miR-4417 targets lncRNA PSMG3-AS1 to suppress cell invasion and migration in cervical squamous cell carcinoma

SHUHONG MAN[1], XIAOHAN LI[2] and WEI ZHU[3]

[1]Department of Gynaecology, Weifang People's Hospital, Weifang, Shandong 261000;
[2]School of Public Health, Shandong University, Jinan, Shandong 250012;
[3]Nursing Department, Weifang People's Hospital, Weifang, Shandong 261000, P.R. China

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Abstract. Although long non-coding RNA (lncRNA) PSMG3-AS1 has been reported to participate in cancer biology, its role in cervical squamous cell carcinoma (CSCC) is unknown. The present study aimed to investigate the role of the lncRNA PSMG3-AS1 in CSCC. The expression levels of PSMG3-AS1 in both CSCC and non-tumor tissues from 64 patients with CSCC were measured by reverse transcription-quantitative PCR. The potential interaction between miR-4417 and PSMG3-AS1 was predicted using IntaRNA 2.0. Overexpression of miR-4417 and PSMG3-AS1 were achieved in CSCC cells to further explore the potential interaction between them. The effects of overexpression of miR-4417 and PSMG3-AS1 on CSCC cell invasion and migration were assessed by Transwell assay. The results revealed that PSMG3-AS1 expression was upregulated in CSCC tissues, and its high expression levels predicted a poor survival in patients with CSCC. miR-4417 expression was downregulated in CSCC tissues and was inversely correlated with PSMG3-AS1 expression. Moreover, miR-4417 was predicted to interact with PSMG3-AS1. In CSCC cells, overexpression of miR-4417 decreased the expression levels of PSMG3-AS1, while overexpression of PSMG3-AS1 did not affect miR-4417 expression. Transwell assay demonstrated that overexpression of PSMG3-AS1 increased CSCC cell invasion and migration. However, overexpression of miR-4417 inhibited CSCC cell invasion and migration, and attenuated the effects of PSMG3-AS1 overexpression in CSCC cells. In conclusion, the present study indicated that miR-4417 may target PSMG3-AS1 to suppress cancer cell invasion and migration in CSCC.

Correspondence to: Dr Wei Zhu, Nursing Department, Weifang People's Hospital, 151 Guangwen Street, Kuiwen, Weifang, Shandong 261000, P.R. China
E-mail: rf7350@163.com

Abbreviations: CSCC, cervical squamous cell carcinoma; HPV, human papillomavirus; lncRNA, long non-coding RNA

Key words: CSCC, lncRNA PSMG3-AS1, microRNA-4417, invasion, migration

Introduction

Cervical cancer is considered as the 4th most common type of cancer in global clinical practice (1). Globally, cervical cancer caused 569,847 new cases, accounting for 3.2% of all new cancer cases, and 311,365 deaths, accounting for 3.3% of all cancer-associated deaths, in 2018 alone (2). Human papillomavirus (HPV) infection is a major risk factor for cervical cancer (3). With the increased understanding of the molecular mechanisms of HPV infection (4,5), as well as the popularization of HPV screening and vaccination (6,7), the incidence and mortality rates of cervical cancer have significantly dropped over the past decades (6,7). However, the HPV screening rate in developing countries, such as China, remains low, and most patients with cervical cancer are diagnosed at advanced stages and have a poor survival (8).

A considerable number of molecular pathways are involved in the pathogenesis of cervical cancer (9). Understanding the roles of these molecular signaling pathways provides novel insights into the development of targeted therapies (10). The development and progression of cancer, such as lung cancer, liver cancer and CSCC, involve the regulation of non-coding RNAs (ncRNAs), which do not encode proteins but regulate the expression of cancer-associated genes at different levels (11). ncRNA-targeted therapies have provided valuable insights into cancer treatment (11). However, the functions of most ncRNAs remain unknown. ncRNAs, such as long (>200 nucleotides) ncRNAs (lncRNAs), have no protein-coding information, but they participate in human diseases by affecting protein synthesis (11). PSMG3-AS1 has been characterized as an oncogenic lncRNA in breast cancer (12), while its role in other types of cancer is unknown. A previous bioinformatics study revealed that PSMG3-AS1 may be a potential target of microRNA (miRNA/miR)-4417, a critical player in cancer biology (13). Therefore, the present study aimed to investigate the interaction between miR-4417 and PSMG3-AS1 in cervical squamous cell carcinoma (CSCC), which is a major subtype of cervical cancer.

