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Original Article

microRNA-361 inhibited prostate carcinoma cell invasion by targeting Gli1

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Abstract: Abnormal expression of transcriptional factor Gli1 in Hedgehog pathway is closely correlated with epithelial mesenchymal transition (EMT) of tumor cells, and invasion/metastasis. MicroRNA (miR)-361 expression level was significantly depressed in prostate carcinoma. Bioinformatics analysis showed targeted binding sites between miR-361 and 3'-UTR of Gli1. This study thus investigated the role of miR-361 and Gli1 abnormal expression in regulating invasion or metastasis of prostate cancer. Prostate cancer tissues and benign prostate hyperplasia tissues were collected to test expression of miR-361, Gli1 mRNA and protein. Dual luciferase reporter gene assay (LRGA) was employed to test relationship between miR-361 and Gli1. Expression of miR-361, Gli1 and EMT related molecules, along with malignant growth and invasion ability were compared between PC3M-1E8 and PC3M-2B4 cell. Cultured PC3M-1E8 cells were treated with miR-361 mimic and/or si-Gli1 to compare expression of miR-361, Gli1 and EMT molecules, as well as malignant growth and invasion capacities. Expression levels of Gli1 were significantly elevated in cancer tissues, whilst miR-361 was down-regulated. MiR-361 targeted and regulated Gli1 expression. PC3M-1E8 cell had significantly elevated Gli1, Snail and N-cadherin expressions compared to PC3M-2B4 cells, whilst miR-361, E-cadherin and Occludin expressions were significantly decreased. Potency for malignant growth of PC3M-1E8 cells was significantly enhanced. The transfection of miR-361 mimic and/or si-Gli1 significantly inhibited Snail and N-cadherin expression in PC3M-1E8 cells, thus elevating E-cadherin and Occludin expression to inhibit invasion and malignant growth of prostate cancer cells. MiR-361 weakened EMT process, thus alleviating invasion of prostate carcinoma cells via inhibiting Gli1.

Keywords: MicroRNA-361, Gli1, endothelial mesenchymal transition, prostate cancer, invasiveness

Introduction

Prostate cancer (PCa) is the most common malignant tumor in male reproductive-urinary system, and is the sixth popular cancer in males [1]. It is estimated that incidence of PCa in China is 60.3 per 100000, with mortality rate at 26.6 per 100000 [2]. Current opinions agreed that endothelial mesenchymal transition (EMT) is one initiating step for tumor invasion and metastasis [3]. Hedgehog signal pathway plays a critical role in regulating human embryonic development [4, 5] as it participates in cell proliferation [6], migration [7] and differentiation [8]. Abundant researches have revealed the close relationship between Hedgehog signal pathway and prostate gland embryonic development and occurrence and progression of PCa, and is the possible novel target of diagnosis and treatment of prostate cancer [9, 10]. For example, National Cancer Institute (NCI) has listed Hedgehog gene as the primary target for treating PCa beyond phosphatase and tensin homology deleted on chromosome ten (PTEN) or androgen receptor gene [11]. Nuclear transcriptional factor glioma-associated oncogene homolog 1 (Gli1) plays as the transcriptional effector in Hedgehog signal transduction. Under the stimulus of extracellular Hedgehog signal molecule, transcriptional factor Gli1 enters the nucleus, where it facilitates transcription and translation of target genes, further mediating cell proliferation, apoptosis, differentiation, migration and motility [12, 13]. Enhancement of Gli1 expression or function may be related with occurrence of pulmonary carcinoma [14], gastric cancer [15], bladder cancer [16] and breast cancer [17]. Previous

study showed the role of abnormal expression/function of Gli1 in PCa pathogenesis [18, 19]. MicroRNA is one small molecule of non-coding single stranded RNA with 22~25 nucleic acids length. By complementary binding with 3'-untranslated region (3'-UTR) of target gene mRNA, it can regulate target gene expression via degrading mRNA or inhibiting mRNA translation, thus mediating biological processes including cell proliferation, differentiation and migration [20]. Previous studies showed significantly depressed miR-361 expression in PCa tumor tissues [21, 22]. Bioinformatics analysis showed satisfactory targeted correlation between miR-361 and 3'-UTR of Gli1 gene. This study thus investigated if dysregulation of miR-361 or Gli1 played a role in regulating PCa invasion and metastasis.

