies are available for metastatic UM patients and treatments for UM are mainly locoregional controls without directed therapies [7,8]. Therefore, understanding the key signals which lead to the potential of UM invasion and migration may help discover new therapies for patients with UM.

It is proverbial that microRNA (miRNA/miR) regulates the expressions of target mRNAs via binding 3'UTRs [9]. Accumulating studies have

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demonstrated the crucial roles of miRs in a variety of tumors, being involved in several processes, including apoptosis, proliferation, differentiation and metastasis [10-12]. Identification of specific miRs, which play key roles in tumorigenesis, is supported by the recognition that abnormally expressed miRs in tumor progression often serve as tumor suppressor or oncogene [13,14]. Previous studies revealed that the aberrantly expressed miRs can serve as therapeutic targets and diagnostic biomarkers for various tumors. For instance, miR-196a was one candidate therapeutic and diagnostic biomarker for laryngeal cancer [15]. In another report, miR-129 downregulation in prostate carcinoma was a diagnostic and prognostic marker [16]. All the above studies indicated that miRs were directly correlated with the genesis and development of tumors. However, the functional relevance of miR-137 with UM remains unclear.

The enhancer of zeste homolog 2 (EZH2) is one member of PcG gene family. According to previous reports, EZH2 had functions in maintaining the silent state of homologous genes through chromosome modifications, and previous studies divided it into the core proteins of PcG family. EZH2 dysregulation in different malignancies has been confirmed to be closely related to the occurrence and development of carcinogenesis [17,18]. In studies by Zhou et al, significantly elevated EZH2 expression in bladder urothelial carcinoma indicated aggressive potential [19]. A previous study unraveled the upregulation of EZH2 in head and neck cancer, which was closely related to lymph node metastasis [20]. In an analysis of multiple myeloma, EZH2 overexpression had close correlation with dysregulation of cell cycle control and poor prognosis [21]. The present study aimed to examine the biofunctions of EZH2 in UM development.

Methods

Tissue samples

UM tissue samples and normal uveal tissues were obtained from patients without preoperative radiotherapy or chemotherapy at Weifang People's Hospital. All subjects provided signed informed consent and this study was approved by the Clinical Research Ethics Committee of Weifang People's Hospital.

Cell cultures and transfections

Melanocyte cell line (D78) and UM cells (MUM-2B, C918, MUM-2C and OCM-1A) were collected from the Chinese Academy of Sciences (Beijing, China). The MUM-2B and C918 cells were cultured in RPMI 1640 (Invitrogen, Carlsbad, CA, USA) containing 10% fetal bovine serum (FBS) while the D78, MUM-2C and OCM-1A cells were maintained in DMEM (Invitrogen). Cells were cultured in 6-well plates overnight, followed by transfection with miR-137 mimic, inhibitors or miR-NC (GenePharma, Shanghai, China) by Lipofectamine 2000 (Invitrogen).

qRT-PCR

Total RNA was extracted by TRIzol (Invitrogen) and reverse transcribed into cDNA using PrimeScript RT-PCR kit (Takara Biotechnology Co., Ltd., Dalian, China). The miR-137 and EZH2 expressions were examined by RT-PCR analysis using the TaqMan miRNA assays (Applied Biosystems, Carlsbad, CA, USA) and One Step SYBR PrimeScript™ RTPCR Kit (Takara) with U6 and GAPDH internal controls on an ABI 7900 system (Applied Biosystems), respectively. The $2^{-\Delta\Delta Ct}$ method was utilized for the relative expression quantification. Primer sequences for qRT-PCR are shown in Table 1.

Western blot

Total protein was isolated from the cells with ice-cold lysis buffer (RIPA; Sigma Chemical Co, MO, USA). Bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA) was used for the protein concentration. Proteins were separated on 10% SDS-PAGE and then electrotransferred onto PVDF membrane. After blocking with non-fat milk, the membrane was incubated overnight at 4°C with the antibody for cyclin D1 (1:1000, Abcam Cambridge, MA, USA), c-Myc (1:1000, Abcam), β-catenin(1:1000, Abcam), p-GSK3β (1:1000, Abcam), total GSK3β (1:1000, Abcam), E-cadherin (1:2000, Abcam), N-cadherin(1:2000, Abcam), Vimentin (1:1000, Abcam) and GAPDH (1:1000, Abcam). The membrane was washed and incubated with HRP-conjugated secondary antibody (1:3,000, Abcam). Detection was visualized using the enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech) system.