Materials and methods

Sample collection. The present study was approved by the Ethics Committee of Weifang People's Hospital (Weifang, China;

approval no. WPH011). A total of 64 patients with CSCC (all females; age range, 42-64 years; mean age, 51.7±6.7 years) admitted at the aforementioned hospital between December 2012 and December 2014 were enrolled in the present study. All patients were newly diagnosed cases, and no other severe clinical disorders, such as chronic diseases, severe infections, heart diseases or other types of cancer, were observed among these patients. No therapy was initiated before the admission of patients. All patients provided written informed consent. Fine-needle aspiration was performed to collect CSCC and paired adjacent (within 3 cm around tumors) non-tumor tissues from all patients before therapy. Histopathological examinations were performed to confirm all tissue samples. Tissue samples were stored in liquid nitrogen before subsequent experiments. The clinical data of all 64 patients are listed in Table I.

Treatment and follow-up. The 64 patients with CSCC included 12, 17, 20 and 15 cases at clinical stage I, II, III and IV, respectively, based on the American Joint Committee on Cancer (AJCC) staging (14). Based on the health conditions and AJCC stage of the patients, anticancer therapies, such as surgical resection, chemotherapy, radiotherapy or combined therapy, were performed. From the day of admission, all patients were followed up for 5 years (through phone call and/or outpatient visit) in a monthly manner to record survival, and patients who died of causes other than CSCC were excluded from the present study (77 patients with CSCC were enrolled at the beginning of the study, and 13 patients who died of causes unrelated to CSCC were excluded).

Cell culture. The CSCC SiHa and C33A cell lines were obtained from the American Type Culture Collection. Cells were cultured in Eagle's Minimum Essential Medium with 10% FBS (both Gibco; Thermo Fisher Scientific, Inc.) at 37˚C with 5% CO_2 at 95% humidity. Cells were harvested at ~85% confluence and used in subsequent transient transfection experiments.

Cell transfection. The PSMG3-AS1 expression vector was constructed using pcDNA3.1 vector (Invitrogen; Thermo Fisher Scientific, Inc.) as the backbone. miR-4417 mimic (5'-GGUGGGCUUCCCGGAGGG-3') and its non-targeting negative control (NC) miRNA (5'-UGCCACGUGGCAUGCA GUG-3') were purchased from Sigma-Aldrich (Merck KGaA). SiHa and C33A cells were transfected with 10 nM PSMG3-AS1 expression vector or 45 nM miR-4417 mimic using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). Incubation with transfection mixture was performed at 37˚C for 6 h. Empty vector- or NC miRNA-transfected cells were used as NC cells. Untransfected cells were used as the control (C) cells. Subsequent experiments were performed 48 h post-transfection.

Luciferase reporter assay. The pGL3-PSMG3-AS1 luciferase reporter vector (Promega Corporation) was established. Cells were transfected with miR-4417 mimic + pGL3-PSMG3-AS1 (miR-4417 group) or NC miRNA + pGL3-PSMG3-AS1 (NC miRNA group) using the aforementioned transfection method. Luciferase activity was measured after 48 h using the Firefly Luciferase Assay kit 2.0 (Biotium, Inc.). Firefly luciferase activity was normalized to *Renilla* luciferase activity.

RNA extraction. Total RNA was extracted from tissue samples and cultivated cells using RNAzol (Sigma-Aldrich; Merck KGaA). To harvest miRNAs, 85% ethanol was used to precipitate and wash RNA samples. RNA concentrations were measured using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, Inc.). RNA samples were digested with DNase I to remove genomic DNA.