Materials and methods

Reagents, materials and equipment

Human PCa cell line with high metastasis PC3M-1E8 and low metastasis cell line PC3M-2B4 were purchased from Jiniou Bio (China). DMEM culture medium, fetal bovine serum (FBS) and penicillin-streptomycin were purchased from Gibco (US). Lipofectamine RNAiMAX was purchased from Invitrogen (US). MiR-361 nucleic acid sequence was designed and synthesized by Ruibo Bio (China). ReverTra Ace qPCR RT Kit and SYBR Green dye were purchased from Toyobo (Japan). Mouse anti-E-cadherin and rabbit anti-N-cadherin antibody, and HRP labelled secondary antibody were all purchased from Abcam (US). Mouse anti-Gli1, rabbit polyclonal antibody against Snail and mouse monoclonal antibody against Occludin were purchased from Santa Cruz (US). Transwell chamber was purchased from Millipore (US). Matrigel was purchased from BD biosciences (US). Dual-Luciferase Reporter assay system and pGL3-promoter were purchased from Promega (US). BCA protein quantification test kit was purchased from Beyotime (China). HERAcell model 2401 CO_2 cell incubator was purchased from Thermo (US). Model CFX96 fluorescent quantitative PCR cycler was purchased from Bio-Rad (US).

Clinical information

A total of 68 PCa patients (aging between 59 and 73 years, average age = 65.7 years) who received treatment in the First Affiliated Hospital of Xinxiang Medical University from April 2015 to June 2016 were recruited. All patients were primarily diagnosed without endocrine or other treatment. Pre-op examination of prostate specific antigen (PSA) showed 5 cases between 4 and 10 μg/L and 63 cases with higher than 10 μg/L. All patients were diagnosed as PCa by prostate biopsy. According to 7th edition of TNM staging by UICC in 2009, there were 19, 14, 20 and 15 cases at phase T1, T2, T3 and T4, respectively. Another cohort of 28 cases of benign prostatic hyperplasia (BPH) patients (aging between 58 and 76 years, average age = 64.5 years) was recruited from our hospital during the same time period. Acquirement of all samples has obtained written consents of all patients, and has been approved by the First Affiliated Hospital of Xinxiang Medical University ethical committee board.

Cell culture

PC3M-1E8 and PC3M-2B4 cells were all culture in DMEM medium containing 10% FBS, 100 U/mL penicillin and 100 μg/mL streptomycin, and were cultured in 37°C incubator with 5% CO_2. Those cells at log-growth phase with good status were used in experiments.

Construction of luciferase reporter gene vector

Using HEK293 genome as the template, full length fragment of 3'-UTR of Gli1 gene was amplified. PCR products were collected from 1.0% agarose gel, and were ligated into pGL-3M luciferase reporter plasmid after XbaI/NotI enzymatic digestion. Recombinant plasmid was then used to transform DH5α competent cells. Positive clones with primary screening were selected and named as pGL3-Gli1-3'UTR.

Luciferase reporter gene assay

Lipofectamine RNAiMAX was used to transfect HEK293 cells with 100 ng pGL3-Gli1-3'UTR plasmid, 50 nmol miR-361 oligonucleotide fragment, and 50 ng pRL-TK. After 6 h transfection, normal DMEM medium containing 10% FBS was used to replace Opti-MEM medium. After 48 h continuous incubation, dual-luciferase assay was performed. In brief, cells were washed twice in PBS, with the addition of 100 μL Passive Lysis Buffer (PLB). With vortex at

room temperature for 15 min, the mixture was centrifuged at 1000 rpm for 5 min. 50 μL cell lysate was mixed with 50 μL luciferase substrate. Activity of luciferase was measured immediately. The enzymatic reaction was stopped in 50 μL Stop & Glo, followed by quantification of sea pansy luciferase activity. The relative expression level of reporter gene was calculated as the ratio of luciferase activity against sea pansy luciferase activity. Oligonucleotide sequences used were: mimic NC, 5'-UUCUC CGAAC GUGUC ACGUT T-3'; miR-361 mimic, 5'-ACCCC UGGAG AUUCU GAUAA UU-3'; inhibitor NC, 5'-CAGGU CAUAG GCAUA CCGU-3'; miR-361 inhibitor, 5'-ACCUU AGCAG GAUUA CUAG-3'.

