lncRNA MIR4435-2HG promotes cancer cell migration and invasion in prostate carcinoma by upregulating TGF-β1

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Abstract. The long noncoding (lnc) RNA MIR4435-2HG is known to promote lung cancer; however, its role in prostate carcinoma (PCa) remains unknown. The aim of the current study was therefore to investigate the role of MIR4435-2HG in PCa by detecting differential gene expression using quantitative PCR and ELISA kits. Furthermore, overexpression experiments were performed to analyze gene interactions and Transwell assays were used to analyze cell invasion and migration. The present study demonstrated that plasma levels of MIR4435-2HG and transforming growth factor-β1 (TGF-β1) were significantly higher in patients with PCa compared with healthy controls. Furthermore, MIR4435-2HG and TGF-β1 plasma levels were positively correlated in patients with PCa, but not in healthy controls. The results from the follow-up study suggested that MIR4435-2HG was closely associated with patient survival. MIR4435-2HG overexpression and treatment with TGF-β1 promoted cancer cell invasion and migration. In addition, TGF-β inhibitor attenuated the enhancing effects of MIR4435-2HG overexpression on cell invasion and migration. MIR4435-2HG overexpression led to upregulation of TGF-β1 expression, whereas TGF-β1 treatment had no effect on MIR4435-2HG expression. These results suggested that MIR4435-2HG may promote PCa by upregulating TGF-β1.

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Introduction

Prostate carcinoma (PCa) is a common male malignancy and a major cause of cancer-associated mortality worldwide (1). An increased incidence of PCa has been observed currently, due to improved life expectancy, lifestyle changes and the existence of cancer screening programs (2). Despite efforts made in the treatment of PCa, the overall 5-year survival rate remains <30% (3), which is mainly due to the lack of radical treatment for metastatic tumors that is commonly observed in patients with PCa at the time of first diagnosis (4). Current knowledge of PCa metastasis remains limited (5). Investigation of the underlying molecular mechanism of PCa metastasis is therefore required.

Transforming growth factor-β (TGF-β) is a well-established signal transduction pathway in the human body. The TGF-β signaling pathway is involved in numerous cellular activities, including cell growth, proliferation, differentiation, apoptosis and homeostasis in developing embryos and adult organisms (6). The TGF-β signaling pathway has a dual role in cancer development as it can inhibit cancer cell proliferation in the early stages, but also promote tumor metastasis through epithelial-mesenchymal transition in the later stages (7-9). It has been reported that the TGF-β signaling pathway can be regulated in several types of cancer (such as breast cancer) by a considerable number of long noncoding RNAs (lncRNAs) (10). lncRNAs represent a group of non-protein-coding RNA transcripts that have pivotal roles in cancer (11). In particular, it has been demonstrated that the lncRNA MIR4435-2HG promotes lung cancer by interacting with β-catenin signaling, which has been shown to crosstalk with TGF-β (12). However, its role in PCa remains unknown. The present study demonstrated that MIR4435-2HG promoted PCa potential through the TGF-β1 signaling pathway.

Materials and methods

Research subjects. A total of 68 patients with PCa who were admitted to The First Affiliated Hospital of Guangzhou University of Chinese Medicine between January 2011 and

January 2013, and 62 healthy volunteers, were enrolled in the present study. A 5-year follow-up was performed on all patients with PCa following discharge. The inclusion criteria were as follows: i) Patients with PCa that was confirmed by pathological biopsy; and ii) patients who had not been treated prior to admission. The exclusion criteria were as follows: i) Patients with PCa who were diagnosed with other diseases; ii) patients who had received any treatments prior to this study; iii) patients who were transferred to other hospitals during treatment; and iv) patients who succumbed to an unrelated cause or clinical disorder during follow-up. All patients received surgical resection and/or radiation and chemotherapy according to their condition. The 62 healthy volunteers received systemic physiological examinations at the same hospital and all indexes were within the normal range. Healthy volunteers with a previous history or family history of malignancies were included. The 62 healthy volunteers were selected to match the age distribution of the patient group. The age of the 68 patients with PCa was in the range of 40-76 years old with a mean age of 56.5±5.8 years. According to the American Joint Committee on Cancer (AJCC) staging system (13), 11, 19, 20 and 18 patients had stage I, II, III and IV PCa, respectively. The age of the 62 controls was in the range of 42-74 years old with a mean age of 57.0±6.2 years. No significant differences in age or other basic clinical data, including the body mass index and disease history, were found between the patient and control groups. The present study was approved by the Ethics Committee of The First Affiliated Hospital of Guangzhou University of Chinese Medicine prior to patient admission. Written informed consent was collected from all patients with PCa and healthy volunteers.