Reverse transcription-quantitative (RT-q)PCR. Preparation of cDNA samples was performed via RT using the QuantiTect Reverse Transcription kit (Qiagen China Co., Ltd.) in accordance with the manufacturer's instructions. The templates were RNA samples from both tissue and cultivated cells. The expression levels of PSMG3-AS1 were measured using BlazeTaq™ One-Step SYBR-Green RT-qPCR kit (GeneCopoeia, Inc.) following the manufacturer's instructions, with β-actin as the internal control. The expression levels of mature miR-4417 were measured using All-in-One™ miRNA qRT-PCR Detection kit (GeneCopoeia, Inc.) with U6 as the internal control. All PCR reactions were performed in triplicate, and the $2^{-\Delta\Delta Cq}$ method (15) was used to normalize Ct values: ΔCT = Ct (target gene) - Ct (internal control). The sample with the biggest ΔCt value was set to 1, and all other samples were normalized to this sample. The primer sequences were as follows: PSMG3-AS1 forward, 5'-GAAGCAGAACCAA CGCACAG-3' and reverse, 5'-GCATAATCCAATCCCTCAA GAA-3'; β-actin forward, 5'-TGACGGGGTCACCCACACTGT GCCCATCTA-3' and reverse, 5'-CTAGAAGCATTTGCGGT GGACGATGGAGGG-3'; miR-4417 forward, 5'-GGTGGGC TTCCCGGA-3'. Universal reverse primer and U6 primers were included in the aforementioned All-in-One™ miRNA qRT-PCR Detection kit. The qPCR conditions consisted of 95˚C for 1 min, followed by 40 cycles of 95˚C for 10 sec and 58˚C for 40 sec.

RNA interaction prediction. The potential interaction between miR-4417 and PSMG3-AS1 was predicted using IntaRNA 2.0 (16). In this program, miR-4417 was used as short sequence and PSMG3-AS1 was used as long sequence.

Transwell assay. SiHa and C33A cells were harvested 48 h post-transfection and subjected to Transwell assay using Transwell® Cell Culture Plate Inserts (8.0-μm pore; Corning, Inc.). Briefly, 600 cells in 0.1 ml serum-free EMEM were seeded in the upper chamber. The lower chamber was filled with EMEM with 20% FBS. Matrigel (EMD Millipore)-coated membranes (6 h at 37˚C) were used in invasion assays, while uncoated membranes were used in migration assays. Cells were cultivated for 12 h at 37˚C, followed by staining of the lower surface of membranes with 1% crystal violet (Sigma-Aldrich; Merck KGaA) for 20 min at room temperature in the dark. Cells were counted under a light microscope (magnification, x20) and images were obtained.

Statistical analysis. GraphPad Prism 6 (GraphPad Software, Inc.) was used to process and analyze data. Data were expressed as the mean ± SEM of three biological replicates. Paired Student's t-test was used to compare gene expression levels between CSCC and non-tumor tissues. One-way ANOVA and

