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Original Article

Long non-coding RNA PSMG3 Antisense RNA 1 is correlated with oral squamous cell carcinoma and regulates cancer cell proliferation by targeting premature microRNA-141

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Abstract: Oral squamous cell carcinoma (OSCC) is common worldwide. In this study, the interaction of microRNA-141 (miR-141) with long non-coding RNA (lncRNA) PSMG3 Antisense RNA 1 (PSMG3-AS1) in OSCC was explored. RT-qPCR was used to analyze the expression of PSMG3-AS1 and miR-141 (both mature and premature) in OSCC. Nuclear fractionation assay was applied to detect PSMG3-AS1 in subcellular locations. RNA pull-down assay was performed to evaluate the binding of miR-141 to PSMG3-AS1. Overexpression assay followed by RT-qPCR was performed to explore the role of PSMG3-AS1 in maturation of miR-141. The function of PSMG3-AS1 and miR-141 in regulating OSCC cell proliferation was assessed by BrdU assay. The results showed that PSMG3-AS1 was highly upregulated in OSCC and miR-141 was downregulated in OSCC. However, no alteration in the expression of premature miR-141 was observed in OSCC. Premature miR-141 was found to directly bind to PSMG3-AS1. Overexpression of PSMG3-AS1 suppressed the maturation of miR-141. PSMG3-AS1 increased OSCC cell proliferation and tumor growth and suppressed the inhibitory role of miR-141 in cell proliferation and tumor growth. Therefore, PSMG3-AS1 may inhibit the maturation of miR-141 to promote OSCC cell proliferation.

Keywords: PSMG3-AS1, miRNA maturation, miR-141, cell proliferation, oral squamous cell carcinoma

Introduction

As a type of malignancy originates from the throat or mouth tissues, oral squamous cell carcinoma (OSCC) is a highly aggressive tumor and is frequently diagnosed with metastatic conditions [1, 2]. Treatment approaches have been developed for OSCC, however, psychological impairments with OSCC remain unimproved, and survival in most cases is poor [3-5]. Therefore, it is of great importance to decipher the molecular mechanisms underlying the disease progression of OSCC.

Non-coding RNAs (ncRNAs), such as long ncRNAs (lncRNAs), participate in cancers by indirectly regulating protein accumulation [6-8]. For example, lncRNA TTN-AS1 regulates the progression of OSCC via microRNA (miR)-199a-3p [9]. PSMG3-AS1 promotes cervical [10] and lung cancers [11]. In cervical cancer, PSMG3-AS1 promotes cell movement and it can be targeted by miR-4417 [10]. Interestingly, in lung adenocarcinoma, overexpression of PSMG3-AS1 is targeted by miR-449b-5p to affect cell proliferation [11]. LncRNAs can absorb miRNAs to suppress their function [12-14]. MiR-141-3p is derived from 3'-end of the miR-141 hairpin structure and plays a suppressive role in esophageal squamous cell carcinoma [15], clear cell renal cell carcinoma [16] and endometrial carcinoma [17]. Previous studies reported a significant downregulation of miR-141 in OSCC compared with the expression levels of miR-141 in normal tissues [18, 19]. In the present study, a potential binding of premature miR-141 to

PSMG3-AS1 was detected, suggesting their potential interaction in OSCC. This study was then carried out to explore the crosstalk between miR-141 and PSMG3-AS1 in OSCC.

Materials and methods

Tissue specimens and cells

This study enrolled a total of 58 OSCC patients (41 males and 17 females, mean age 46.9±5.2 years old) at Oral and Maxillofacial Surgery, Second Affiliated Hospital of Nanchang University. Ethics approval was obtained from the Ethic Committee of this hospital. All patients signed the informed consent. Both OSCC and adjacent non-cancer tissues were collected from the patients during surgery or through biopsies. OSCC patients included in this study had no history of OSCC treatment before admission. Histological analysis of patients' disease conditions revealed that the 58 patients included 32 cases at stage I or II (AJCC) and 26 cases at stage III or IV, respectively.

Cell experiments were performed using OSCC cell lines SCC090 and SCC25 (ATCC, USA). Cells were cultivated in EMEM medium supplemented with 2 mM L-Glutamine and 10% FBS. All other conditions were the same as described in the manufacturer's instructions.

OSCC transfection

SCC090 and SCC25 cells were transfected with PSMG3-AS1 (Genebank Accession: NR_027329.1) expression vector (pcDNA3.1 backbone vector) or miR-141 mimic (5'-UAACACUGUCUGGUAAAGAUGG-3') to overexpress PSMG3-AS1 or miR-141. Expression vectors and miRNAs were obtained from Invitrogen (Shanghai, China). A neon Transfection System (Thermo Fisher Scientific) was used for transfection. All steps of transfection were performed following the manufacturer's instructions. The same method was used to transfect empty vector or negative control (NC) miRNA to serve as NC groups. Transfection was confirmed every 24 h until 72 h.