Specimens and cell lines. Blood samples (5 ml) were extracted from the elbow vein of all fasting participants on day 1 following admission. Plasma samples were obtained using conventional methods and were stored in liquid nitrogen prior to further experiments. The 22Rv1 human PCa cell line was used for all *in vitro* experiments and was purchased from the American Type Culture Collection. Cells were cultured in DMEM supplemented with 10% FBS and placed at 37°C in a humidified incubator containing 5% CO_2. For experiments involving TGF-β1, cells were treated with exogenous TGF-β1 (Sigma-Aldrich; Merck KGaA) at 5, 10 and 20 ng/ml at 37°C for 24 h before use.

ELISA. Plasma levels of TGF-β1 were measured using a human TGF-β1 ELISA kit (cat. no. ab108912; Abcam), according to the manufacturer's instructions.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from plasma and 22Rv1 cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) to detect MIR4435-2HG. cDNA was synthesized using Reverse Transcriptase AMV (Sigma-Aldrich; Merck KGaA) using the following conditions: 5 min at 25°C, 25 min at 52°C and 10 min at 80°C. PCR reaction systems were prepared using the Applied Biosystems PowerUp™ SYBR™ Green Master Mix (Thermo Fisher Scientific, Inc.). The PCR reaction conditions were as follows: 1 min at 95°C, followed by 10 sec at 95°C, 30 sec at 55°C and 40 sec at 72°C. The primers for MIR4435-2HG and the endogenous control GAPDH were synthesized by Sangon Biotech Co., Ltd. and were designed as

follows: MIR4435-2HG forward, 5'-CGGAGCATGGAACTC GACA-3' and reverse, 5'-CAAGTCTCACACATCCGGG-3'; and GAPDH forward, 5'-AAGGTGAAGGTCGGAGTCA-3' and reverse, 5'-AATGAAGGGGTCATTGATG-3'. The relative expression levels of MIR4435-2HG were normalized to the GAPDH endogenous control and expressed as $2^{-\Delta\Delta Cq}$ (14).

Vectors and cell transfection. pcDNA3.1 vectors expressing MIR4435-2HG were constructed by Sangon Biotech Co., Ltd. Lipofectamine™ 2000 reagent (Thermo Fisher Scientific, Inc.) was used transfect 10 nM vectors into 10^5 cells. Cells were incubated with the vectors for 5 h and fresh culture medium was added. Cells were harvested 24 h following transfection for subsequent experiments. Control (C) cells were untransfected cells and negative control (NC) cells were cells transfected with empty vectors.

Transwell migration and invasion assay. Cells were harvested for Transwell migration and invasion assays only if the MIR4435-2HG overexpression rate was >200% (assessed by RT-qPCR). Briefly, cell suspensions were prepared in serum-free culture medium. In cases of TGF-β1 treatment, TGF-β1 (10 ng/ml) was added into the medium. Cell density was normalized to $5x10^4$ cells/ml, 0.1 ml cell suspension was transferred into the upper chamber of the Transwell (pore size, 8 μm), and DMEM supplemented with 10% FBS was added into the lower chamber. After 3 h at 37°C and 5% CO_2, cells in the upper chamber were stained for 15 min with 0.5% crystal violet (Sigma-Aldrich; Merck KGaA) at room temperature. For the invasion assay, Matrigel (cat. no. 356234; EMD Millipore) was used to coat the upper chamber at 37°C for 6 h and the steps described for the migration assay were performed. Stained cells were counted in five randomly-selected fields using a light microscope (magnification, x40).