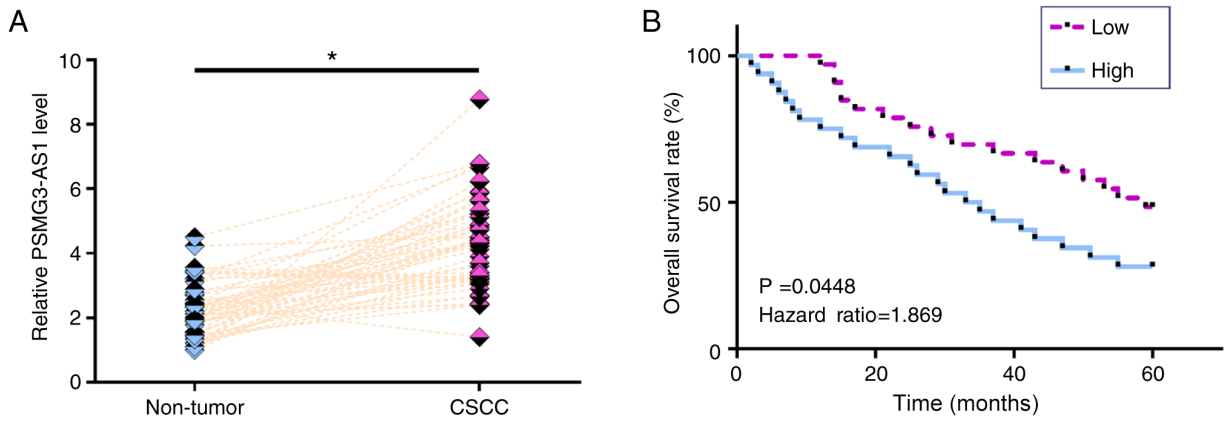

Figure 1. Upregulation of PSMG3-AS1 in CSCC tissues predicts a poor survival. (A) PSMG3-AS1 expression in paired CSCC and non-tumor tissues from 64 patients with CSCC was measured via reverse transcription-quantitative PCR. PCR reactions were performed in three technical replicates, and average values are presented and compared. (B) Patients with CSCC (n=64) were divided into high and low PSMG3-AS1 expression groups (n=32 each), with the median relative level of PSMG3-AS1 expression in CSCC tissues set as the cut-off value (4.27). Survival curves were plotted based on the 5-year follow-up data and compared using the log-rank test. *P<0.05. CSCC, cervical squamous cell carcinoma.

Tukey's post-hoc test were used to compare multiple groups. The 64 patients with CSCC were divided into high and low PSMG3-AS1 expression groups (n=32) based on the median relative PSMG3-AS1 expression level (4.27) in CSCC tissues. Survival curves were plotted based on the 5-year follow-up data using GraphPad Prism 6 with the 'Survival' panel and 'Comparing two groups' function. Survival curves were compared using the log-rank test. Correlations were analyzed using Pearson's correlation coefficient. χ^2 test was performed to analyze the association between the expression levels of miR-4417, PSMG3-AS1 and the patients' clinical data. P<0.05 was considered to indicate a statistically significant difference.

Results

Upregulation of PSMG3-AS1 expression in CSCC tissues predicts a poor survival. The expression levels of PSMG3-AS1 in both CSCC and non-tumor tissues from the 64 patients with CSCC were measured via RT-qPCR. Compared with non-tumor tissues, the expression levels of PSMG3-AS1 were significantly increased in CSCC tissues (Fig. 1A; P<0.05). Survival curve analysis revealed that the overall survival rate of patients in the high PSMG3-AS1 expression group was significantly lower than that in the low PSMG3-AS1 expression group (Fig. 1B; P=0.0448). χ^2 test revealed that the expression levels of PSMG3-AS1 were not significantly associated with age, smoking habit, HPV infection and clinical stage (data not shown).

miR-4417 expression is downregulated in CSCC tissues and inversely correlated with PSMG3-AS1 expression. The expression levels of miR-4417 in both CSCC and non-tumor tissues from the 64 patients with CSCC were measured via RT-qPCR. Compared with non-tumor tissues, the expression levels of miR-4417 were significantly lower in CSCC tissues (Fig. 2A; P<0.05). Pearson's correlation coefficient analysis revealed that the expression levels of miR-4417 were inversely and significantly correlated with the expression levels of PSMG3-AS1 in both CSCC tissues (Fig. 2B) and non-tumor tissues (Fig. 2C). χ^2 test revealed that the expression levels of miR-4417 were not significantly associated with age, smoking habit, HPV infection and clinical stage (data not shown).

Table I. Clinical data of the 64 patients with cervical squamous cell carcinoma.

Characteristic	Value
Clinical stage, n	
I or II	29
III or IV	35
Mean age ± SD, years	51.7±6.7
Tumor multiplicity, n	
Single	40
Multiple	24
Differentiation, n	
G1	22
G2	24
G3	18
Body mass index, n	
≥24	20
<24	44
Smoking, n	
Yes	18
No	46

miR-4417 targets PSMG3-AS1 in CSCC cells. The potential interaction between miR-4417 and PSMG3-AS1 was predicted using IntaRNA 2.0 (14). It was observed that miR-4417 and PSMG3-AS1 may form multiple base pairing (Fig. 3A). Luciferase reporter assay revealed that, compared with the NC miRNA group, the miR-4417 group exhibited significantly decreased luciferase activity, indicating a direct interaction between miR-4417 and PSMG3-AS1 (Fig. 3A). SiHa and C33A cells were transfected with either miR-4417 mimic or PSMG3-AS1 expression vector, and the overexpression of

