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MDM4 is targeted by miR-449b-5p to promote the proliferation of endometrial carcinoma

J. ZHAO[1], X. DONG[2], S.-J. TAO[3], X.-L. LIU[1], Z. LI[1], J.-M. LIU[4], Y. CHEN[5]

[1]Department of Reproductive Center, Jinan Second Women's Protection Hospital, Jinan, China
[2]Department of Obstetrics and Gynecology Emergency, Jinan Second Women's Protection Hospital, Jinan, China
[3]Department of Obstetrics and Gynecology, Jinan Second Women's Protection Hospital, Jinan, China
[4]Department of Neonatal, Jinan Second Women's Protection Hospital, Jinan, China
[5]Department of Pediatrics, Jinan Second Women's Protection Hospital, Jinan, China

Jing Zhao and Xue Dong contributed equally to this work

Abstract. – OBJECTIVE: Previous studies have shown the involvement of microRNA-449b-5p (miR-449b-5p) and MDM4 in tumor development. This study aims to illustrate the role of miR-449b-5p in inhibiting proliferative capacity of endometrial carcinoma (EC) by targeting MDM4.

PATIENTS AND METHODS: Expression levels of miR-449b-5p and MDM4 in tumor tissues and paracancerous ones of EC patients were determined. Relationships between their levels and clinical parameters of EC patients were analyzed. Subsequently, regulatory effects of miR-449b-5p and MDM4 on proliferative capacities in KLE and HEC-1B cells were assessed by cell counting kit-8 (CCK-8) and colony formation assay, respectively. Thereafter, *in vivo* xenograft models were established in nude mice administrated with KLE cells overexpressing MDM4 or those with miR-449b-5p knockdown. Then, tumor weight and tumor volume were measured after mouse sacrifice. Finally, the interaction between miR-449b-5p and MDM4 was explored by Luciferase assay.

RESULTS: It was found that MDM4 was upregulated and miR-449b-5p was downregulated in EC tissues. Highly expressed MDM4 and lowly expressed miR-449b-5p were unfavorable to prognosis in EC patients, manifesting as a larger tumor size, more advanced tumor stage and lower overall survival. Besides, overexpression of MDM4 enhanced in vitro proliferative capacity in EC cells and *in vivo* tumorigenesis in nude mice bearing EC. Similarly, knockdown of miR-449b-5p yielded similar results. Luciferase assay confirmed that MDM4 was the target gene binding to miR-449b-5p, and its level was negatively correlated with miR-449b-5p level in EC.

CONCLUSIONS: MiR-449b-5p and MDM4 are downregulated and upregulated in EC species, respectively. They are closely linked to tumor size, tumor stage and overall survival in EC patients. Through negatively regulating MDM4 level, miR-449b-5p inhibits proliferative capacity in EC cells.

Key Words:
MiR-449b-5p, MDM4, Endometrial carcinoma, Proliferation.

Introduction

Endometrial carcinoma (EC) is the fourth leading malignancy in females and it is secondary to breast cancer, colorectal cancer and lung cancer[1-3]. The incidence of EC becomes higher and its onset shows a younger trend in recent years[1,2]. It is generally considered that endocrine and genetic factors are the two major parts that influence the development of EC[4,5]. Although therapeutic strategies have been greatly improved, clinical outcomes of advanced or recurrent EC are far away from satisfication[5,6]. Tumorigenesis is a complicated process that involves oncogenes, tumor suppressors and relevant signals[7,8]. It is of significance to clarify the mechanisms underlying the growth, metastasis and recurrence of EC[8,9].