Western blotting. 22Rv1 cells were lysed with RIPA solution (Thermo Fisher Scientific, Inc.) to extract the protein. Protein concentrations were measured using a bicinchoninic acid assay (Thermo Fisher Scientific, Inc.). Proteins were separated by 10% SDS-PAGE (30 μg per lane) and transferred onto polyvinylidene fluoride membranes. The membranes were blocked with 5% skimmed milk dissolved in FBS for 2 h at room temperature. The membranes were then incubated with the primary antibodies against TGF-β1 (1:1,600; cat. no. ab92486; Abcam) and GAPDH (1:1,400; cat. no. ab9485; Abcam) for 16 h at 4°C, followed by horseradish peroxidase-conjugated anti-rabbit immunoglobulin G secondary antibody (1:1,000; cat. no. MBS435036; MyBioSource) for 2 h at 24°C. Enhanced chemiluminescence reagent (Sigma-Aldrich; Merck KGaA) was used to detect the signal on the membrane. Data were analyzed via densitometry using Image J V1.34 software (National Institutes of Health) and normalized to the expression of the internal control (GAPDH).

Statistical analysis. Data are presented as the means ± standard deviation of three independent replicates. Comparisons between patients with PCa and healthy controls were performed by unpaired t-test. Comparisons amongst patients and cell transfection groups were analyzed by one-way ANOVA followed by Tukey's test. Correlations between

Table I. Correlation between MIR4435-2H levels and clinical characteristics of patients with prostate carcinoma.

Characteristics	Cases, n	High (n=31)	Low (n=37)	χ^2 value	P-value
AJCC stage					
I	11	6	5	0.46	0.93
II	19	8	11		
III	20	9	11		
IV	18	8	10		
Age, years					
≥55	36	17	19	0.08	0.77
<55	32	14	18		

AJCC, American Joint Committee on Cancer.
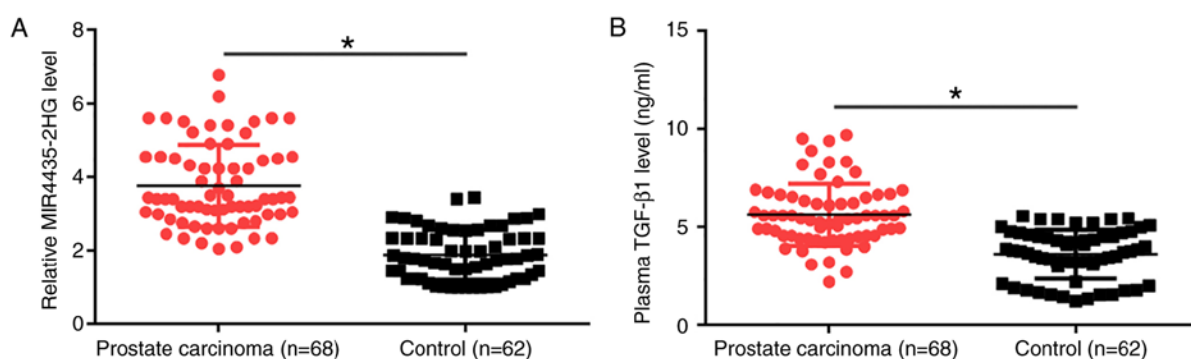
Figure 1. Plasma levels of MIR4435-2HG and TGF-β1 are upregulated in patients with PCa. The reverse transcription-quantitative PCR and ELISA results revealed that the plasma levels of (A) MIR4435-2HG and (B) TGF-β1 were significantly upregulated in patients with PCa compared with healthy controls. *P<0.05. TGF-β1, transforming growth factor-β1; PCa, prostate carcinoma.
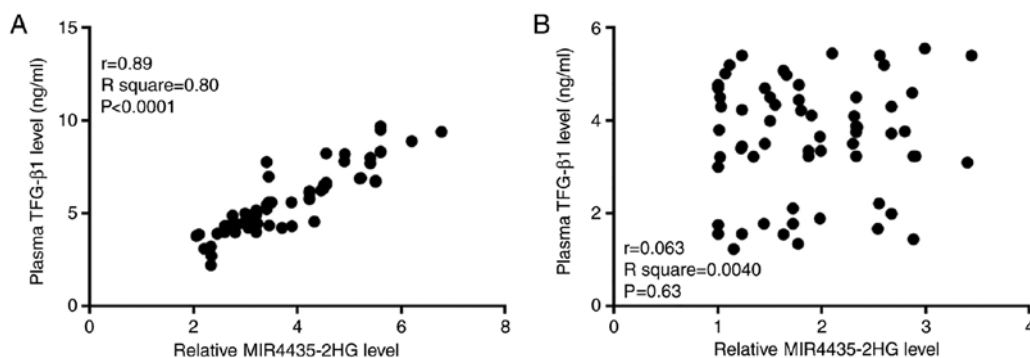