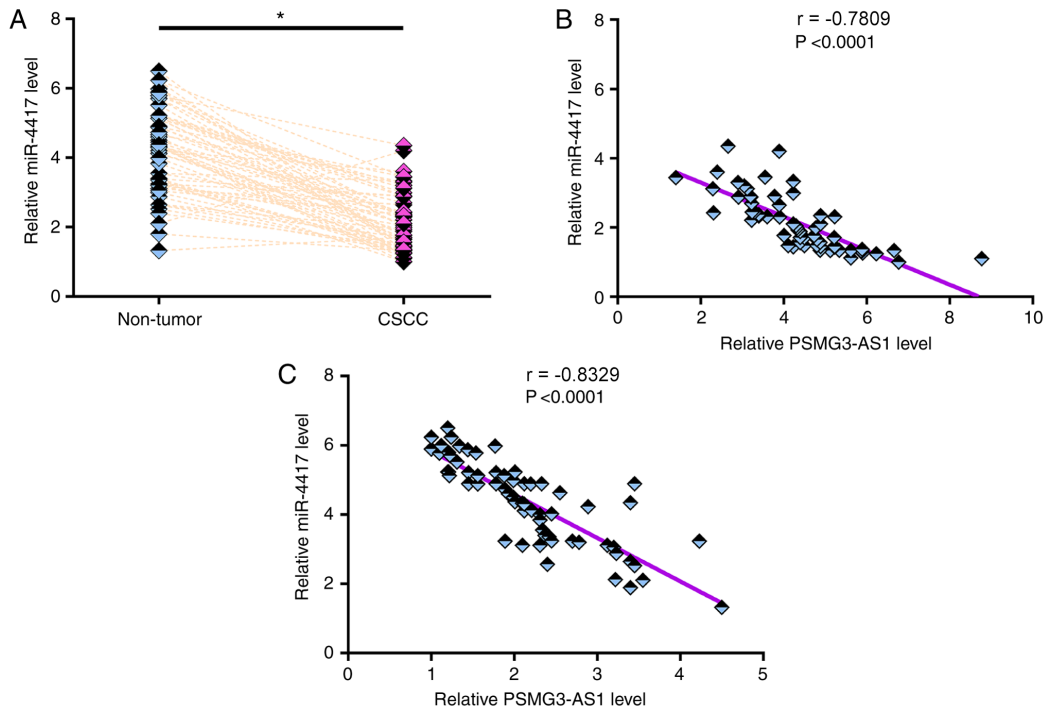

Figure 2. miR-4417 expression is downregulated in CSCC and inversely correlated with PSMG3-AS1 expression. (A) miR-4417 expression in paired CSCC and non-tumor tissues from 64 patients with CSCC was measured via reverse transcription-quantitative PCR. PCR reactions were performed in three technical replicates and mean values are presented and compared. Pearson's correlation coefficient analysis was performed to analyze the correlation between the expression levels of miR-4417 and PSMG3-AS1 in both (B) CSCC and (C) non-tumor tissues. *P<0.05. CSCC, cervical squamous cell carcinoma; miR, microRNA.