Epigenetics displays a general and important role in tumor occurrence and development[10,11]. Epigenetics regulates gene expressions and functions [i.e., DNA methylation, histone modifications, and microRNA (miRNA) regulation] without changing DNA sequences[9,12]. MiRNAs are non-coding RNAs with 19-24 nucleotides, which are extensively distributed in eukaryotes and highly conserved. They are transcribed into pri-miRNAs by nuclear RNA polymerase. Under the guidance of Drosha enzyme (ribonuclease), newly formed pre-miRNAs are transported into the cytoplasm and thus mature miRNAs are produced after Di-

Corresponding Author: Jing Zhao, BM; e-mail: jnsdefybjybgs@jn.shandong.cn

cer enzyme cleavage[13-15]. Through complementary base pairing, miRNAs, which only account for 2% human genomes, are able to regulate expressions and functions of over one third genes[16,17]. Yin et al[18] and Cheng et al[19] have reported the involvement of miR-449b-5p in tumor development. Its potential roles in EC, however, remains unclear.

MDM4 locates on human chromosome 1q32, where many tumor-associated genes are located in this locus, and it contains 10 introns and 11 exons. It is highly suggested that MDM4 is closely linked to tumor development[20,21]. Potential binding sequences in the 3'UTR of miR-449b-5p and MDM4 are identified by bioinformatics method[22,23]. This study aims to uncover the regulatory effects of miR-449b-5p and MDM4 on the proliferative capacity in EC.

Patients and Methods

Patients and EC Samples

A total of 56 paired EC tissues and paracancerous ones (5 cm away from tumor tissues) were surgically resected from EC patients undergoing surgery or biopsy. Their clinical data were recorded. In this research, tumor staging was conducted based on the guidelines proposed by the Union for International Cancer Control (UICC). Every included patient was followed up for general condition, clinical symptom and imaging examination through telephone and outpatient review. This investigation was approved by Ethics Committee of Jinan Second Women's Protection Hospital. Signed written informed consents were obtained from all participants before the study.

Cell Culture

Human EC cell lines (HEC-1A, HEC-1B, KLE, and Ishikawa) and endometrial stromal cell line (T-HESC) were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS, HyClone, South Logan, UT, USA), 100 U/mL penicillin and 100 μg/mL streptomycin in a 5% CO_2 incubator at 37°C.

Transfection

Cells were cultured to 60-70% confluence in 6-well plates and transfected with plasmids constructed by GenePharma (Shanghai, China), using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). 48 hours later, cells were collected for the following use.

Cell Proliferation Assay

Cells were inoculated in a 96-well plate with 2×10^3 cells per well. At the appointed time points, absorbance value at 490 nm of each sample was recorded using the cell counting kit-8 (CCK-8) kit (Dojindo Laboratories, Kumamoto, Japan) for plotting the viability curves.

Colony Formation Assay

Cells were inoculated in 6-well plates with 200 cells per well and cultured for 2 weeks. Culture medium was replaced once in the first week and twice in the second week. Then, visible colonies were washed in phosphate-buffered saline (PBS), fixed in methanol for 20 min and dyed in 0.1% crystal violet (Solarbio, Beijing, China) for 20 min, which were finally captured and calculated.

Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Extracted RNAs by TRIzol reagent (Invitrogen, Carlsbad, CA, USA) were purified by DNase I treatment, and reversely transcribed into complementary deoxyribose nucleic acids (cDNAs) using Primescript RT Reagent (TaKaRa, Dalian, China). The obtained cDNAs underwent qRT-PCR using SYBR®Premix Ex Taq™ (TaKaRa, Dalian, China). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and U6 were the internal references. Each sample was performed in triplicate, and relative level was calculated by $2^{-\Delta\Delta Ct}$. MiR-449b-5p: forward: 5'-TGGCAGTGTATTGTTAGCTGGT-3' and reverse: 5'-CGCAAGGATGACACGCAAATTC-3', U6: forward: 5'-GCTCGCTTCGGCAGCACAT-3' and reverse: 5'-AAAATATGGAACGCTTCACG-3', MDM4: forward: 5'-TGCGTGTGGAGTATTTGGATG-3' and reverse: 5'-TGGTACAGTCAGAGCCAACCAG-3', and GAPDH: forward: 5'-GCACCACACCTTCTACAATG-3', and reverse: 5'-TGCTTGCTGATCCACATCTG-3'.