Cell viability

Cell proliferation abilities were detected by the MTT assay. Briefly, transfected cells were incubated for indicated time in 96-well plates. Then, MTT (5 mg/mL) (Sigma, USA) was added into each well for 4-h incubation. Then the culture medium was removed and the remaining crystal was dissolved by 150μl DMSO (Sigma). The OD_{490} was measured by a microplate reader (Molecular Devices, Sunnyvale, CA, USA).

Table 1. Primer sequences for qRT-PCR

Primer	*Sequence*
miR-137 forward	5'-GCGCGCTTATTGCTTAAGAATAC-3'
miR-137 reverse	5'-CGGCCCAGTGTTCAGACTAC-3'
U6 forward	5'-CTCGCTTCGGCAGCACA-3'
U6 reverse	5'- AACGCTTCACGAATTTGCGT-3'
EZH2 forward	5'-AATCCCTTGACATCTGCT-3'
EZH2 reverse	5'-TTTCTAAATTGCCCACAG-3'
GAPDH forward	5'-ACCTGACCTGCCGTCTAGAA-3'
GAPDH reverse	5'-TCCACCACCCTGTTGCTGTA-3'

U6: small nuclear RNA, snRNA, EZH2: enhancer of zeste homolog 2, GAPDH: glyceraldehyde-3-phosphate dehydrogenase

Cell migration and invasion assay

For invasion assays, transfected UM cells in serum-free medium were inserted into the top chambers of an 8-μm pore membrane transwell chamber (BD Biosciences, San Jose, CA, USA) which was coated with Matrigel (BD Biosciences) and medium containing 10% FBS was added into the lower chamber. 48h after incubation, cells remained on the top chamber were scraped. The invasive cells were then fixed and stained. The cells were counted using inverted microscope (Olympus, Tokyo, Japan). The transwell migration assays were conducted with the same procedures, but without Matrigel.

Luciferase reporter assay

The fragment containing the predicted miR-137 binding regions of EZH2 (3'-UTR-WT) or a mutant reporter (3'-UTR-MUT) were cloned into luciferase reporter and co-transfected with miR-137 mimics into UM cells. After 24h, the luciferase activities were determined with a dual-luciferase reporter assay system (Promega Corporation, Fitchburg, WI, USA).

Statistics

All the experiments were performed in triplicate. The survival rate was analyzed by Kaplan-Meier method and log-rank test. One-way ANOVA followed by Scheffe's *post-hoc* analysis or Student's t-test were applied for the statistical analysis by the SPSS software version 17.0 (SPSS Inc., Chicago, IL). P<0.05 showed statistically significant difference.

Results

MiR-137 expressions were negatively correlated with the prognosis and clinicopathologic features of UM patients

To investigate the possibility of miR-137 to be implicated in UM progression, we examined miR-137 expressions in UM tissues. MiR-137 levels were reduced in UM tissues compared to non-tumor uveal tissues as indicated by qRT-PCR (Figure 1A). Then, the prognostic value of miR-137 in UM patients was analyzed. Firstly, the enrolled patients were divided into high and low miR-137 level groups according to the median miR-137 level. As demonstrated by the Kaplan-Meier analysis and log-rank test, there was a significant shorter overall survival (OS) rate in low miR-137 group than high miR-137 group (Figure 1B). Moreover, Table 2 summarizes the correlation between clinicopathological features of UM patients and miR-137 expression. UM patients with low miR-137 expressions presented more adverse characteristics than those with high miR-137 expressions. The results suggested that low miR-137 expressions in UM patients predicted a poor prognosis.