Cell transfection and grouping

In vitro cultured human PCa high migration cell line PC3M-1E8 were divided into five groups, including mimic NC group, miR-361 mimic transfection group, si-NC transfection group; si-Gli1 group and miR-361 mimic + si-Gli1 group. 72 h after transfection, cells were collected for further assays. Oligonucleotide sequences for transfection were: si-Gli1 sense, 5'-CCAGG AAUUU GACUC CCAAT T-3'; si-Gli1 anti-sense, 5'-UUGGG AGUCA AAUUC CUGGC T-3'; si-NC sense, 5'-UUCUC CGAAC GUGUC ACGUT T-3'; si-NC anti-sense, 5'-ACGUG ACACG UUCGG AGAAT T-3'.

qRT-PCR for gene expression

ReverTra Ace qPCR RT Kit was to synthesize cDNA in a 10 μL system including 2 μg total RNA extracted by Trizol reagent, 4 μL RT buffer (5×), 1 μL dNTP, 1 μL RT enzyme mix, 1 μL RNase inhibitor, 2 μL RT enzyme and ddH_2O. The reaction conditions were: 95°C for 15 s, followed by 60°C 30 s and 74°C 30 s. PCR was performed on Bio-Rad CFX96 fluorescent quantitative PCR cycler for 40 cycles to collect data. Primer sequences were: miR-361P_F: 5'-ATAAA GTGCTG ACAGT GCAGA TAGTG-3'; miR-361P_R: 5'-TCAAG TACCC ACAGT GCGGT-3'; U6P_F: 5'-ATTGG AACGA TACAG AGAAG ATT-3'; U6P_R: 5'-GGAAC GCTTC ACGAA TTTG-3'; Gli1P_F: 5'-AGCGT GAGCC TGAAT CTGTG-3'; Gli1P_R: 5'-CAGCA TGTAC TGGGC TTTGA A-3'; Snail: 5'-ACCCC ACATC CTTCT CACTG-3'; SnailP_R: 5'-TACAA AAACC CACGC AGACA-3'; β-actinP_F: 5'-GAACC CTAAG GCCAA C-3'; β-actinP_R: 5'-TGTCA CGCAC GATTT CC-3.

Western blot

Total proteins were extracted and tested for quality and concentration by BCA approach. After boiling for 5 min in 4× loading buffer, 50 μg protein samples were separated in 10% SDS-PAGE (45 V, 200 min), and were transferred to PVDF membrane (300 mA current, 110 min time). The membrane was blocked in 5% defatted milk powder for 60 min, followed by primary antibody (anti-E-cadherin at 1:400, anti-N-cadherin at 1:400, anti-Gli1 at 1:100, anti-Snail at 1:400 or anti-β-actin at 1:1000) incubation at 4°C for 12 h. By PBST washing (3 times), HRP-labelled secondary antibody (anti-mouse or anti-rabbit at 1:10000 dilution) was added for 60 min incubation under room temperature. After PBST rinsing for three times, ECL reagent was added for 1~3 min dark incubation. The membrane was then exposure in dark and scanned for data analysis using Quantity One software.

Plate clonal assay to test malignant growth ability

Cells at log growth phase were digested in trypsin and prepared into single cell suspension using culture medium. Cells were then inoculated into 10 cm culture medium at 100 cells per dish density. Cells were incubated in a humidified chamber for 2~3 weeks. Incubation was stopped when visible clones were formed in the culture dish. The medium was discarded. Cells were rinsed twice in PBS, and fixed by 4% paraformaldehyde for 20 min at room temperature. Giemsa staining buffer was added for 20 min incubation after discarding the fixation buffer. The staining was removed by slow tap water and the plate was air dried. The number of clones with more than 10 cells was counted under low magnification objectives. Clonal formation rate was calculated as (clone number/inoculated cell number) ×100%.

Transwell assay

100 μL Matrigel was paved on the upper surface of Transwell chamber (8 μm pore size). The chamber was incubated at 37°C for 30 min. 200 μL PC3M-1E8 cell suspension in serum-free medium was added into the upper chamber, while the lower chamber was filled with