Western Blot

Cells were lysed for isolating cellular protein and electrophoresed. Protein samples were loaded on polyvinylidene difluoride (PVDF) membranes (Roche, Basel, Switzerland). Subsequently, non-specific antigens were blocked in 5% skim milk for 2 hours. Membranes were reacted with primary and secondary antibodies for indicated time. Band exposure and analyses were finally conducted.

In Vivo Xenograft Model

Experimental procedures of establishing *in vivo* xenograft models in nude mice were approved by the Animal Ethics and Use Committee. A total of 20 male nude mice were randomly assigned into four groups, with 5 mice in each group. They were subcutaneously administrated with KLE cells transfected with pcDNA3.1-NC, pcDNA3.1-MDM4, NC inhibitor or miR-449b-5p inhibitor, respectively. Tumor size was recorded every 5 days. Five weeks later, mice were sacrificed for collecting EC tissues. Tumor volume = (width^2×length)/2. Ultimately, the positive expression of MDM4 in EC tissues was determined using S-P method.

Luciferase Assay

KLE and HEC-1B cells were co-transfected with NC mimic/miR-449b-5p mimic and MDM4-WT/MDM4-MUT, respectively. Cells were lysed for determining relative Luciferase activity (Promega, Madison, WI, USA) 48 h later.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 19.0 (IBM, Armonk, NY, USA) was used for data analyses. Data were expressed as mean ± standard deviation. Differences between two groups were analyzed by the *t*-test. Chi-square test was conducted for analyzing the relationship between relative levels of miR-449b-5p and MDM4, and clinical data of EC patients. Pearson's correlation test was applied for evaluating the relationship between two genes in EC species. $p<0.05$ suggested that the difference was statistically significant.

Results

Relative Levels of MiR-449b-5p and MDM4 in EC Species

A total of 56 paired EC tissues and paracancerous ones were collected. It was shown that MDM4 was upregulated (Figure 1A) and miR-449b-5p was downregulated (Figure 1D) in EC tissues. Identically, MDM4 and miR-449b-5p showed the same trends in EC cell lines (Figure 1B, 1E).

Based on the median level of MDM4 in 56 EC patients, they were divided into high and low expression groups, respectively. MDM4 level was positively correlated with tumor size and tumor stage in EC patients, while it was unrelated to age and metastasis (Table I). Highly expressed