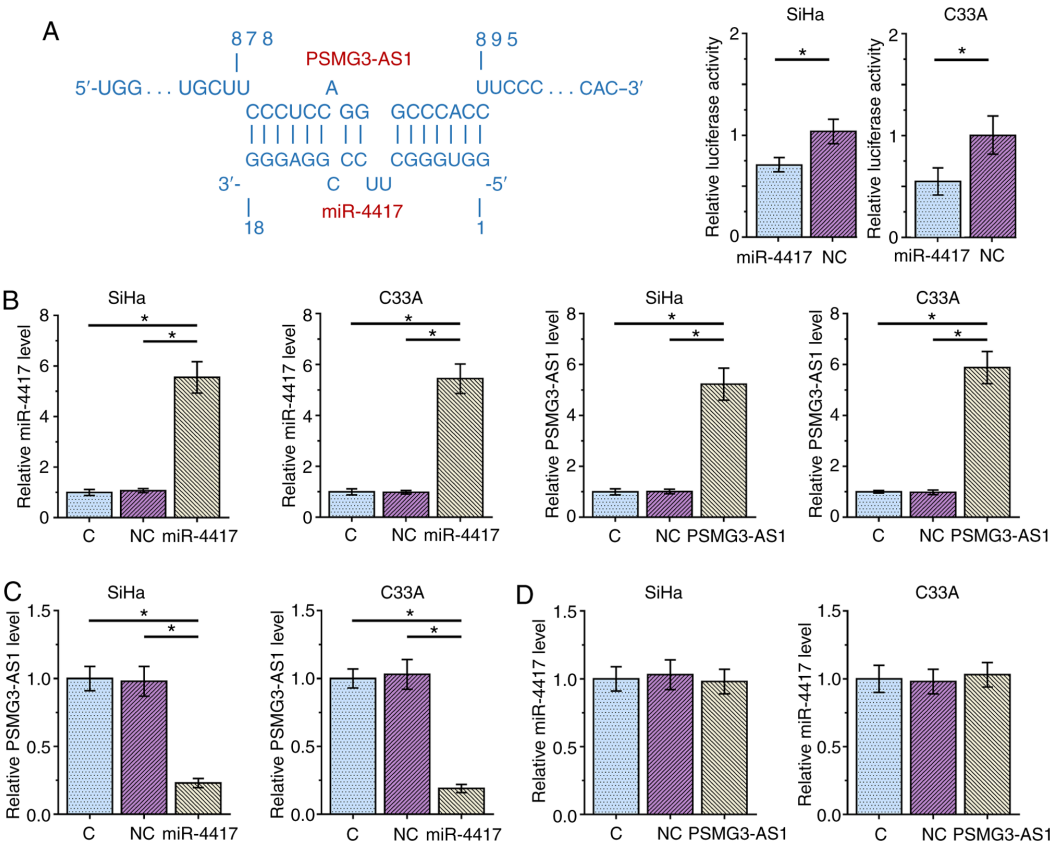

Figure 3. miR-4417 targets PSMG3-AS1 in CSCC cells. (A) Potential interaction between miR-4417 and PSMG3-AS1 was predicted using IntaRNA 2.0. Luciferase assay was performed to further confirm the direct interaction between miR-4417 and PSMG3-AS1. (B) SiHa and C33A cells were transfected with either miR-4417 mimic or PSMG3-AS1 expression vector, and the overexpression of miR-4417 and PSMG3-AS1 was confirmed 48 h after transfection via RT-qPCR. (C) Effects of miR-4417 overexpression on PSMG3-AS1 expression and (D) effects of PSMG3-AS1 overexpression on miR-4417 expression were also analyzed by RT-qPCR 48 h post-transfection. All experiments were repeated in three biological replicates, and the data are presented as the mean ± SEM. *P<0.05. RT-qPCR, reverse transcription-quantitative PCR; miR, microRNA; C, control; NC, negative control.