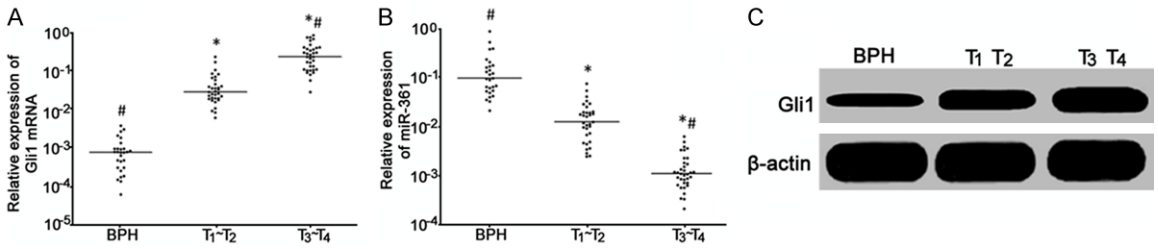

Figure 1. Expression of miR-361 and Gli1 in PCa tissues. A: qRT-PCR for Gli1 mRNA expression. B: qRT-PCR for miR-361 expression. C: Western blot for Gli1 protein expression. *P<0.05 compared to BPH tissues; #P<0.05 compared to T1~T2.

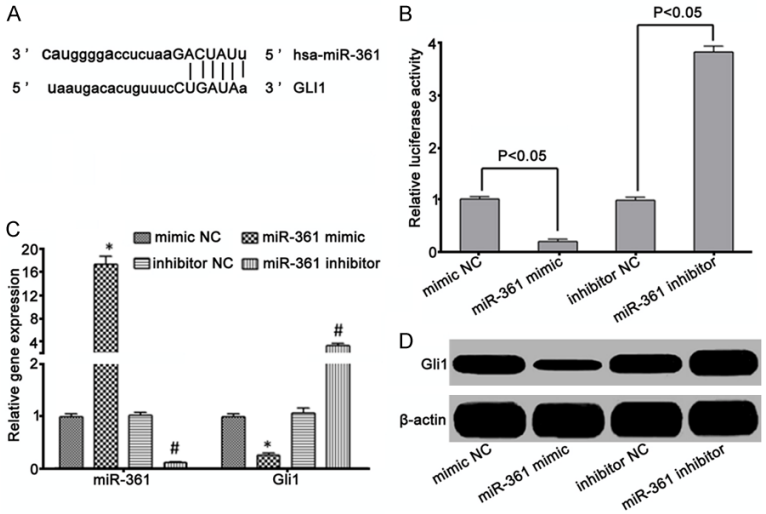

Figure 2. Targeted inhibition on Gli1 expression by miR-361. A: Binding sites between miR-361 and 3'-UTR of Gli1 mRNA. B: Dual luciferase reporter gene assay. C: aRT-PCR for miR-361 and Gli1 expression. D: Western blot for Gli1 protein expression. *P<0.05 compared to mimic NC group; #P<0.05 compared to inhibitor NC group.

600 μL complete medium containing 10% FBS. After 48 h incubation, culture medium in the upper chamber was discarded. Unpenetrated cells were removed by sterilized cotton swabs, followed by methanol fixation for 30 min. The membrane was washed twice in PBS and air-dried, and was stained by 0.1% crystal violet for 20 min. Under the microscope, five randomly selected fields were counted for the number of cells penetrating the micro-pores and averaged.

Statistical analysis

SPSS18.0 software was used for data analysis. Measurement data were presented as mean ± standard deviation (SD), and were compared by student t-test. A statistical significance was defined when P<0.05.

Results

Expressional profiles of Gli1 and miR-361 in PCa

qRT-PCR results showed significantly elevated Gli1 mRNA level in PCa tissues compared to BPH tissues (**Figure 1A**), whilst miR-361 expression was significantly lower (**Figure 1B**). T3~T4 phase PCa patients had significantly higher Gli1 mRNA expression compared to T1~T2 phase patients, whilst miR-361 level was remarkably lower. Western blot test result showed significantly elevated PTEN protein expression in PCa tissues compared to BPH tissues (**Figure 1C**). Those with advanced pathology grade had higher PTEN expression than those at lower grade. These results suggested that abnormally decreased miR-361 expression might play a role in up-regulating Gli1 expression and facilitating prostate cancer pathogenesis.

miR-361 targeted and inhibited Gli1 expression

Online prediction of MicroRNA.org showed the existence of targeted binding sites between miR-361 and 3'-UTR of Gli1 mRNA (**Figure 2A**). The transfection of miR-361 mimic or miR-361 inhibitor significantly decreased or elevated relative activity of luciferase, indicating that miR-361 could targeted 3'-UTR of Gli1 gene (**Figure 2B**). The potentiation or inhibition of miR-361 expression remarkably decreased

