

miR-138 modulates prostate cancer cell invasion and migration via Wnt/ β -catenin pathway

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Received August 14, 2016; Accepted May 22, 2017

DOI: 10.3892/mmr.2017.8273

Abstract. The prognosis for prostate cancer patients with distant metastasis is poor, with an average survival rate of 24-48 months. The exact mechanisms underlying prostate cancer metastasis remain to be elucidated, despite previous research efforts. The present study aimed to reveal the regulatory roles of miR-138 via Wnt/ β -catenin pathway in prostate cancer cell migration and invasion. Reverse transcription-quantitative polymerase chain reaction was used to examine the mRNA and protein expression levels and transwell assay was conducted to determine cell invasion and migration. A luciferase reporter assay was used to determine the target association between miR-138 and β -catenin. The present study identified microRNA (miR)-138 as an invasion and migration regulator in prostate cancer. miR-138 was downregulated in aggressive prostate cancer cell lines. Furthermore, following miR-138 overexpression, prostate cancer cells exhibited impaired invasive and migratory abilities. E-cadherin was upregulated and vimentin was downregulated. In addition, it was demonstrated that miR-138 negatively regulated the Wnt/ β -catenin pathway activation in prostate cancer. The pathway was then activated via β -catenin overexpression and this reversed the effects of miR-138. The results suggest that miR-138 downregulation may contribute to prostate cancer progression and metastasis. The findings provide a novel molecular therapeutic target in the treatment of prostate cancer metastasis.

Introduction

Prostate cancer is the most frequently occurring cancer and the second leading cause of cancer-associated mortalities in men in the United States. In 2014, estimated newly diagnosed

cases and fatalities from the disease were 233,000 and 29,480, respectively (1). The rate of tumor growth varies from very slow to moderately rapid, and various patients may have prolonged survival even following cancer metastasis to distant sites, including bone (2,3). The 5-year relative survival rate for men diagnosed in the United States from 2001-2007 with local or regional disease (where tumors have not metastasized from their point of origin) was 100%, and the rate for distant disease (where tumors that have metastasized to further sites around the body) was 28.7%. The great survival rate gap between local or regional tumors and advanced stages of the disease where the tumors have metastasized, is of primary concern, and has resulted in various research efforts to investigate the metastatic process of the disease and the mechanisms underlying it. Considerable progress has been made in the last decade to understand the disease at the molecular and genetic level (4,5), however understanding of the process of metastasis remains to be fully elucidated.

miRNAs are non-protein-coding RNAs that regulate genes and genomes. This regulation may occur at various important levels of genome functioning, including chromatin structure, chromosome segregation, transcription, RNA processing, RNA stability and translation (6). miRNAs may regulate the expression of up to 90% of human genes (7). They bind by complementary base pairing to the 3'-untranslated region (3'-UTR) of their target mRNA to post-transcriptionally suppress gene expression (8).

The downregulation of miR-138 may be associated with cancer progression, and the tumor suppressive role has been reported in lung, kidney, tongue, head and neck malignant diseases (9-12). miR-138 modulates tumor growth, migration and invasion. The molecular mechanism underlying the functions of miR-138 remain to be elucidated, despite current research efforts. Furthermore, whether miR-138 exhibits the ability to act as a tumor suppressor in prostate cancer is as of yet unknown.

The present study aimed to investigate the function of miR-138 in prostate cancer and to identify the associated molecular mechanism by which it may exhibit tumor suppressive effects. It was demonstrated that miR-138 was downregulated in PC3 and C4-2B aggressive prostate cancerous cell lines, compared with cancerous cell lines that are less metastatic. Furthermore, following miR-138 overexpression, PC3 and C4-2B cell lines exhibited impaired invasion and migration. Additionally, E-cadherin was upregulated and vimentin was

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Key words: microRNA-138, β -catenin, metastasis, prostate cancer

downregulated. It was revealed that miR-138 overexpression suppressed β -catenin activation, and inhibition of the activation of the β -catenin pathway reversed the anti-tumor effect of miR-138. These results suggest that miR-138 is a tumor suppressor in prostate cancer and its associated anti-tumor effect is dependent on β -catenin pathway inhibition.