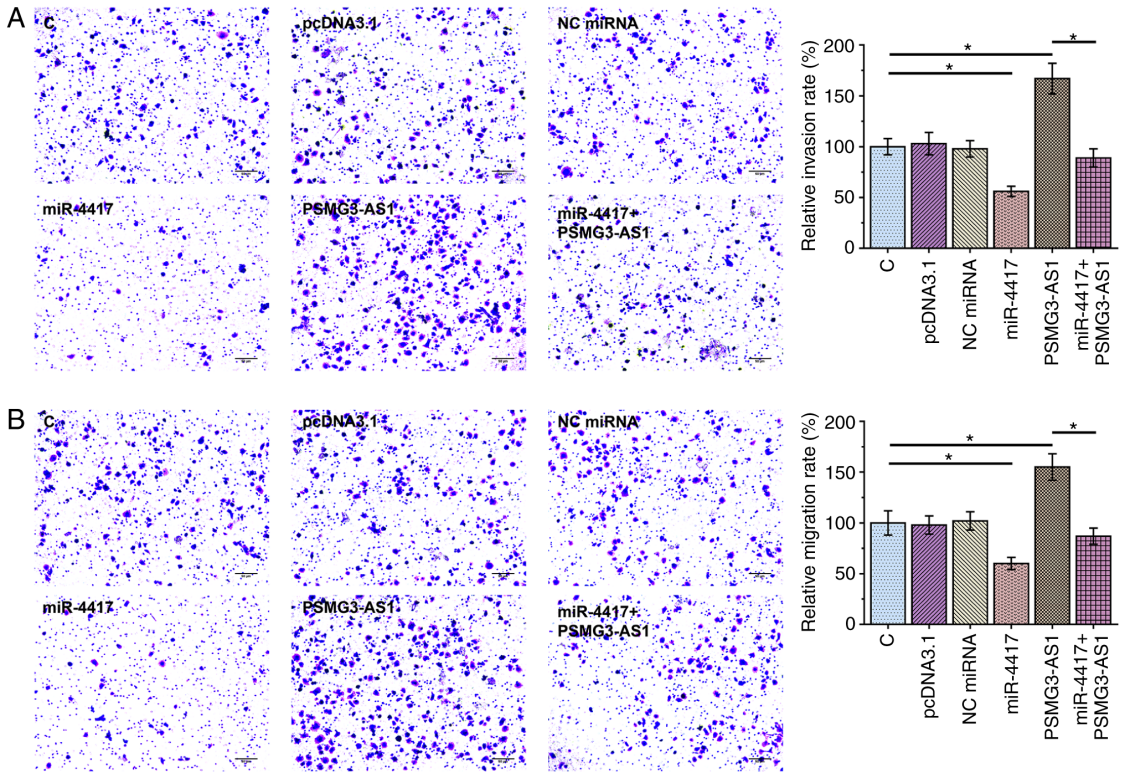

Figure 4. miR-4417 targets PSMG3-AS1 to suppress cancer cell invasion and migration. Transwell assays were performed to analyze the effects of PSMG3-AS1 and miR-4417 overexpression on (A) the invasion and (B) migration of SiHa and C33A cells (magnification, x20). All experiments were performed in three biological replicates, and the data are presented as the mean ± SEM. *P<0.05. miR/miRNA, microRNA; C, control; NC, negative control.

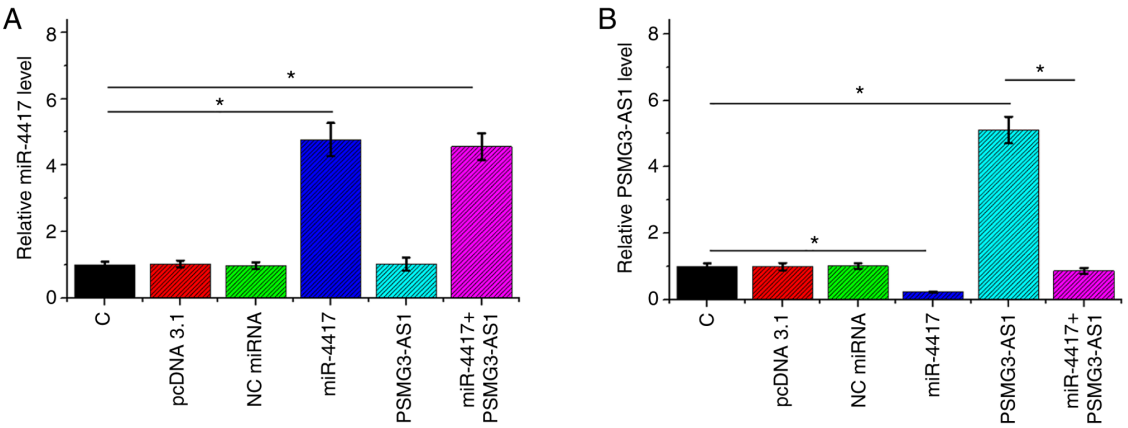

Figure 5. miR-4417 and PSMG3-AS1 expression in C, pcDNA 3.1, NC miRNA, miR-4417, PSMG3-AS1 and miR-4417+PSMG3-AS1 groups included in Transwell assay. Expression levels of (A) miR-4417 and (B) PSMG3-AS1 in C, pcDNA 3.1, NC miRNA, miR-4417, PSMG3-AS1 and miR-4417+PSMG3-AS1 groups involved in Transwell assays were also measured by reverse transcription-quantitative PCR. All experiments were performed in three biological replicates, and the data are presented as the mean ± SEM. *P<0.05. miR/miRNA, microRNA; C, control; NC, negative control.

miR-4417 and PSMG3-AS1 were confirmed 48 h post-transfection via RT-qPCR (Fig. 3B; P<0.05). Compared with the C and NC groups, overexpression of miR-4417 significantly decreased the expression levels of PSMG3-AS1 (Fig. 3C; P<0.05), while overexpression of PSMG3-AS1 did not affect miR-4417 expression (Fig. 3D) in both cell lines. These results suggested that miR-4417 may target PSMG3-AS1 to downregulate its expression, while PSMG3-AS1 did not regulate miR-4417 expression.