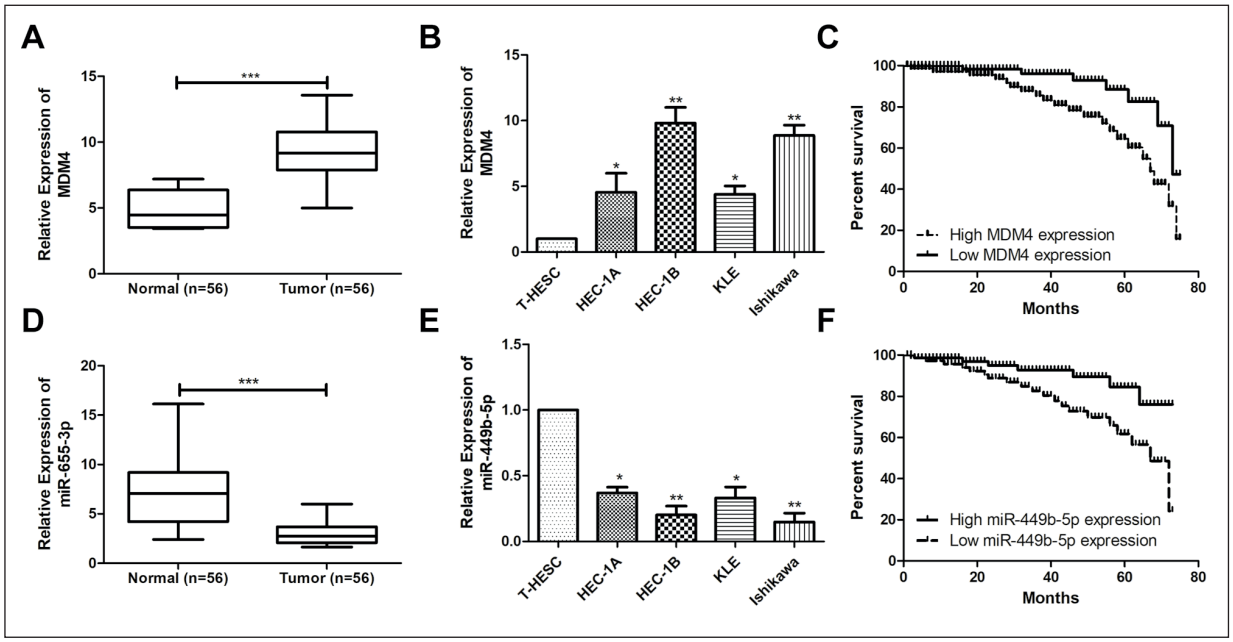

Figure 1. Relative levels of miR-449b-5p and MDM4 in EC tissues. **A**, MDM4 levels in EC tissues (n=56) and paracancerous ones (n=56). **B**, MDM4 levels in EC cell lines. **C**, Overall survival in EC patients based on MDM4 expression. **D**, MiR-449b-5p levels in EC tissues (n=56) and paracancerous ones (n=56). **E**, MiR-449b-5p levels in EC cell lines. **F**, Overall survival in EC patients based on miR-449b-5p expression. Data are expressed as mean ± SD, *$p<0.05$, **$p<0.01$, ***$p<0.001$.

Table I. Association of miR-449b-5p and MDM4 expression with clinicopathologic characteristics of endometrial carcinoma.

Parameters	No. of cases	miR-449b-5p expression		*p*-value	MDM4 expression		*p*-value
		High (%)	Low (%)		Low (%)	High (%)	
Age (years)				0.415			0.805
<60	23	13	10		9	14	
≥60	33	15	18		14	19	
Tumor size				0.033			0.014
<4 cm	28	18	10		16	12	
≥4 cm	28	10	18		7	21	
T stage				0.014			0.014
T1-T2	33	21	12		18	15	
T3-T4	23	7	16		5	18	
Lymph node metastasis				0.265			0.114
No	36	20	16		12	24	
Yes	20	8	12		11	9	
Distance metastasis				0.174			0.159
No	33	19	14		11	22	
Yes	23	9	14		12	11	

MDM4 was unfavorable to overall survival in EC patients (Figure 1C). The relationship between miR-449b-5p level and clinical features of EC patients was analyzed. It was found that its level was negatively related to tumor size and tumor stage (Table I), and lowly expressed miR-449b-5p indicated a poor prognosis in EC patients (Figure 1F).

MDM4 Stimulated Proliferative Capacity and Tumorigenesis in EC