Figure 2. Plasma levels of MIR4435-2HG and TGF-β1 were positively correlated in patients with PCa, but not in the control group. Pearson's correlation coefficient was used to analyze the correlations between the expression levels of MIR4435-2HG and TGF-β1 in (A) patients with PCa and (B) controls. TGF-β1, transforming growth factor-β1.

plasma levels of TGF-β1 and MIR4435-2HG in patients with PCa and healthy controls were analyzed by Pearson's correlation coefficient. Patients were divided into high (n=31) and low (n=37) MIR4435-2HG plasma level groups based on Youden's index, an index used to define an optimized cut-off value (15). Kaplan-Meier analysis and the log-rank test were used to compare survival curves. The association between MIR4435-2H plasma levels and clinical characteristics of patients with PCa were analyzed by χ^2 test. P<0.05 was considered to indicate a statistically significant difference.

Results

Plasma levels of MIR4435-2HG and TGF-β1 are upregulated in patients with PCa. MIR4435-2HG and TGF-β1 plasma levels were measured by RT-qPCR and ELISA, respectively. Plasma levels of MIR4435-2HG (Fig. 1A) and TGF-β1 (Fig. 1B) were significantly upregulated in patients with PCa compared with healthy controls (P<0.05). In addition, MIR4435-2HG and TGF-β1 levels showed increasing trends with clinical stages (I-IV, data not shown); however,
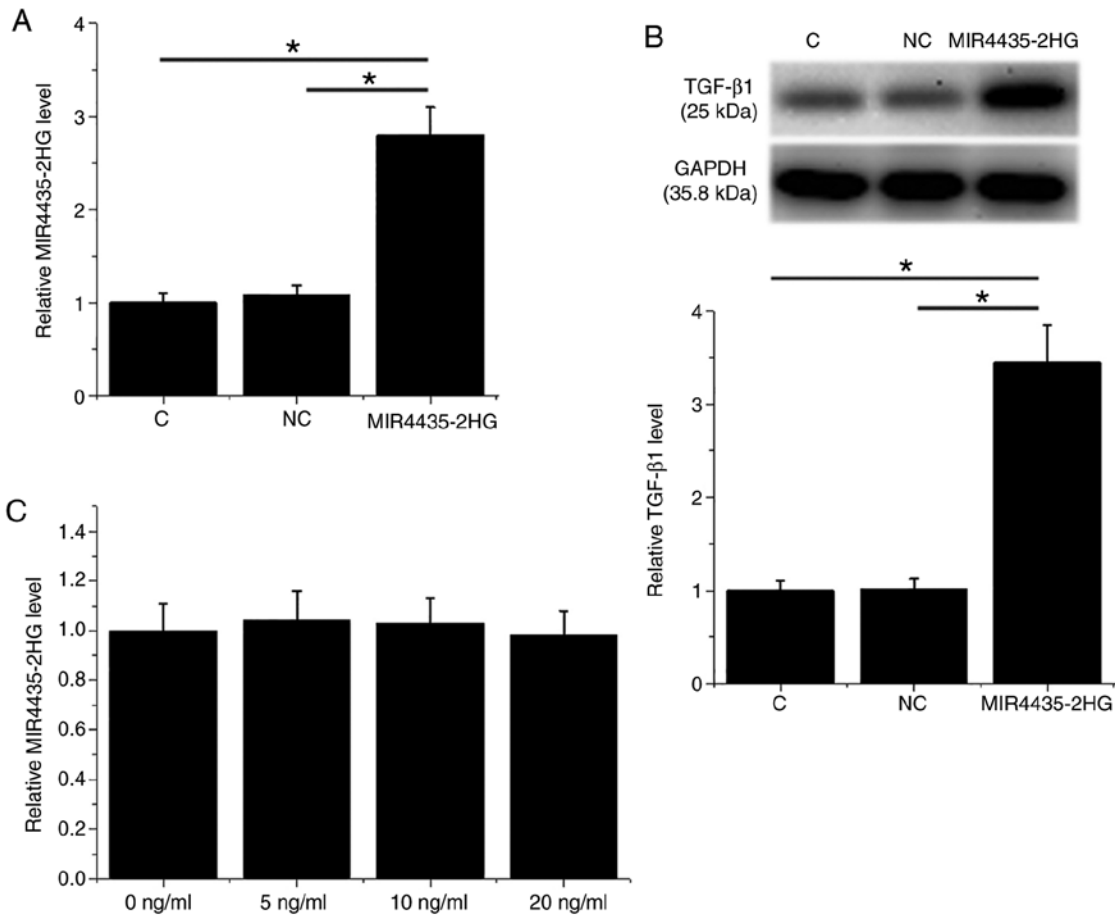
Figure 3. MIR4435-2HG overexpression induces TGF-β1 upregulation in the 22Rv1 cell line. Results from the reverse transcription-quantitative PCR demonstrated that (A) MIR4435-2HG overexpression was efficient in the 22Rv1 cell line following transfection. (B) Western blotting demonstrated that MIR4435-2HG overexpression induced TGF-β1 upregulation in the 22Rv1 cell line. (C) Treatment with exogenous TGF-β1 at 5, 10 and 20 ng/ml for 24 h had no effect on MIR4435-2HG expression. *P<0.05. C, control; NC, negative control; TGF-β1, transforming growth factor-β1.