In vitro effects of miR-137 on UM cell viability

MiR-137 expressions were also measured in UM cells and correlated with the results from the tissue samples. The miR-137 levels were remarkably decreased in UM cells compared with the counterpart (Figure 2A). To manipulate miR-137 expressions in UM cells, miR-137 mimics or inhibitor were transfected into MUM-2B or MUM-2C cells according to their relatively lower or higher endogenous miR-137 expressions. The significant increase or decrease of miR-137 expression was confirmed by qRT-PCR (Figure 2B and 2C). MTT assays indicated that miR-137 upregulation in MUM-2B cells dramatically impaired the proliferation (Figure 2D). On the contrary, miR-137 inhibition promoted MUM-2C cell viability (Figure 2E).

In vitro impacts of miR-137 on UM cell invasion and migration

Moreover, transwell assay was conducted to determine the impact of miR-137 on UM cell inva-

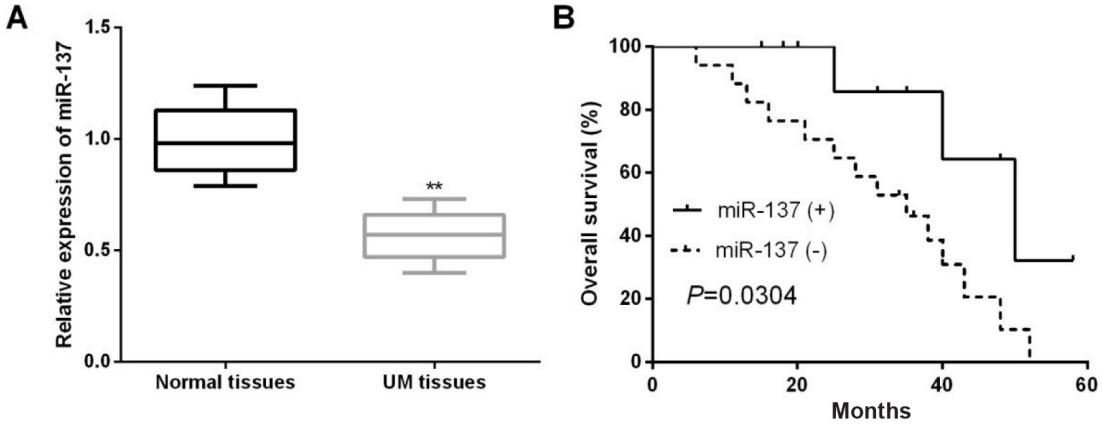

Figure 1. Decreased miR-137 in UM tissues correlated with a poor prognosis. **A:** qRT-PCR demonstrated lower miR-137 expressions in UM tissues than normal tissues. **B:** Prognostic signatures of UM patients with different miR-137 levels were analyzed by Kaplan-Meier plus log-rank test survival curves (**p<0.01).

Table 2. Correlation of miR-137 expression with the clinicopathological characteristics of the UM patients

Clinicopathological characteristics	*Cases (n=27)*	*miR-137# expression*		*p value*
		High(n=10)	*Low(n=17)*	
Age (years)				0.5311
>60	13	4	9	
≤60	14	6	8	
Gender				0.4205
Male	15	5	10	
Female	12	5	7	
Eye				0.3316
Right	12	4	8	
Left	15	6	9	
Tumor basal diameter, mm				0.2351
<15	11	4	7	
>15	16	6	10	
Recurrence				0.0136*
Yes	14	2	12	
No	13	8	5	
Histopathology				0.3687
Spindle cell	8	3	5	
Epithelioid	10	4	6	
Mixed	9	3	6	

UM: uveal melanoma; TNM: tumor-node-metastasis. The mean expression level of miR-137 was used as the cutoff. *Statistically significant.