Materials and methods

Cell lines and transfection. PC3, DU145 and LNCaP cells were obtained from Type Culture Collection of Chinese Academy of Sciences (Shanghai, China); PC3 is an aggressive prostate cancerous cell line whereas DU145 and LNCaP cells are non-aggressive prostate cancerous cell lines. The cells were maintained in F-12, modified Eagle's medium and RPMI-1640 (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc., USA) and penicillin/streptomycin (Gibco; Thermo Fisher Scientific, Inc.). The cells were maintained in an incubator containing 5% CO₂ at 37°C and 100% humidity. C4-2B cell lines were purchased from UroCor, Inc. (Shanghai, China) and maintained in RPMI-1640 supplemented with 10% FBS. miR-138 mimics (GCCGGACUAAGUGUUGUGGUCGA; 50 nM) were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany) and β -catenin overexpression plasmid (100 nM) was obtained from Addgene Inc. (Cambridge, MA, USA), (Plasmid 17,198). Transient transfection was performed using the Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol.

Invasion and migration assays. Assays were performed in BioCoat transwell chambers (BD Pharmingen; BD Biosciences, Franklin Lakes, NJ, USA) with uncoated porous filters (pore size 8 μ m) to evaluate cell migration, or Matrigel coated porous filters to exam cell invasion. C4-2B and PC3 cells (5 \times 10⁵/ml) in serum-free medium were seeded into the upper chamber of each insert, and complete medium was added to the lower chamber. Following 12 h of incubation at 37°C, cells in the upper chambers were removed using cotton tips. Filters were fixed in 4% paraformaldehyde and stained at room temperature for 24 h with 0.1% crystal violet (Sigma-Aldrich; Merck KGaA) for counting. Independent experiments were repeated at least three times. Values for cell migration or invasion are expressed as the average number of cells per microscopic field (CX23 microscope; Olympus Corporation, Tokyo, Japan) over five fields for each filter.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from C4-2B and PC3 cells using TRIzol (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The relative expression levels of miR-138 were determined by RT-qPCR using mirVana™ qPCR microRNA Detection kit, according to the manufacturer's protocol. An Applied Biosystems® 7500 thermal cycler, (Thermo Fisher Scientific, Inc.) was used and the reaction conditions were as follows: 95°C for 10 min, 40 cycles at 95°C for 15 sec and 60°C for 30 sec. For the PCR reaction, 1 μ l cDNA solution, 10 μ l PCR master mix, 2 μ l of primers and 5 μ l H₂O were mixed to obtain a final reaction

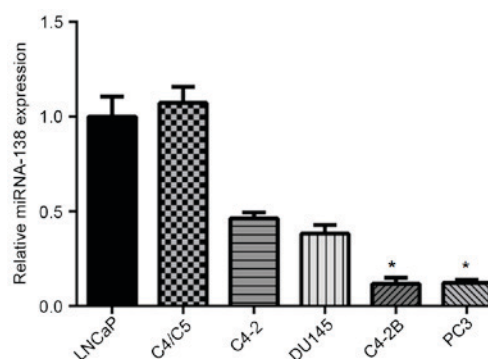


Figure 1. miR-138 is downregulated in aggressive prostate cancer cell lines. miR-138 expression in six prostate cancer cell lines was analyzed using reverse transcription-quantitative polymerase chain reaction *P<0.01 vs. LNCaP, C4/C5, C4-2 and DU145 cell lines. Data is presented as the mean \pm standard deviation, n=3. miR-138, microRNA-138.

volume of 20 μ l. Specific primer sets for miR-138 and U6 were obtained from Genecopoeia (Applied Biosystems; Thermo Fisher Scientific, Inc.). The mRNA expression levels of LIMK1 were detected by RT-qPCR using the standard SYBR-Green RT-PCR kit (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Primers were synthesized by Invitrogen (Thermo Fisher Scientific, Inc.) were as follows: miR-138; sense 5'-GCGAAC TGTTTGACAG AGG-3', antisense 5'-CAGTGCCTGTCTGTGG AGT-3'; U6 forward, 5'-TGCGGGTGCTCGCTTCGGCAGC-3' and reverse, 5'-CCAGTGCAGGGTCCGAGGT-3' and GAPDH forward, 5'-TGTGGGCATCAATGGATTGG-3' and reverse, 5'-ACACCATGTATTCCGGGTCAAT-3'. The relative expression levels miR-138 were quantified using GraphPad Prism 5.0 software (GraphPad Software, Inc., San Diego, CA, USA), and the 2- $\Delta\Delta$ Cq method (13).