these associations were not significant. The associations between MIR4435-2H levels and the clinical characteristics of patients with PCa were analyzed by χ^2 test. The results demonstrated that the plasma levels of MIR4435-2HG were not associated with the disease stage or age of patients (Table I).

MIR4435-2HG and TGF-β1 are positively correlated in patients with PCa. Correlations between TGF-β1 and MIR4435-2HG plasma levels in patients and healthy controls were analyzed by Pearson's correlation coefficient. As presented in Fig. 2A, MIR4435-2HG and TGF-β1 plasma levels were positively correlated in patients with PCa. Conversely, there was no correlation between MIR4435-2HG and TGF-β1 plasma levels in healthy controls (Fig. 2B).

MIR4435-2HG overexpression induces TGF-β1 upregulation in the 22Rv1 cell line. Following transfection, MIR4435-2HG overexpression was confirmed in the 22Rv1 cell line (Fig. 3A; P<0.05). Furthermore, MIR4435-2HG overexpression induced TGF-β1 upregulation (Fig. 3B; P<0.05) compared with the negative control (NC) and control (C). However, treatment with exogenous TGF-β1 (Sigma-Aldrich; Merck KGaA) at 5, 10 and 20 ng/ml for 24 h had no effect on MIR4435-2HG expression level (Fig. 3C).
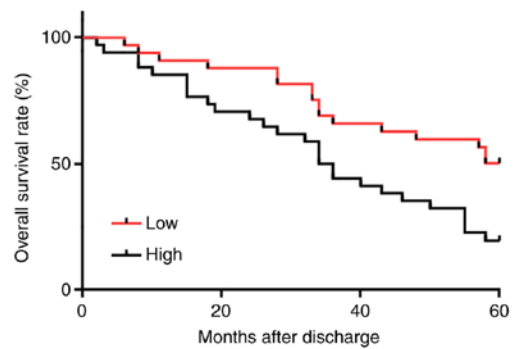

Figure 4. High plasma levels of MIR4435-2HG are closely associated with poor survival in patients with PCa. Patients with a high level of MIR4435-2HG exhibited a lower overall survival rate compared with patients with low levels of MIR4435-2HG.