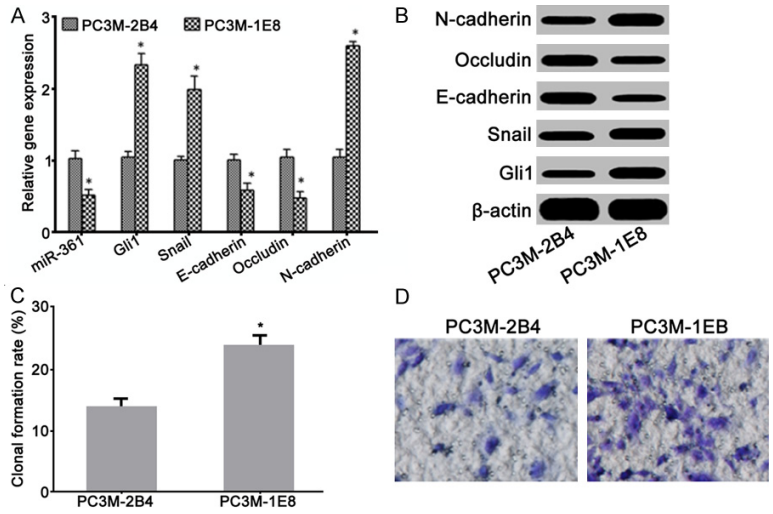

Figure 3. Correlation between miR-361/Gli1 expression and cell invasiveness. A: qRT-PCR for gene expression; B: Western blot for protein expression; C: Plate clonal formation assay; D: Transwell assay for cell invasion potency. *P<0.05 compared to PC3M-2B4 cells.

and increased Gli1 mRNA (**Figure 2C**) and protein (**Figure 2D**) expression in PC3M-1E8 cells.

Correlation between miR-361/Gli1 expression and cell invasion

qRT-PCR test showed significantly higher Gli1 and Snail expression in human highly metastatic PCa cell line PC3M-1E8 compared to low metastatic PCa cell line PC3M-2B4, whilst miR-361 expression was significantly lower (**Figure 3A**). Western blot results showed remarkably decreased E-cadherin and Occludin expression in PC3M-1E8 cells compared to PC3M-2B4 cells, accompanied with elevated N-cadherin expression, indicating that PC3M-1E8 cells had more potent EMT feature compared to PC3M-2B4 cells (**Figure 3B**). Moreover, clonal formation number (**Figure 3C**) and membrane perforated cell number (**Figure 3D**) in PC3M-1E8 cells were significantly higher than PC3M-2B4 line.

miR-361 regulated malignant growth and invasiveness of PC3M-1E8 cells via Gli1

The transfection of miR-361 mimic and/or si-Gli1 significantly depressed expression of Gli1, Snail, N-cadherin in PC3M-1E8 cells (**Figure 4A** and **4B**), and significantly elevated expression of E-cadherin and Occludin (**Figure 4A** and **4B**). Cell clonal formation was remarkably decreased (**Figure 4C**) whilst invasion potency was weakened (**Figure 4D**).

Discussion

As one urinary-reproductive tumor commonly occurred in aged males, over 90% of PCa patients were aging between 60 and 80 years old [23]. Various risk factors affect PCa pathogenesis. Due to different ethical groups, life habitat and diet factors, PCa incidence have significant geographic-specific distribution. China and other Asian countries have relatively lower incidence, whilst European and developed countries had higher rate of occurrence, which can be as high as 25-fold of the former regions [24]. It is surveyed that about 417000 patients in Europe were newly diagnosed with PCa, making it the third common tumor after colorectal carcinoma and breast cancer [25]. It is reported that mortality rate of PCa is the second high in Europe and Western developed countries, only lower than pulmonary carcinoma [26]. With aging of the whole society, plus life style and diet habit transition, PCa occurrence rate in China is also increasing by years, and has become the most popular malignant tumor in male urinary-reproductive system. Besides higher incidence, mortality rate of PCa is also relatively higher. In 2012, over 308000 people worldwide died from PCa (overall mortality = 69.5 per 100000). Among those developed countries had 142000 (mortality rate = 10.0 per 100000), and developing countries had 165000 deaths (mortality rate = 6.6 per 100000) [27].