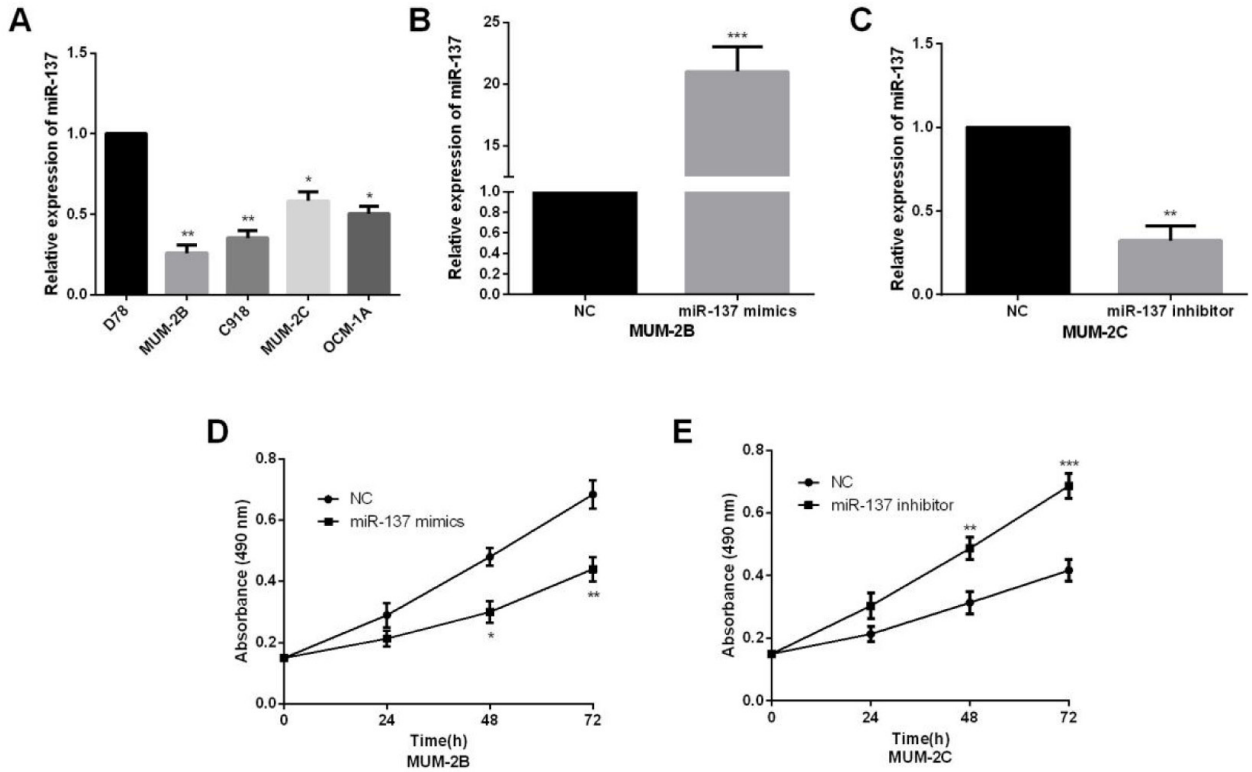

Figure 2. MiR-137 upregulation inhibited UM cell viability. **A:** Downregulated miR-137 was demonstrated in UM cell lines by qRT-PCR. **B,C:** MiR-137 was overexpressed or inhibited in MUM-2B or MUM-2C cells by transfecting with miR-137 mimics or inhibitor. **D,E:** MTT assays were applied to determine the influence of miR-137 on UM cell viability (*p<0.05, **p<0.01, ***p<0.001).

sion and migration. In miR-137 mimics-transfected MUM-2B cells, the invasion and migration abilities were inhibited (Figure 3A and 3B). Additionally, the facilitated roles of miR-137 inhibition in MUM-2C invasion and migration were also confirmed (Figure 3C and 3D).

Effect of miR-137 on expression of its target gene EZH2

We sought the putative targets of miR-137 by TargetScan to clarify the underlying mechanisms of miR-137 in UM. EZH2 was confirmed to contain conserved binding regions of miR-137(Figure 4A). This correlation was also verified by luciferase reporter assay. In brief, the luciferase activity of EZH2-3'UTR-MT was dramatically decreased following miR-137 upregulation while luciferase activity of EZH2-3'UTR-MUT was not influenced with transfection of miR-137 mimics (Figure 4B). Next, qRT-PCR was performed to examine the impacts of miR-137 on EZH2 expressions. In MUM-2B cells, miR-137 overexpression obviously suppressed the EZH2 expressions (Figure 4C). In addition, the EZH2 expressions in MUM-2C cells were markedly promoted by miR-137 inhibition (Figure 4D). These results confirmed that EZH2 was a target of miR-137 in UM cells.