MDM4 overexpression and knockdown models were constructed in KLE and HEC-1B cells, respectively (Figure 2A). Transfection with pcDNA3.1-MDM4 markedly enhanced viability (Figure 2B) and clonality (Figure 2C) in KLE cells. Conversely, knockdown of MDM4 inhibited proliferative capacity in HEC-1B cells. Subsequently, potential function of MDM4 in regulating *in vivo* proliferation of EC was analyzed. Nude mice administrated with KLE cells overexpressing MDM4 showed a higher positive expression of MDM4 in EC tissues, suggesting the successful construction of xenograft model (Figure 2F). Nude mice overexpressing MDM4 showed a larger tumor volume (Figure 2D) and tumor weight (Figure 2E) than those of controls.

MiR-449b-5p Suppressed Proliferative Capacity and Tumorigenesis in EC

To uncover the role of miR-449b-5p in the malignant development of EC, miR-449b-5p inhibitor and mimic were constructed (Figure 3A). The results showed that knockdown of miR-449b-5p remarkably enhanced viability (Figure 3B) and colony number (Figure 3C) in KLE cells. Overexpression of miR-449b-5p yielded the opposite trends in HEC-1B cells. In the same way, nude mice were administrated with KLE cells transfected with NC inhibitor or miR-449b-5p inhibitor, respectively. Compared with control mice, those with miR-449b-5p knockdown showed a larger tumor volume (Figure 3D) and tumor weight (Figure 3E). Positive expression of MDM4 was higher in EC tissues extracted from nude mice with miR-449b-5p knockdown (Figure 3F).