Immunoblotting assay. Whole-cell extracts were obtained by lysis of cells in an appropriate volume of ice-cold radioimmunoprecipitation assay buffer (Thermo Fisher Scientific, Inc.). The BCA Protein Assay kit (Thermo Fisher Scientific, Inc.) was used to determine protein concentration. Protein samples (20 μ g) were separated by 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and were transferred to a polyvinylidene difluoride (PVDF) membrane (Thermo Fisher Scientific, Inc.) and blocked in phosphate-buffered saline/Tween-20 containing 5% non-fat milk for 1 h at room temperature. Subsequently, the PVDF membrane was incubated with mouse anti-E-cadherin antibody (ab1416; 1:200; Abcam, Cambridge, UK), mouse anti-vimentin antibody (ab8978; 1:200; Abcam), mouse anti- β -catenin antibody (ab22656; 1:200; Abcam), mouse anti-active- β -catenin antibody (05-665; 1:200; Sigma-Aldrich; Merck KGaA), anti-lamin B1 (ab8982; 1:200; Abcam) and mouse anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) monoclonal antibody (ab8245; 1:50; Abcam) at 4°C overnight. The membranes were washed with TBS containing 0.1% Tween-20, and incubated with a corresponding horseradish peroxidase conjugated secondary antibody (ab6789; 1:3,000; Abcam) at 37°C for 1 h. Following extensive washing, proteins were visualized by enhanced chemiluminescence (ab5801; Abcam) and exposure to film (Fujifilm, Tokyo, Japan).