Hedgehog signal pathway consists of extracellular signal ligand Hh, transmembrane protein receptor complex Patched (Ptch) and Smoothened (Smo), intermediate signal transducing molecule and downstream transcriptional factor Gli family, forming a Hh-Ptc-Smo-Gli process. Mammalian Hedgehog signal pathway includes three extracellular signal proteins, including Sonic hedgehog (Shh), Indian hedgehog (Ihh) and Desert hedgehog (Dhh), among which Shh ligand is the most potent one [28]. Under the direction of extracellular signal ligand Hh, it can bind with membrane receptor Ptch, and change the conformation of receptor com-

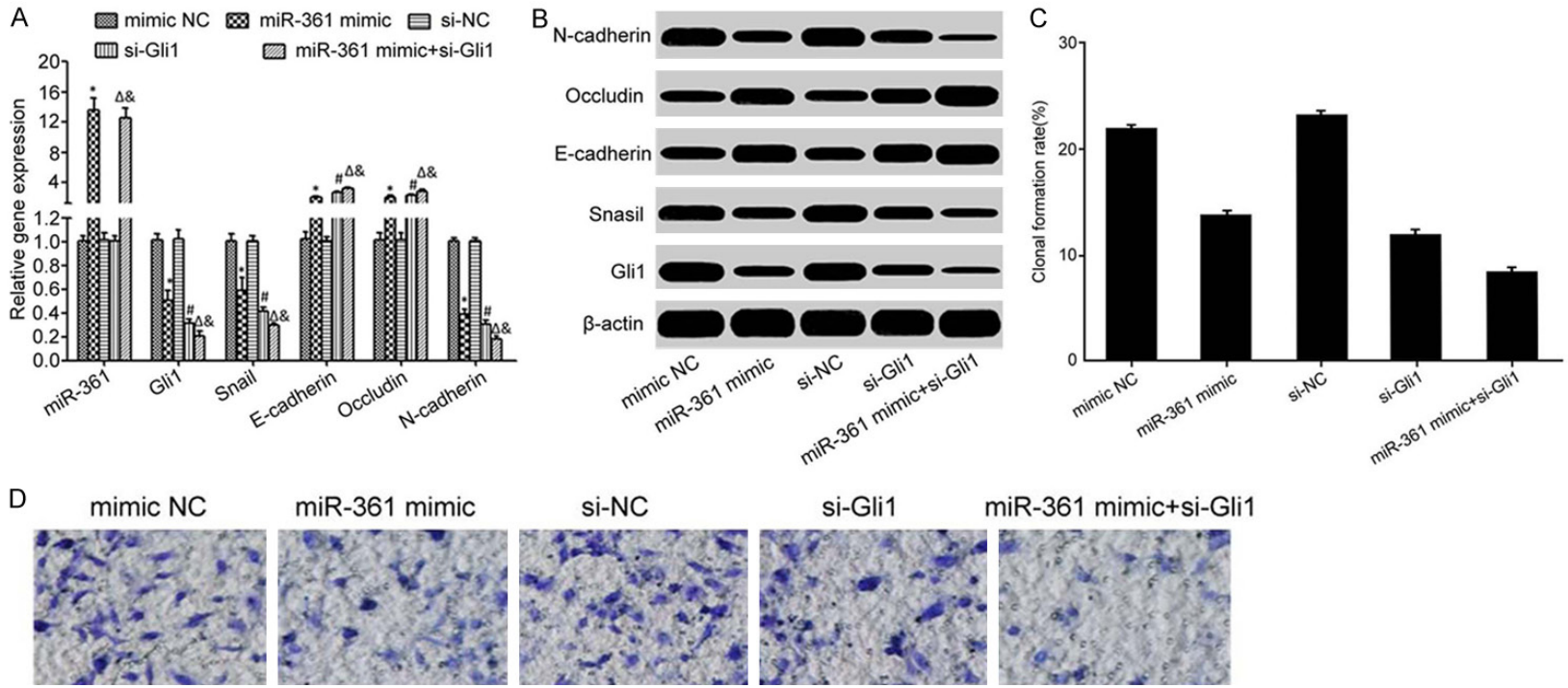

Figure 4. Regulation of malignant growth and invasion potency in PC3M-1E8 cells by miR-361 via Gli1. A: qRT-PCR for gene expression; B: Western blot for protein expression; C: Plate clonal formation assay; D: Transwell assay for cell invasion potency. *P<0.05 when comparing between miR-361 mimic and mimic NC group; #P<0.05 when comparing between si-Gli1 group and si-NC group; ΔP<0.05 when comparing between miR-361 mimic + si-Gli1 group and mimic NC group; &P<0.05 when comparing between miR-361 + si-Gli1 and si-NC group.