RNA isolation

Total RNAs were extracted from tissue samples that were ground in liquid nitrogen as well as *in vitro* cultivated cells using Ribozol reagent (Invitrogen). RNA samples were digested by DNase I (Sigma-Aldrich) to remove genomic DNA. To make sure pure RNA samples were obtained, the ratio of OD 260/280 of RNA samples was measured and a ratio of about 2.0 was considered as pure for RNA. Urea-PAGE gel (5%) electrophoresis was performed to analyze RNA integrity.

RNA pull-down assay

Biotin was used to label premature miR-141, and the label miRNAs including bio-miR-141 (pre) and bio-NC miRNA were provided by Sigma-Aldrich. SCC090 and SCC25 cells were transfected with either bio-miR-141 (pre) or bio-NC miRNA, followed by RNA extraction and RT-qPCRs to determine the expression of PSMG3-AS1 in pull-down samples.

Dual luciferase activity assay

PSMG3-AS1 vector was established using the pGL3 luciferase reporter gene vector. Two transfection groups, including PSMG3-AS1 vector + NC miRNA (NC group) and PSMG3-AS1 vector + miR-141 (pre) (miR-141 (pre) group), were conducted to further explore the interaction between PSMG3-AS1 and miR-141 (pre). Luciferase activity was analyzed at 24 h later.

Xenograft experiments

Animal experiments were approved by the Second Affiliated Hospital of Nanchang University Animal Care and Use Committee. Stably transfected cells (1×10^7) were subcutaneously injected into the flanks of each of the five BALB/c nude mice (4-6 weeks old) included in each group. Mice were sacrificed 4 weeks later and tumor volume was calculated every week using the following formula: Tumor volume = $½LW^2$, where L is the maximum length and W is the minimum length.

Nuclear fractionation assay

The expression of PSMG3-AS1 in subcellular locations of OSCC cells was detected by nuclear fractionation assay using the Nuclear/Cytosol Fractionation Kit (BioVision, # K266). Nucleus and cytoplasm samples were prepared, followed by the isolation of total RNAs. After that, reverse transcriptions (RTs) and routine PCRs were performed to detect the expres-

sion of PSMG3-AS1. PCR products were separated using 1% Agarose gel electrophoresis. After EB staining, gel images were captured.

RT-qPCR

The expression of PSMG3-AS1 and miR-141 (both mature and premature miRNAs) was determined. RTs were performed on all RNA samples to synthesize cDNA samples, followed by qPCR to determine the expression of PSMG3-AS1 and premature miR-141 with 18S rRNA as an internal control. Expression of mature miR-141 was determined with the All-in-One™ miRNA qRT-PCR Detection Kit (Genecopoeia) with U6 as the internal control. The $2^{-\Delta\Delta CT}$ method was used to process all Ct values [20]. Primer sequences were: PSMG3-AS1 forward, 5'-GAAGCAGAACCAACGCAC-3' and reverse, 5'-GCATAATCCAATCCCTCAAG-3'; premature miR-141 forward, 5'-CGGCCGGCCCTGGGTCCATCT-3' and reverse, 5'-GAACCCACCCGGGAGCCCA-GC-3'; mature miR-141 forward, 5'-TAACACTG-TCTGGTAAAG-3' and reverse, universal reverse primer; 18S rRNA forward, 5'-CTACCACATCCA-AGGAAGCA-3' and reverse, 5'-TTTTCGTCACTA-CCTCCC-3'; U6 forward, 5'-CGCTTCGGCAGCA-CATAT-3' and reverse, 5'-CACGAATTTGCGTGTC-ATC-3'.

IntaRNA prediction of RNA interaction

IntaRNA 2.0 (http://rna.informatik.uni-freiburg.de/IntaRNA/Input.jsp) [21] was used to predict the binding of premature miR-141 to PSMG3-AS1. PSMG3-AS1 was inputted as the long sequence and premature miR-141 was the short sequence.

BrdU (5-Bromo-2-deoxyUridine)

BrdU assay was carried out at 48 h post-transfection to determine BrdU incorporation, which reflects cell proliferation. Cells were incubated with 10 μM BrdU (BD Pharmingen) for 24 h, followed by incubation with peroxidase-coupled anti-BrdU-antibody (Sigma-Aldrich) for 60 min. After that, cells were washed with PBS, and tetramethylbenzidine (peroxidase substrate) incubation was performed for 30 min. Finally, OD values were measured at 450 nm.