Ectopic expressions of miR-137 regulated Wnt/β-catenin and EMT in UM cells

Next, the expression levels and prognostic significance of EZH2 in UM cells were examined. As demonstrated by qRT-PCR, EZH2 expression was prominently decreased in UM tissues and cells (Figure 5A and 5B). Moreover, Kaplan-Meier analysis and log-rank test demonstrated significantly shorter OS of EZH2 highly-expressed patients than

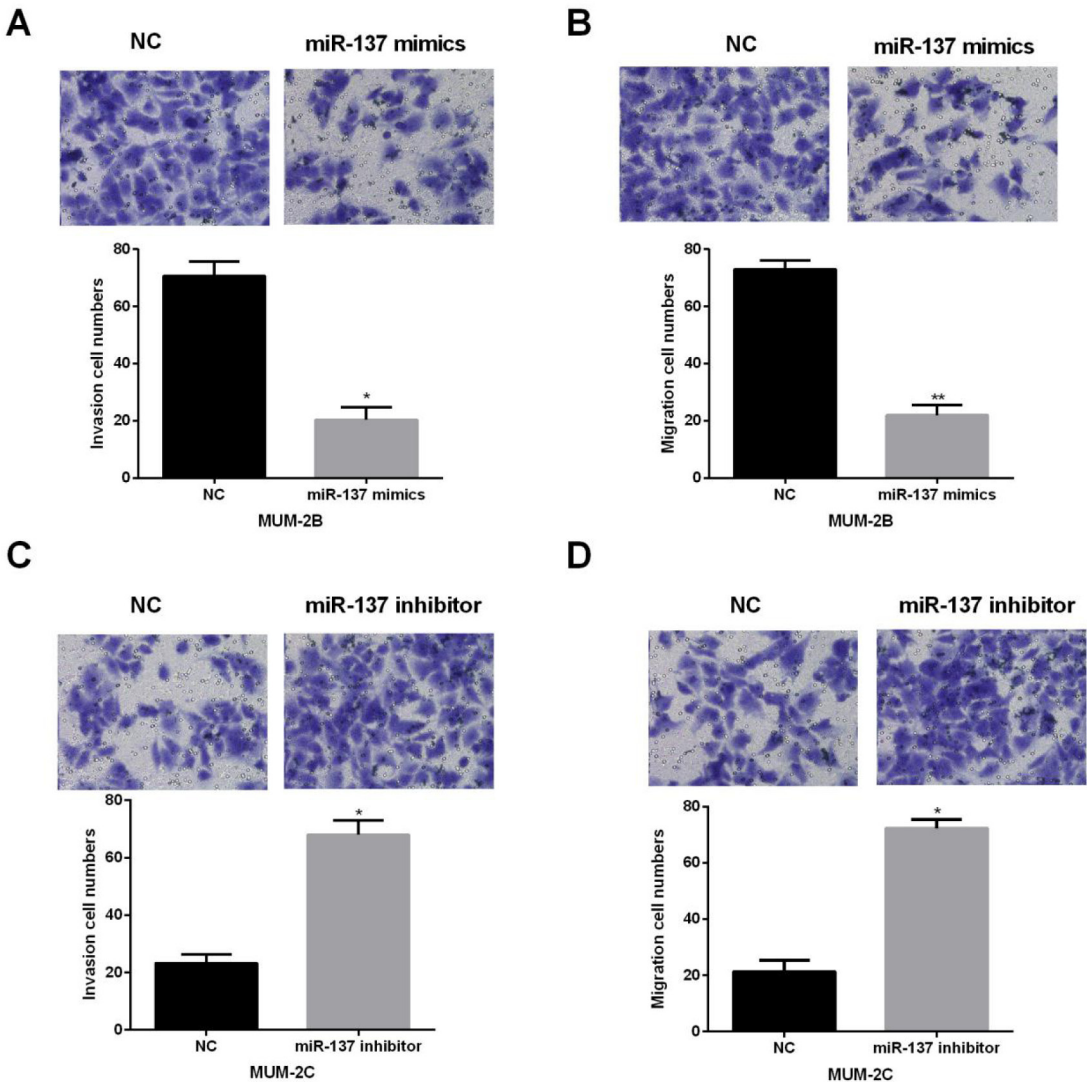

Figure 3. MiR-137 inhibited UM cell invasion and migration. **A,B:** The influence of miR-137 overexpression on MUM-2B cell invasion and