miR-4417 downregulates PSMG3-AS1 to suppress cancer cell invasion and migration. Transwell assay was performed to assess the effect of overexpression of PSMG3-AS1 and miR-4417 on invasion (Fig. 4A) and migration (Fig. 4B) of SiHa and C33A cells. Overexpression of PSMG3-AS1 significantly increased invasion and migration rates of cancer cells compared with control cells, while overexpression of miR-4417 significantly inhibited invasion and migration of cancer cells and attenuated the effect of PSMG3-AS1 overexpression (Fig. 4A and B; P<0.05). The expression levels of miR-4417 (Fig. 5A; P<0.05) and PSMG3-AS1 (Fig. 5B; P<0.05) in all groups included in Transwell assays were also measured via RT-qPCR. It was observed that the cell

invasion and migration patterns were consistent with the expression pattern of PSMG3-AS1, with the highest expression level of PSMG3-AS1 being observed in the PSMG3-AS1 group and the lowest PSMG3-AS1 level in the miR-4417 group (Fig. 5B).

Discussion

The present study investigated the interaction between miR-4417 and PSMG3-AS1 in CSCC. It was observed that the expression levels of miR-4417 and PSMG3-AS1 were altered in CSCC, and that miR-4417 may target PSMG3-AS1 to suppress the invasion and migration of CSCC cells.

It has been previously demonstrated that PSMG3-AS1 expression is upregulated in breast cancer and may sponge miR-143-3p to promote migration and proliferation of breast cancer cells (12). To the best of our knowledge, the present study was the first to report the upregulation of PSMG3-AS1 expression in CSCC. In addition, increased cell invasion and migration rates of CSCC cells were observed after the overexpression of PSMG3-AS1. Therefore, PSMG3-AS1 may serve an oncogenic role in CSCC.

HPV infection screening has significantly increased the early diagnostic rate of CSCC (6,7). However, in China, the HPV screening rate remains low and HPV vaccination is not beneficial for patients who have been already infected (8). Consequently, a considerable number of patients with CSCC are diagnosed at advanced stages and their survival is generally poor (6-8). In the present study, it was demonstrated that high expression levels of PSMG3-AS1 in cancer tissues of patients with CSCC may predict a poor survival. Therefore, measuring the expression levels of PSMG3-AS1 in cancer tissues before therapy may guide the determination of treatment strategies, thereby improving the survival of patients. However, the viability of this approach needs to be further studied.

miR-4417 serves different roles in different types of cancer (13,17). It has been reported that miR-4417 expression is downregulated in triple-negative breast cancer, and overexpression of miR-4417 suppresses cancer cell migration and invasion, indicating its tumor suppressive role in this disease (13). By contrast, miR-4417 expression is upregulated in hepatocellular carcinoma and regulates the phosphorylation of pyruvate kinase muscle 2 to suppress apoptosis and promote proliferation of cancer cells, suggesting its oncogenic role (14). In the present study, it was observed that miR-4417 expression was downregulated in CSCC tissues and miR-4417 overexpression had inhibitory effects on cancer cell invasion and migration. Therefore, miR-4417 may be a tumor suppressor in CSCC. In addition, it was revealed that miR-4417 may target PSMG3-AS1 to exert its tumor suppressive role and that miR-4417 expression was inversely correlated with PSMG3-AS1 expression in both CSCC and non-tumor tissues. However, the present study is limited by the small number of patients. Future studies with more patients are required to further confirm the current findings.

In conclusion, miR-4417 expression was downregulated and PSMG3-AS1 expression was upregulated in CSCC. miR-4417 may target PSMG3-AS1 to suppress cell invasion and migration in CSCC.

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Availability of data and materials

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

Authors' contributions

WZ designed the experiments and drafted the manuscript. SM, XL and WZ performed the experiments. SM and WZ analyzed the data. All authors confirmed the authenticity of the data, and have read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Ethics Committee of Weifang People's Hospital (Weifang, China; approval no. WPH011). The research was performed in accordance with the World Medical Association Declaration of Helsinki. All patients provided written informed consent prior to their inclusion in the study.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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