MDM4 Was the Target Gene of MiR-449b-5p

Bioinformatics analysis revealed potential binding sequences in the 3'UTR of miR-449b-5p and MDM4. Luciferase activity was markedly reduced in wild-type MDM4 vector after overexpression of miR-449b-5p, verifying the binding relationship between miR-449b-5p and MDM4 (Figure 4A). Protein level of MDM4 was found to be negatively regulated by miR-449b-5p in EC cells (Figure 4B). As expected, miR-449b-5p level was negatively influenced by MDM4 as well (Figure 4C). A negative correlation between expression levels of miR-449b-5p and MDM4 was identified in EC tissues (Figure 4D).

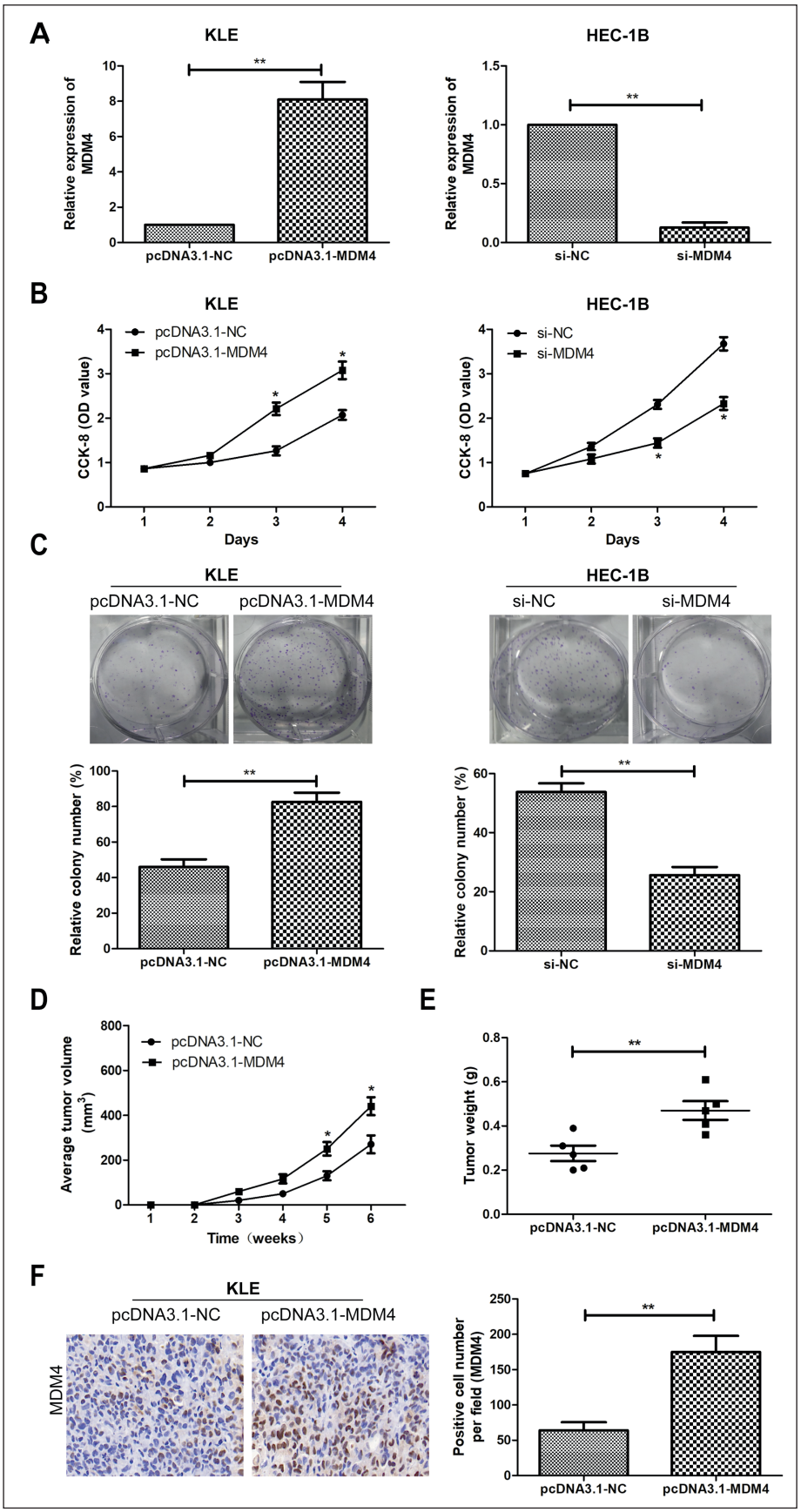

Figure 2. MDM4 stimulates proliferative capacity and tumorigenesis in EC. **A**, Transfection efficacy of pcDNA3.1-MDM4 and si-MDM4 in KLE and HEC-1B cells, respectively. **B**, Viability in KLE and HEC-1B cells tested by MDM4. **C**, Colony number in KLE and HEC-1B cells tested by MDM4 (magnification: 10×). **D**, Tumor volume in nude mice bearing EC regulated by MDM4. **E**, Tumor weight in nude mice bearing EC regulated by MDM4. **F**, Positive expression of MDM4 in nude mice bearing EC regulated by MDM4 (magnification: 40×). Data are expressed as mean ± SD, *p<0.05, **p<0.01.