migration was confirmed by Transwell assays. **C,D:** Transwell assay was performed to investigate the effects of miR-137 silence on MUM-2C cell invasion and migration (*p<0.05, **p<0.01).

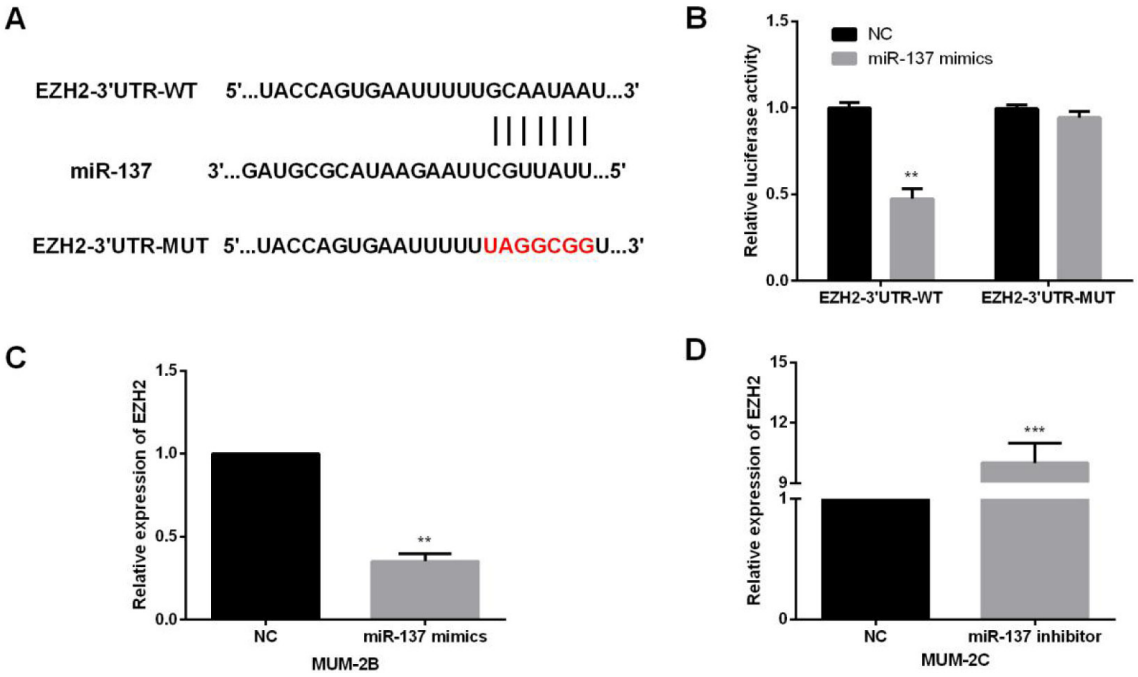

Figure 4. EZH2 was a downstream target of miR-137 in UM cells. **A:** The binding sites of miR-137 in EZH2 3'UTR. **B:** Luciferase activity of EZH2-3'UTR-WT was reduced by miR-137 mimics while the luciferase activity of EZH2-3'UTR-MUT had no changes. **C,D:** The functions of miR-137 in EZH2 expressions were determined by qRT-PCR (**p<0.01, ***p<0.001).