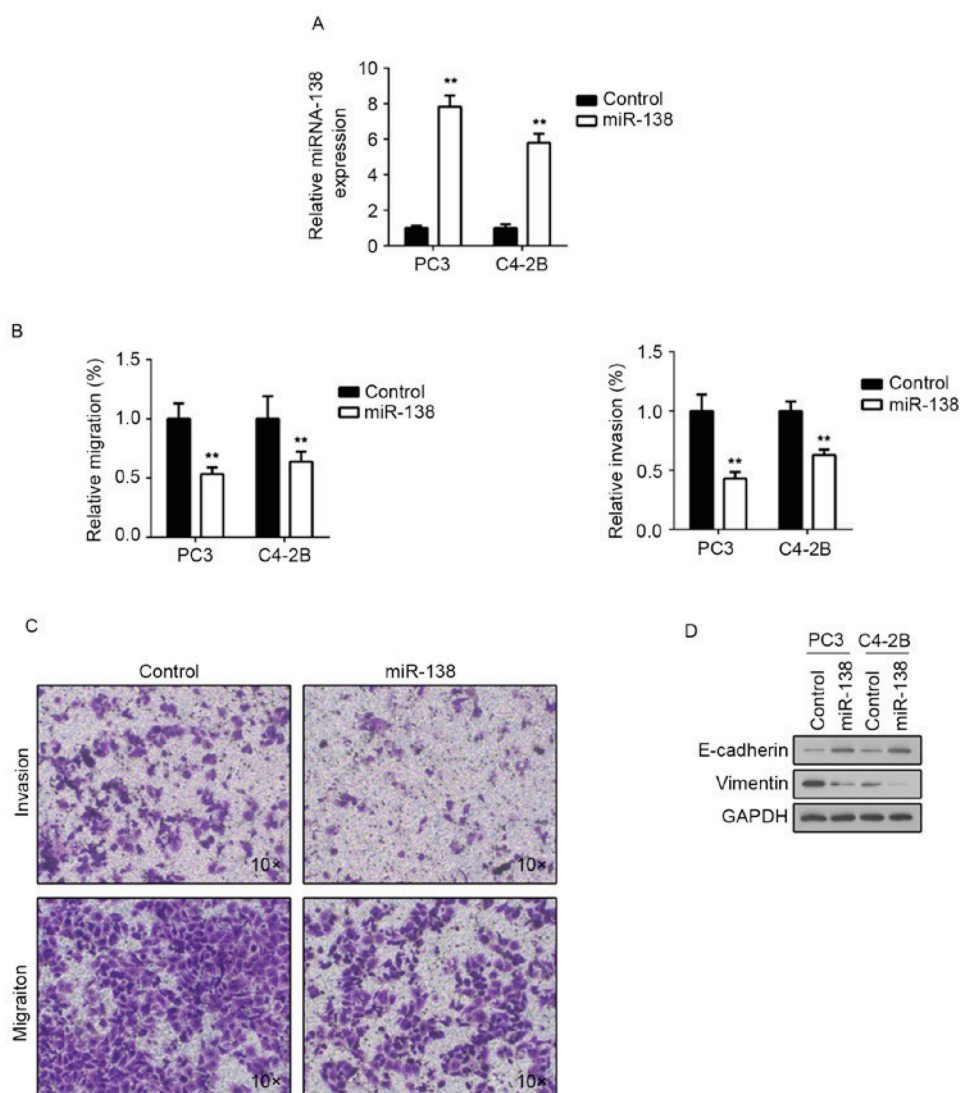


Figure 2. miR-138 negatively regulates invasion and migration in prostate cancer cells. (A) PC3 and C4-2B cells were transfected with miR-138 mimics or control miRNA. Following a 24 h incubation period, miR-138 expression was measured by reverse transcription-polymerase chain reaction. (B) PC3 and C4-2B cells were transfected with miR-138 mimics or control miRNA, then were subjected to migration and invasion assays. (C) Representative images of PC3 cells in migration and invasion assays. (D) Expression of E-cadherin or vimentin was detected by an immunoblotting assay in PC3 and C4-2B cells transfected with miR-138 mimics or control miRNA. Data is presented as the mean \pm standard deviation, n=3. **P<0.01 vs. control. miR-138, microRNA-138.

TOP/FOP Luciferase reporter assay. Cells were transiently transfected with 1 μ g of a constitutively active vector encoding Renilla luciferase (Promega Corporation, Madison, WI, USA) and 10 μ g of β -catenin-responsive firefly luciferase reporter plasmid TopFlash (EMD Millipore, Billerica, MA, USA) or the negative control FopFlash (EMD Millipore) using the Lipofectamine 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Cells were harvested following 24 h in culture and firefly and Renilla luciferase activity was measured using a Dual-Luciferase Reporter Assay System (Promega Corporation) according to the manufacturer's protocol. The firefly luciferase activity was normalized against the Renilla luciferase activity and fold increase in TOPFlash activity compared to FOPFlash is reported.

Statistical analysis. Data are presented as the mean \pm standard deviation. Analysis was performed using SPSS software version 20.0 (IBM Corp, Armonk, NY, USA). All data were

assessed using the Student's t-test or one-way analysis of variance followed by Newman Keuls post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-138 is downregulated in aggressive prostate cancer cell lines. To determine the role of miR-138 in prostate cancer, the present study firstly investigated miR-138 expression in six prostate cancer cell lines (LNCaP, C4/C5, C4-2, DU145, C4-2B and PC3) using RT-qPCR. It was demonstrated that miR-138 expression was associated with the aggressiveness, or, metastatic capabilities of tumor cell lines. In the prostate cancer cell lines C4-2B and PC3 with metastatic abilities, miR-138 expression levels were significantly decreased, compared with LNCaP, C4/C5, C4-2 and DU145 cell lines (Fig. 1). These results suggested that miR-138 expression may be associated with prostate cancer progression, and miR-138 may function as a tumor suppressor.