plex including Ptch and Smo, thus relieving the inhibition on Smo by Ptch. Smo can pass signals to transcriptional factor Gli via function of intermediate protein. Such activated Gli enter the nucleus to exert the role of transcriptional activator for facilitating transcription of target genes such as c-myc and VEGF, thus modulating various biological processes of cells [29]. Abnormal activation of Hedgehog signal pathway can promote transcription of multiple oncogenes such as c-myc [30] and EIF5A2 [31], leading to cell transformation and malignancy, causing tumor pathogenesis [32, 33]. Gli1 is one member of Gli transcriptional factor family (including Gli1, Gli2 and Gli3), and can exert regulatory role on transcription of nuclear target gene via its differential lengths [34]. Previous studies showed the correlation between Gli1 expression or function abnormality with PCa pathogenesis [18, 19]. It is also reported that miR-361 expression level was significantly down-regulated in PCa tumor tissues [21, 22]. Bioinformatics analysis revealed satisfactory targeted complementary relationship between miR-361 and 3'-UTR of Gli1. This study thus investigated if miR-361 and Gli1 dysregulated expression played a role in regulating metastasis and migration of PCa.

Our results showed significantly higher Gli1 mRNA and protein expression in PCa tissues compared to BPH tissues, with further higher expression level in advanced pathology grade tissues. Expression of miR-361, however, was remarkably lower in PCa tissue compared to BPH tissues. Results indicated that abnormal down-regulation of miR-361 could play a role in elevating Gli1 expression and facilitating PCa pathogenesis. Sanchez et al found significantly higher expression of Gli1, Gli2 and Gli3 in PCa tumor tissues compared to BPH tissues [19]. This study observed significantly elevated Gli1 expression in PCa tissues, as consistent with Sanchez et al results [19]. Guzel et al found lower miR-361 expression in PCa tissues compared to BPH samples [22]. Liu et al found the possible tumor suppressor gene role of miR-361 in occurrence of PCa [21]. This study observed significantly lowered miR-361 in PCa tissues, as consistent with Guzel et al [22] and Liu et al [21] results. LRGA assay showed that the transfection of miR-361 mimic or miR-361 inhibitor significantly up-regulated or down-regulated relative luciferase activity of HEK293 cells and Gli1 expression in PC3M-1E8 in PC3M-1E8 cells, demonstrating that Gli1 was the target gene of miR-361 and was under its regulation. EMT is one biological process referring to the transformation of epithelial-like cells into mesenchymal like cells, and plays important roles in embryonic development, tissue reconstruction, tumor metastasis and organ fibrosis. The decreased expression of cell adhesion molecules such as E-cadherin or cell tight junction protein Occludin, and elevated N-cadherin expression were all important features during EMT process [35]. Occurrence of EMT is one initiating step for tumor cells to acquire mobility and motility, and facilitating their invasion and metastasis [3]. Previous study showed the involvement of Hedgehog signal pathway in regulating EMT process of tumor cells [36], indicating its correlation with tumor invasion and metastatic properties. Previous study showed that Gli1 could affect expressions of important regulatory molecules during EMT process, such as E-cadherin, Occludin and N-cadherin via regulating Snail expression, thus perhaps participating in tumor cell EMT regulation [37]. Karhadkar et al found significantly higher Gli1 expression in non-metastatic PCa tissues compared to non-metastatic Pca tissues [38], indicating the participation of Gli1 up-regulation in facilitating the acquirement of invasiveness of PCa. Our results showed significantly higher Gli expression in highly metastatic PCa cell line PC3M-1E8 compared to those in low metastatic PCa cell line PC3M-2B4, indicating the possible correlation between Gli1 expression level and invasive potency of PCa cells. Therefore, this study further tested the role of miR-361 and PCa invasiveness property along with the role of Gli1. Results showed that the transfection of miR-361 mimic and/or si-Gli1 significantly inhibited Snail and N-cadherin expression in PC3M-1E8 cells, whilst elevated E-cadherin and Occludin protein expression, and inhibited invasiveness and malignant growth of PCa cells simultaneously.

Conclusion

MiR-361 could weaken EMT process via targeted inhibition on Gli1 expression, thus compromising invasiveness of PCa tumor tissues.

Disclosure of conflict of interest

None.

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