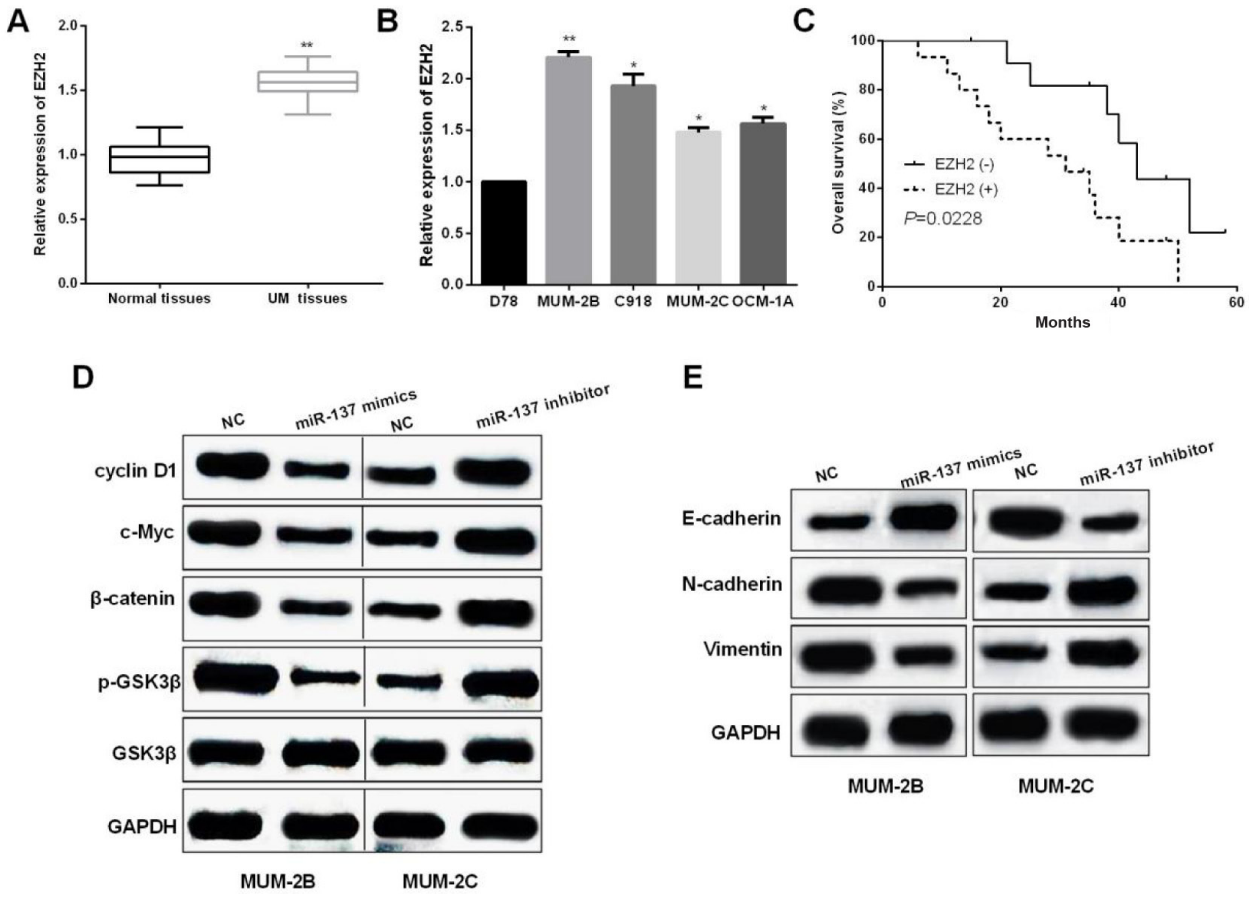

Figure 5. miR-137 inactivated Wnt/β-catenin pathway and EMT in UM cells. **A,B:** EZH2 expression levels in UM tissues and cells were examined by qRT-PCR. **C:** Prognostic signatures of UM patients with different EZH2 levels were analyzed by Kaplan-Meier and log rank survival curves. **D,E:** Western blot was used to detect the influence of miR-137 on Wnt/β-catenin pathway and EMT in UM cells (*p<0.05, **p<0.01).