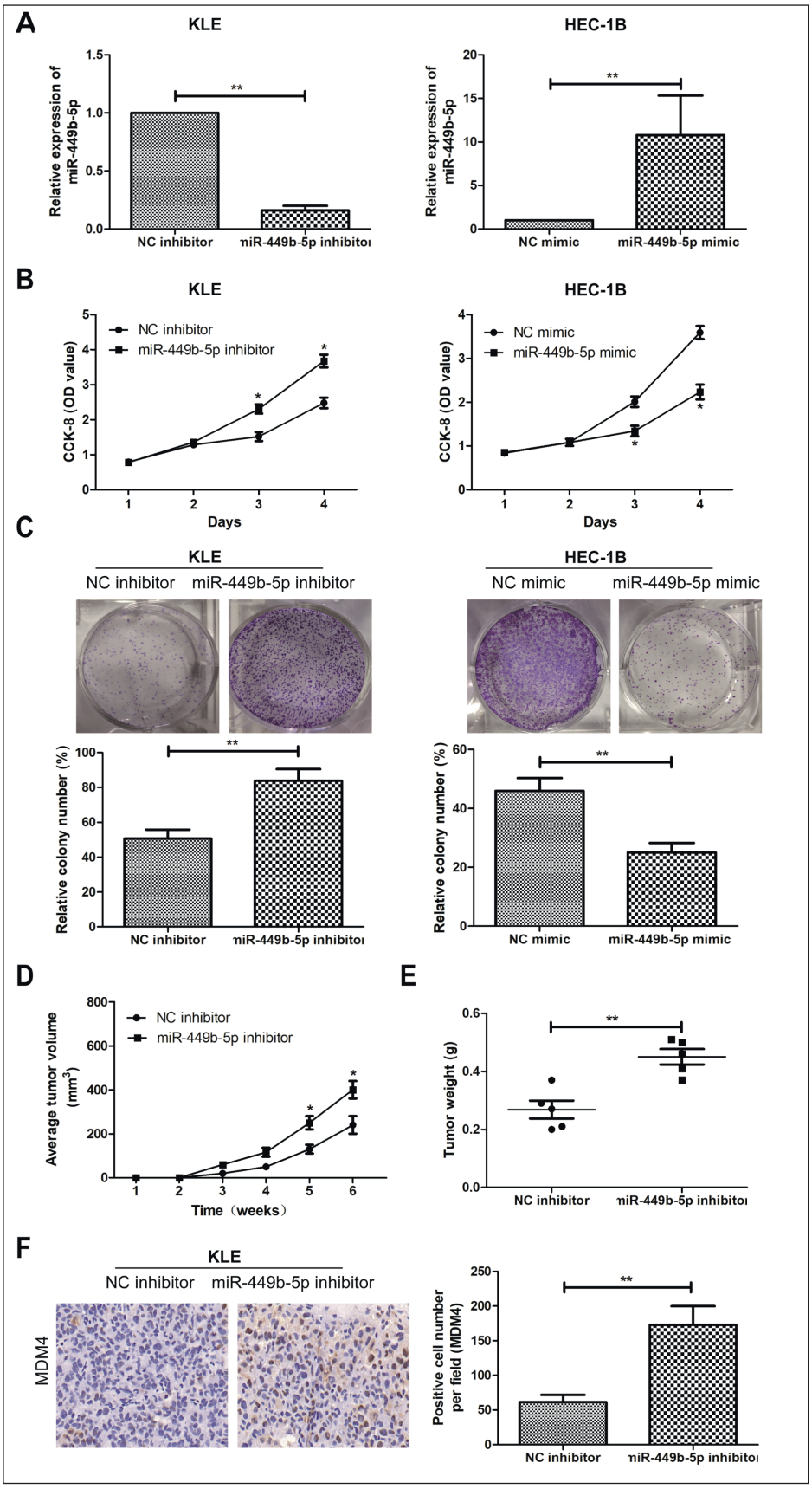

Figure 3. MiR-449b-5p suppresses proliferative capacity and tumorigenesis in EC. **A**, Transfection efficacy of miR-449b-5p inhibitor and mimic in KLE and HEC-1B cells, respectively. **B**, Viability in KLE and HEC-1B cells assessed by miR-449b-5p. **C**, Colony number in KLE and HEC-1B cells regulated by miR-449b-5p (magnification: 10×). **D**, Tumor volume in nude mice bearing EC regulated by miR-449b-5p. **E**, Tumor weight in nude mice bearing EC analyzed by miR-449b-5p. **E**, Positive expression of MDM4 in nude mice bearing EC analyzed by miR-449b-5p (magnification: 40×). Data are expressed as mean ± SD, *p<0.05, **p<0.01.