Statistical analysis

The Heml 1.0 [22] software was used to analyze qPCR data to generate heatmaps showing differential gene expression. Correlations were analyzed by the Pearsons' correlation coefficient. The 58 patients were divided into high and low PSMG3-AS1/mature miR-141 level groups (n = 29). Associations between patients' data and the expression of PSMG3-AS1/mature miR-141 were analyzed by Chi-squared test. Student's t-test was used for comparisons. $P < 0.05$ was considered as statistically significant.

Results

PSMG3-AS1 was located in both cytoplasm and nucleus and it directly interacted with premature miR-141

The potential binding of premature miR-141 to PSMG3-AS1 was predicted using IntaRNA 2.0 to explore their potential crosstalk. It was predicted that PSMG3-AS1 and premature miR-141 may form multiple base pairs (**Figure 1A**). Detection of the subcellular location of certain genes may provide information to speculate their functions. The expression of PSMG3-AS1 in subcellular locations of OSCC cells was evaluated by nuclear fractionation assay. The results illustrated that PSMG3-AS1 was located in both the cytoplasm and nucleus of both SCC090 and SCC25 cells (**Figure 1B**). The direct interaction between PSMG3-AS1 and premature miR-141 was detected by RNA pull-down assay. Compared to bio-NC miRNA pull-down samples, bio-miR-141 (pre) pull-down samples showed significantly higher expression levels of PSMG3-AS1 (**Figure 1C**). Therefore, PSMG3-AS1 may directly interact with premature miR-141. The direct interaction between PSMG3-AS1 and premature miR-141 was further confirmed by dual-luciferase activity assay (**Figure 1D**).

Altered expression of PSMG3-AS1 and mature miR-141 was observed in OSCC tissues

The altered expression suggests potential interaction. The mean values of qPCRs (three technical replicates of the same tissue sample) were inputted into Heml 1.0 software to plot heatmaps. Heatmap analysis revealed that PSMG3-AS1 was highly expressed in OSCC (**Figure 2A**), and mature miR-141 was down-regulated in OSCC (**Figure 2B**). Interestingly, premature miR-141 showed similar expression levels in paired OSCC and non-cancer tissues

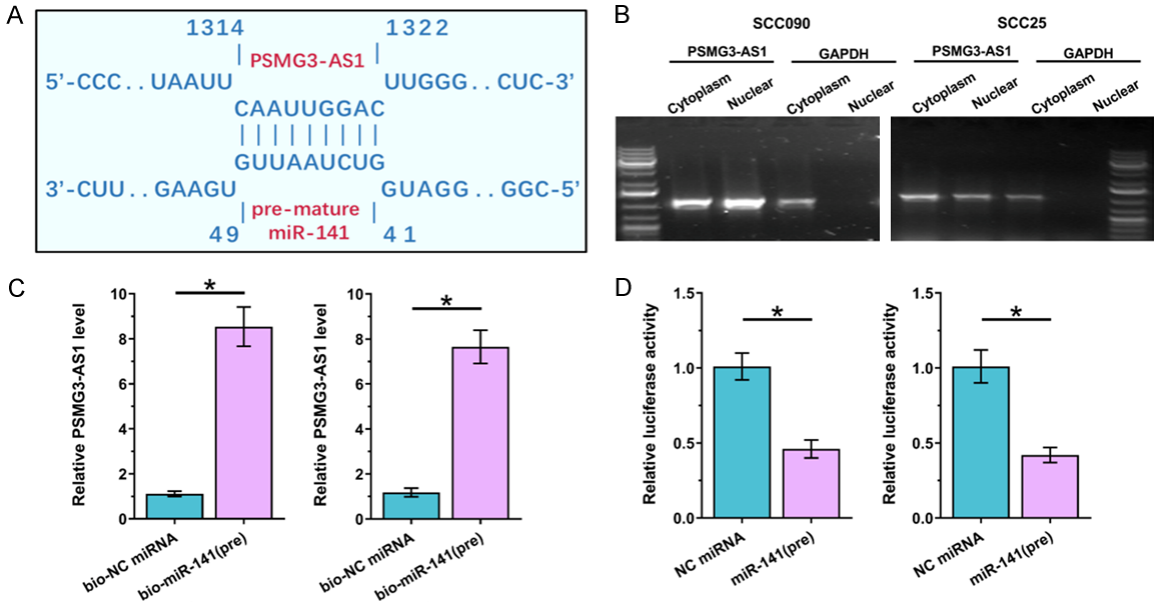

Figure 1. PSMG3-AS1 was located in both cytoplasm and nucleus and it directly interacted with premature miR-141. IntaRNA 2.0 was used to predict the interaction between PSMG3-AS1 and premature miR-141 (A). The expression of PSMG3-AS1 in subcellular locations of OSCC cells was analyzed by nuclear fractionation assay (B). The direct interaction between PSMG3-AS1 and premature miR-141 was analyzed by RNA pull-down assay (C) and dual-luciferase activity assay (D). *, $P < 0.05$.