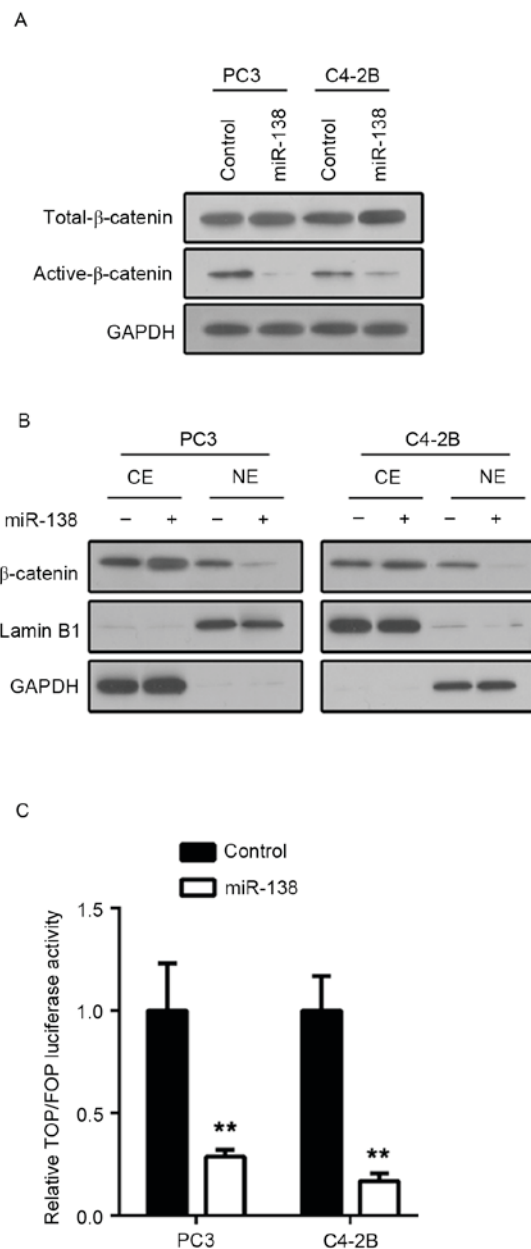


Figure 3. miR-138 negatively regulates β -catenin activation in prostate cancer cells. (A) Active form of β -catenin in PC3 and C4-2B cells transfected with miR-138 mimics or control miRNA was analyzed by immunoblot assay. (B) Nuclear protein and cytoplasmic protein were extracted and analyzed for β -catenin by immunoblot assay. (C) Lymphoid enhancer factor/T-cell factor transcription activity in PC3 and C4-2B cells transfected with miR-138 mimics or control miRNA was analyzed by TOP/FOP Luciferase reporter assay. Data is presented as the mean \pm standard deviation, $n=3$. ** $P<0.01$ vs. control. miR-138, microRNA-138; NE, nuclear extract; CE, cytoplasmic extract.

miR-138 negatively regulates invasion and migration in prostate cancer cells. To further investigate the miR-138 function in prostate cancer, the present study transfected miR-138 mimics into C4-2B and PC3 cells. As presented in Fig. 2A, following transfection, miR-138 expression was significantly upregulated in the two cell lines. A Transwell assay was then performed to examine whether there was an invasive or migratory alteration following miR-138 overexpression. It was demonstrated that C4-2B and PC3 cells transfected with miR-138 mimics exhibited impaired invasion and migration (Fig. 2B and C),

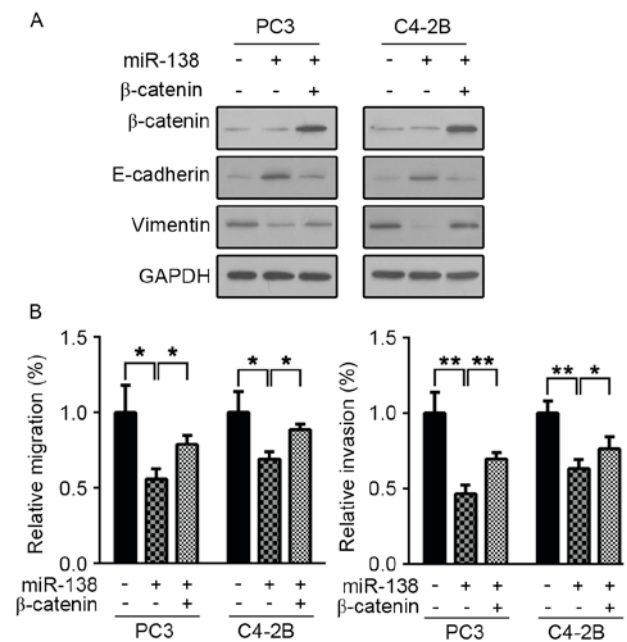


Figure 4. miR-138 tumor suppressor role is dependent on Wnt/ β -catenin pathway inhibition. PC3 and C4-2B cells were transfected with miR-138 mimics, control miRNA alone or miR-138 and β -catenin overexpression together. A total of 48 h following this, (A) expression of β -catenin, E-cadherin or vimentin was detected by immunoblot assay. (B) Assays for cell migration (left) or invasion (right) were performed. Data is presented as the mean \pm standard deviation, $n=3$. * $P<0.05$; ** $P<0.01$. miR-138, microRNA-138.

compared with transfected control miRNA. Epithelial marker E-cadherin expression was then investigated, and it was revealed that miR-138 overexpression induced E-cadherin expression in the two cell lines (Fig. 2D). Furthermore, expression of the mesenchymal marker vimentin was investigated, following miR-138 overexpression. Consistent with E-cadherin alteration, vimentin expression was downregulated (Fig. 2D). These results suggested that miR-138 may participate in tumor metastasis regulation.