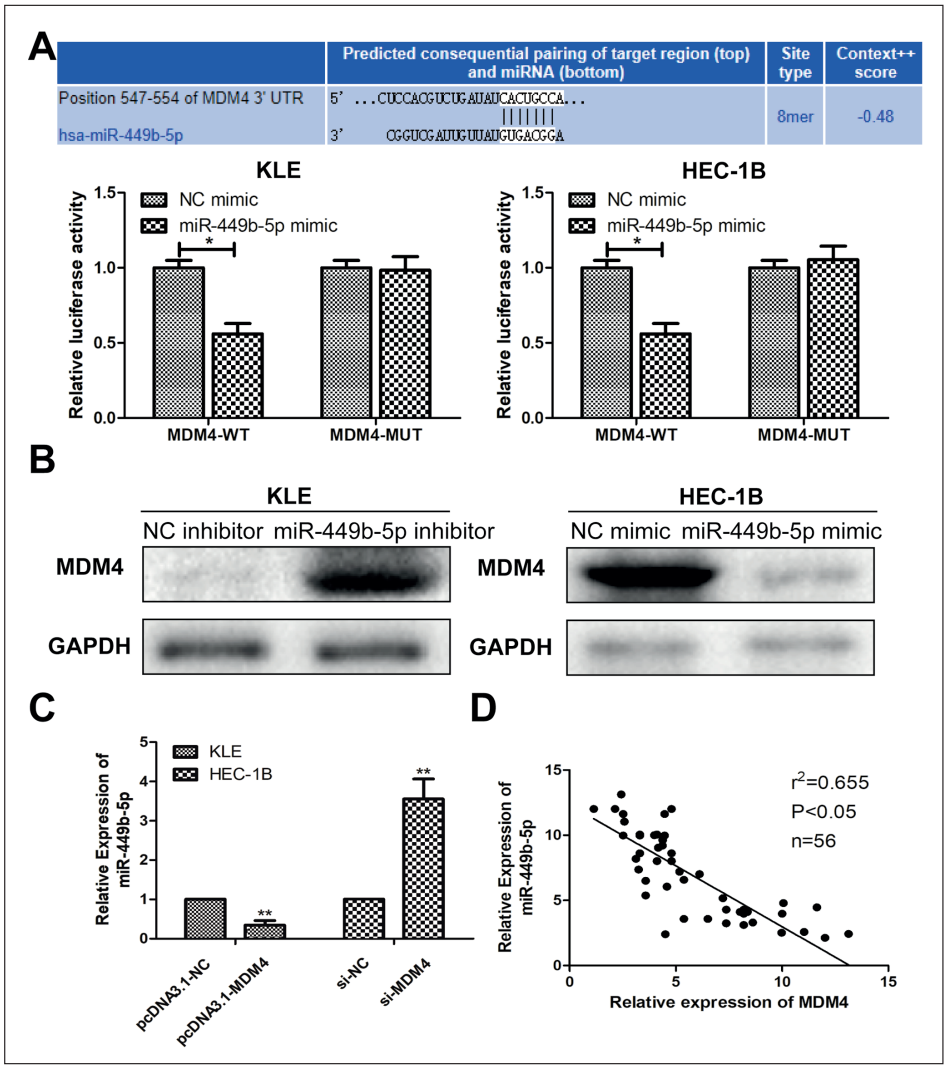

Figure 4. MDM4 is the target gene of miR-449b-5p. **A**, Luciferase activity in KLE and HEC-1B cells co-transfected with NC mimic/miR-449b-5p mimic and MDM4-WT/MDM4-MUT, respectively. **B**, Protein level of MDM4 in KLE and HEC-1B cells assayed by miR-449b-5p. **C**, Relative level of miR-449b-5p in KLE and HEC-1B cells regulated by MDM4. **D**, A negative correlation between expression levels of miR-449b-5p and MDM4 in EC species. Data are expressed as mean ± SD, *p<0.05, **p<0.01.

Discussion

EC is a prevalent malignancy of the female genital tract and is used to mainly affect older women. Nevertheless, the onset age of EC becomes younger and younger[1-3]. Estrogen level is validated to be closely linked to the occurrence of EC[4,5]. In addition, obesity, hypertension, diabetes mellitus, infertility and late menopause are risk factors for EC. About 1% of EC cases are attributed to genetic factors[5-7]. Surgery combined with chemotherapy, radiotherapy or hormone therapy is applied in EC patients[7,8]. Early screening and intervention will greatly improve the therapeutic efficacy of EC.

The occurrence and development of tumors are multi-factor, multi-step and complicated[9-11]. Previous studies[9-12] have focused on oncogenes or tumor-suppressor genes that encode proteins. Recently, non-coding RNAs have been well concerned due to their potential functions in tumor diagnosis and treatment[16,17]. MiR-449b-5p participates in the malignant development of nasopharyngeal carcinoma and cervical cancer[18,19]. In our research, miR-449b-5p was downregulated in EC species and its low level was unfavorable to prognosis. Knockdown of miR-449b-5p remarkably enhanced proliferative capacity in EC cells and tumorigenesis ability in nude mice bearing EC. On the contrary, MDM4 was upregulated in EC as an oncogene, and it promoted both *in vitro* and *in vivo* proliferative capacity in EC.

A novel ceRNA theory proposes that miRNAs display their functions through targeting the corresponding genes. The interaction between miRNAs and proteins may influence tu-

mor development. According to the predicted binding sequences in the 3'UTR of miR-449b-5p and MDM4, our Luciferase assay indicated that MDM4 was the downstream gene binding to miR-449b-5p. Moreover, MDM4 level was negatively regulated by miR-449b-5p.

Conclusions

To sum up, we demonstrate a negative feedback loop miR-449b-5p/MDM4 responsible for alleviating the malignant development of EC.

Conflict of Interests

The authors declare that they have no conflict of interests.

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