High plasma levels of MIR4435-2HG are closely associated with poor survival in patients with PCa. Survival curves of patients with PCA were plotted and compared using the Kaplan-Meier method and log-rank test, respectively. As presented in Fig. 4, patients with high MIR4435-2HG plasma levels exhibited a significantly lower overall survival rate compared with patients with a low MIR4435-2HG plasma level (χ^2=7.238; P=0.0071).
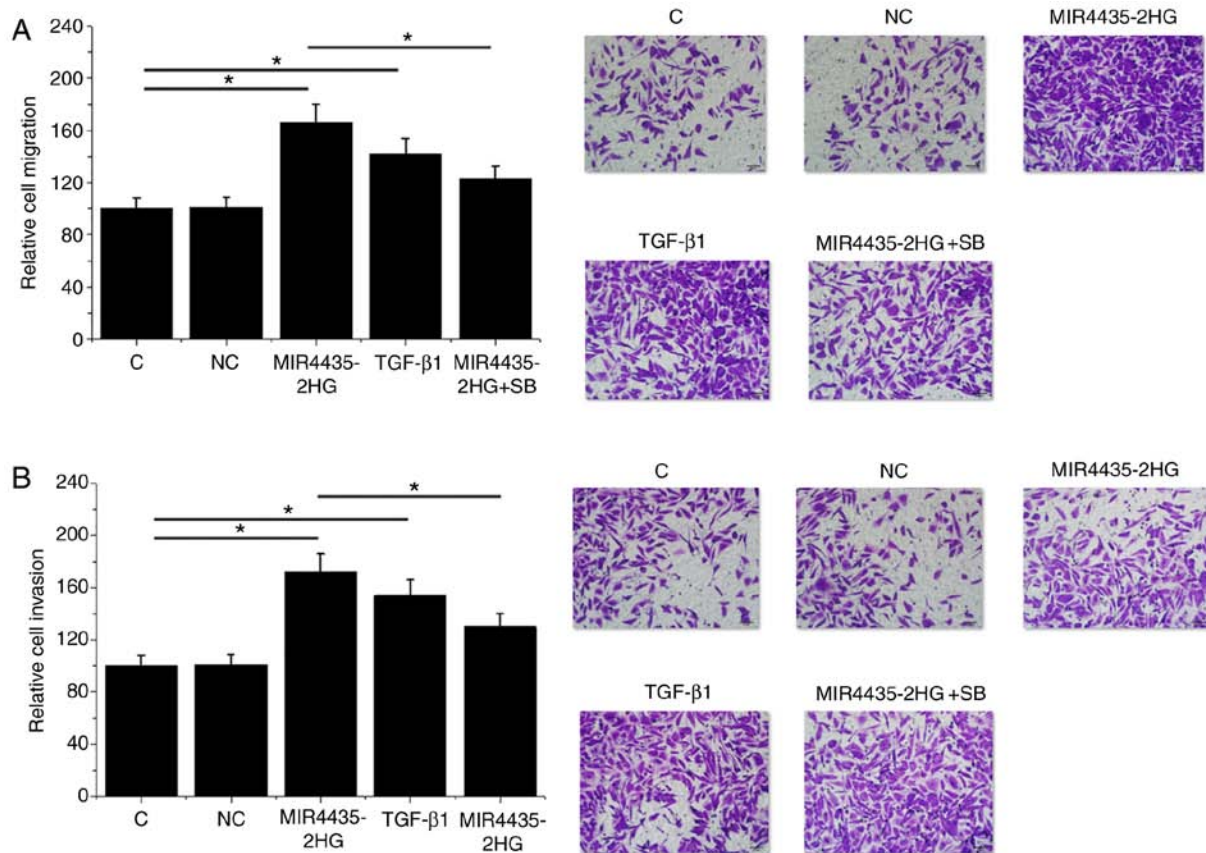
Figure 5. MIR4435-2HG overexpression stimulates cancer cell migration and invasion through TGF-β1. MIR4435-2HG overexpression and treatment with exogenous TGF-β1 (10 ng/ml) stimulated the (A) migration and (B) invasion of 22Rv1 cells. Treatment with the TGF-β inhibitor SB at 10 nM attenuated the stimulating effects of MIR4435-2HG overexpression on cell migration and invasion (magnification, x40). *P<0.05. C, control; NC, negative control; SB, SB431542; TGF-β1, transforming growth factor-β1.