miR-138 negatively regulates β -catenin activation in prostate cancer cells. The Wnt/ β -catenin pathway is involved in tumor metastasis (14). To further investigate whether miR-138 affects the Wnt/ β -catenin pathway in prostate cancer, the present study examined the Wnt/ β -catenin pathway activation status. β -catenin dephosphorylation and translocation to the nucleus are the core events in Wnt/ β -catenin pathway activation. β -catenin phosphorylation status was investigated. It was demonstrated that the active form of β -catenin (dephosphorylated) was downregulated in C4-2B and PC3 cells transfected with miR-138 mimics (Fig. 3A). To determine β -catenin localization, nuclear protein and cytoplasmic proteins from C4-2B and PC3 cell lines transfected with either miR-138 mimics or control mimics were extracted. Then, β -catenin expression was examined by immunoblot assay. In accordance with previous results, nuclear localization of β -catenin in C4-2B and PC3 cells transfected with miR-138 mimics was downregulated compared with controls (Fig. 3B). Furthermore, lymphoid enhancer factor/T-cell factor (LEF/TCF) transcription activity was examined via luciferase activity assay. Following miR-138 overexpression, C4-2B and PC3 cells indicated weakened

LEF/TCF transcription activity (Fig. 3C). These results suggested that miR-138 suppresses the Wnt/ β -catenin pathway in prostate cancer.

miR-138 tumor suppressor role is dependent on Wnt/ β -catenin pathway inhibition. To further investigate whether the miR-138 anti-tumor effects were Wnt/ β -catenin pathway dependent, β -catenin was overexpressed in C4-2B and PC3 cells, to activate the Wnt/ β -catenin signaling pathway. It was then examined whether miR-138 overexpression induced an anti-tumor effect. As presented in Fig. 4A, β -catenin was overexpressed efficiently in C4-2B and PC3 cells. β -catenin overexpression restored C4-2B and PC3 cell invasion and migration capacity (Fig. 4B). Furthermore, E-cadherin and vimentin expression were examined following β -catenin overexpression. In accordance with previous results, the expression levels of the two proteins were restored (Fig. 4B). These results suggested that the miR-138 tumor suppressor role is dependent on Wnt/ β -catenin pathway inhibition.

Discussion

The acquired capability of tumor cells to exhibit tissue invasion and metastasis has been defined as a 'hallmark of cancer' (15,16). Localized prostate tumors may be treated and do not frequently result in patient mortality. However, patients with prostate cancer may develop metastatic tumors in different organs, including bone, lung, brain and liver which may be fatal (3,17). Therefore, understanding the process of prostate cancer metastasis is of primary concern, and a prerequisite for improving prostate cancer therapeutics.

The present study revealed a novel invasion and migration regulator in prostate cancer. miR-138 was downregulated in aggressive tumor cell lines compared with those which were less aggressive in their metastatic capabilities. miR-138 overexpression induced impaired invasion and migration in C4-2B and PC3 cells. Notably, E-cadherin and vimentin, the epithelial mesenchymal transition (EMT) markers, were additionally regulated by miR-138. EMT is a process where epithelial cells acquire mesenchymal characteristics with defined morphology, protein expression and gene signatures. This process has been reported in a wide range of cancers and enables malignant cells to acquire the ability of invasion and migration (18-21). EMT has additionally been reported in prostate cancer (22-25). The results of the present study suggested that miR-138 may be an EMT regulator and therefore regulate invasion and migration in prostate cancer.

Wnt/ β -catenin pathway activation has been widely reported in prostate cancer and its aberrant activation may participate in numerous processes associated with tumor progression (26-28), including invasion and migration. The results demonstrated that miR-138 overexpression significantly inhibited the activation of the Wnt/ β -catenin pathway in C4-2B and PC3 cells. The active form of β -catenin was downregulated in C4-2B and PC3 cells transfected with miR-138 mimics. β -catenin localization in the nucleus was additionally reduced. Furthermore, miR-138 overexpression weakened LEF/TCF transcription activity. Therefore, the present study reported a novel Wnt/ β -catenin pathway inhibitor. Downregulation of miR-138 in prostate cancer may

contribute to the aberrant activation of the Wnt/ β -catenin pathway in the disease. However, further investigation is necessary in order to verify if miR-138 is downregulated and elucidate the underlying molecular mechanisms regarding the association between the downregulation of miR-138 and the Wnt/ β -catenin pathway activation in prostate cancer.

The results revealed the importance of Wnt/ β -catenin signaling in prostate cancer. Identification of these therapeutic targets may be of clinical relevance in the future. These results indicate that drugs and inhibitors targeting this pathway may have clinical potential in the treatment of prostate cancer. Various inhibitors are currently at developmental stages (29-32), however require further evaluation regarding their role in prostate cancer.

In conclusion, the present study demonstrated a novel mechanism that regulates invasion and migration in prostate cancer. miR-138 acts as a Wnt/ β -catenin pathway inhibitor to maintain prostate cancer cell epithelial status. However, the exact targets of miR-138 and its application in tumor therapy require further investigation.

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