EZH2 lowly-expressed patients (Figure 5C). Subsequently, the influence of miR-137 on UM cell Wnt/β-catenin and EMT was determined by western blot. In brief, for Wnt/β-catenin, the increased miR-137 levels in MUM-2B cells had evident inhibitory functions in activated β-catenin, c-Myc, cyclin D1, and p-GSK3β expressions; in miR-137 inhibited MUM-2C cells, it turned out just the opposite (Figure 5D). For EMT, the expression of epithelial-related marker E-cadherin was obviously increased while mesenchymal-related markers Vimentin and N-cadherin were dramatically downregulated by miR-137 mimics in MUM-2B cells (Figure 5E).

Discussion

The extremely poor prognosis of metastatic UM presents an unmet medical need [22]. Although the diagnosis and therapy of UM has made significant advances, the prognosis of UM is still unsatisfactory [23]. There is currently no powerful biomarker for identification of high-risk patients, therefore, screening for tumor-specific prognostic factors, and improving treatment outcomes are dramatically needed for UM patients [24]. Like many other malignant tumors, dysfunctions of genes and signaling pathways are also exhibited in UM [25]. Recently, genomic and gene expression analysis has identified different subtypes of UM, which may drive the design of more personalized and effective treatments in the future [26]. The accumulating knowledge of tumor biology has made it possible to develop new therapeutic strategies. Although protein-coding genes have received increasing attentions, miRs are currently emerging as a promising predictor for UM prognosis.

In recent years, miR-mediated epigenetic events have been closely related to the development of UM. The dynamic interactions among UM-specific regulators, such as miRs, are implicated in UM progression [27]. Moreover, the abnormal expressions of miRs have close relationship with UM suppressor activities and oncogenesis. For example, miR-224-5p functioned as a tumor suppressor through regulation of PIK3R3/AKT3 in UM [28]. On the other hand, miR-367 served as an oncogenic miR promoting UM migration and proliferation [29]. Epithelial to mesenchymal transition (EMT) results in strong trend for distant metastasis and local invasion. In the process of EMT, cells will lose the epithelial features and gain mesenchymal invasion and migration properties. Finally, the increase of mesenchymal markers and loss of epithelial markers promote tumor invasion and metastasis [30]. Therefore, detection of EMT-related genes is of great significance for inhibition of the malignant UM behaviors. Wnt/β-catenin is reported to play key roles in tumor proliferation and development, being frequently activated in multiple cancers [31,32]. Recent studies have indicated that Wnt/β-catenin is also involved in UM [33].

It is well known that miRs could not encode proteins and their biofunctions depend on the negative regulations of their targets. Multiple tumor-suppressive genes or oncogenes are identified as target genes of different miRs and the pivotal functions of miR in tumor progression have been widely recognized. One cancer-associated miR is miR-137. The downregulation of miR-137 was observed in hepatocellular carcinoma and neuroblastoma [34,35], where it served as a potential anti-tumor miR. In addition, miR-137 was upregulated in lung adenocarcinoma and bladder cancer [36,37], suggesting that the roles of miR-137 may vary with tumor types and tumor etiologies. However, the functions and the downstream targets of miR-137 in UM remain unknown. In our study, miR-137 was dramatically downregulated in UM, indicating a poor prognosis and aggressive phenotypes of UM patients. Moreover, miR-137 restoration was confirmed to repress UM cell viability, invasion and migration as demonstrated by functional assays. Findings also indicated that miR-137 overexpression could block Wnt/β-catenin pathway and EMT in UM cells, thus resulting in obstruction of malignant behaviors. We also identified EZH2 as a downstream target of miR-137 in UM cells.

In conclusion, the results of the current study clearly demonstrated that miR-137 expression was decreased in UM. Furthermore, the suppressive functions of miR-137 in UM cell proliferation, invasion and migration implicated Wnt/β-catenin and EMT as well as the regulation of EZH2. Thus, all these findings suggested that miR-137/EZH2 may function as promising therapeutic targets in UM patients.

Conflict of interests

The authors declare no conflict of interests.

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