MIR4435-2HG overexpression promotes 22Rv1 cell migration and invasion, potentially through TGF-β1. MIR4435-2HG overexpression and treatment with exogenous TGF-β1 (10 ng/ml) stimulated 22Rv1 cell migration (Fig. 5A) and invasion (Fig. 5B; P<0.05) compared with the C and NC groups. In addition, treatment with the TGF-β inhibitor SB431542 at 10 nM attenuated the stimulatory effects of MIR4435-2HG overexpression on cell migration and invasion (P<0.05).

Discussion

Tumor metastasis is a major challenge in the treatment of PCa. The present study demonstrated that MIR4435-2HG, which is characterized as an oncogenic lnRNA in lung cancer (12), promotes the migration and invasion of PCa cells *in vitro*. Furthermore, the effects of MIR4435-2HG in PCa may be mediated by TGF-β1 upregulation.

The involvement of TGF-β in cancer biology has been intensively explored (16). During the development of PCa, the TGF-β signaling pathway promotes the migration and invasion of cancer cells through interactions with numerous downstream targets, including the PI3K/AKT/mTOR pathway (17). Conversely, inhibition of TGF-β signaling by inhibitors such as PMEPA1 leads to tumor metastasis inhibition (18). Similarly, the present study demonstrated that the TGF-β1 plasma level was upregulated in patients with PCa compared with healthy controls. In addition, treatment with

exogenous TGF-β1 accelerated the migration and invasion of PCa cells. These results suggested that TGF-β1 may stimulate tumor metastasis in PCa.

The TGF-β signaling pathway may participate in cancer biology by mediating the expression of lncRNAs (19). In addition, activation of TGF-β signaling can be regulated by certain lncRNAs, such as cancer susceptibility 9 and Angelman syndrome chromosome region (20). To the best of our knowledge, the associations between TGF-β signaling and lncRNAs in PCa have been poorly investigated. The present study demonstrated that MIR4435-2HG was upregulated in patients with PCa. MIR4435-2HG may therefore serve as an upstream activator of TGF-β1 signaling pathway. In addition, TGF-β1 upregulation by MIR4435-2HG may be involved in the regulation of PCa cell migration and invasion. The results from this study enrich the understanding of PCa pathogenesis. It has been reported that MIR4435-2HG can interact with the β-catenin signaling pathway to promote lung cancer (12), and that β-catenin can interact with TGF-β signaling (21). β-catenin may therefore be considered as a mediator between MIR4435-2HG and TGF-β. However, β-catenin was not investigated in this study, which represents a limitation. In addition, the present study failed to detect secreted TGF-β1 in the cell culture medium, which is another limitation. Future investigations will focus on these points.

Tumor metastasis is a major cause of mortality in patients with PCa. The present study demonstrated that the circulating level of MIR4435-2HG may serve as a prognostic biomarker for

PCa. However, more clinical trials are needed to further confirm this hypothesis. In addition, it is noteworthy that the TGF-β inhibitor only partially attenuated the effects of MIR4435-2HG on cancer cell migration and invasion. MIR4435-2HG may therefore interact with other downstream effectors to regulate PCa cell migration and invasion. The present study did not investigate the role of the TGF-β inhibitor in regulating PCa cell behavior, since previous studies had already revealed that TGF-β signaling inhibition suppresses PCa by affecting cancer cell behavior, including invasion and migration (18,22,23).

The present study did not investigate the genes involved in the regulation of PCa cell migration and invasion through MIR4435-2HG. Future studies will perform a deeper analysis.

In conclusion, the results from the present study suggested that MIR4435-2HG may stimulate PCa cell migration and invasion by promoting TGF-β signaling.

Acknowledgements

Not applicable.

Availability of data and materials

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

Authors' contributions

MC, HZ and HM designed the experiments. HZ, HM, XH and WT performed the experiments. XL, JL, and CZ collected and analyzed data. MC drafted the manuscript. All authors approved the final version of the manuscript.

Ethics approval and consent to participate

This study was approved by the Ethics Committee of The First Affiliated Hospital of Guangzhou University of Chinese Medicine prior to patient admission. Written informed consent was collected from all patients with PCa and healthy volunteers.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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