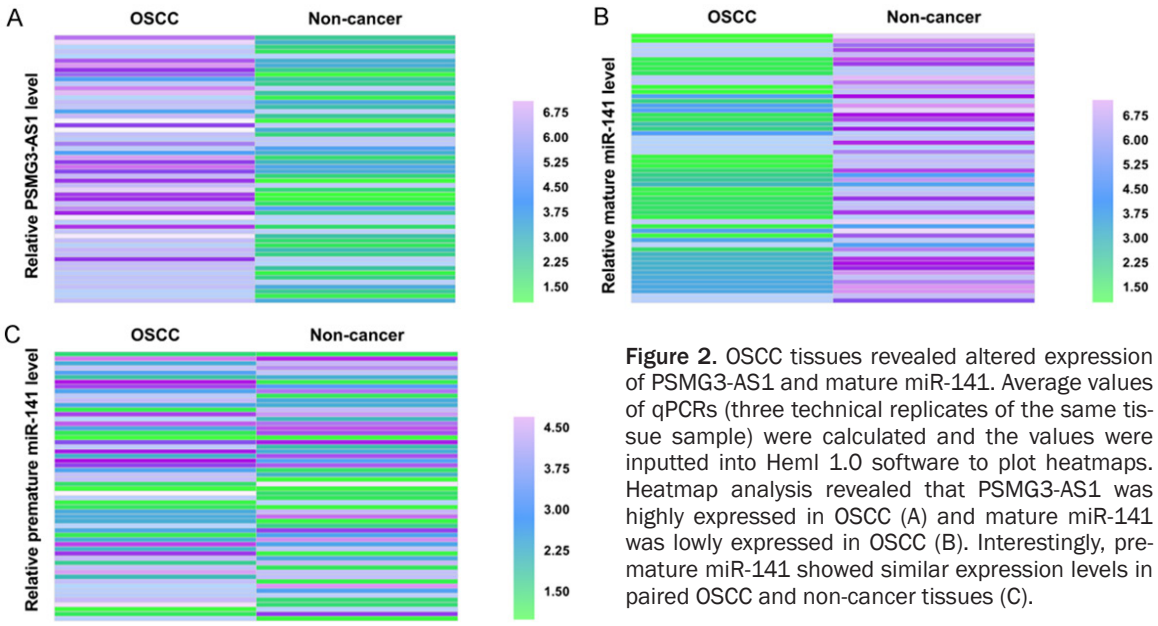

Figure 2. OSCC tissues revealed altered expression of PSMG3-AS1 and mature miR-141. Average values of qPCRs (three technical replicates of the same tissue sample) were calculated and the values were inputted into Heml 1.0 software to plot heatmaps. Heatmap analysis revealed that PSMG3-AS1 was highly expressed in OSCC (A) and mature miR-141 was lowly expressed in OSCC (B). Interestingly, premature miR-141 showed similar expression levels in paired OSCC and non-cancer tissues (C).

(**Figure 2C**). Therefore, altered expression of PSMG3-AS1 and maturation of miR-141 may participate in OSCC. Chi-squared t-test analysis showed that PSMG3-AS1 and mature miR-141 were closely correlated with patients' clinical stage and tumor size, but not distant metastasis, age, and gender (**Table 1**).

PSMG3-AS1 regulated the expression of mature miR-141

Correlations between two genes may suggest interactions. The correlations between PSMG3-AS1 and miR-141 (both mature and premature miRNAs) across OSCC and non-tumor tissues

Table 1. The correlation between PSMG3-AS1, mature miR-141 and patients' clinical data

Features	Cases	PSMG3-AS1		*P*	Mature miR-141		*P*
		High	Low		High	Low	
Age				> 0.05			> 0.05
≥ 45	30	14	16		16	14	
< 45	28	15	13		13	15	
Gender				> 0.05			> 0.05
Male	41	20	21		22	19	
Female	17	9	8		7	10	
Stage				0.002			0.0002
I/II	32	10	22		23	9	
III/IV	26	19	7		6	20	
Tumor size				< 0.00001			< 0.00001
≤ 2 cm	36	9	27		27	9	
> 2 cm	22	20	2		2	20	
Distant metastasis				> 0.05			> 0.05
Yes	48	23	25		26	22	
No	10	6	4		3	7	

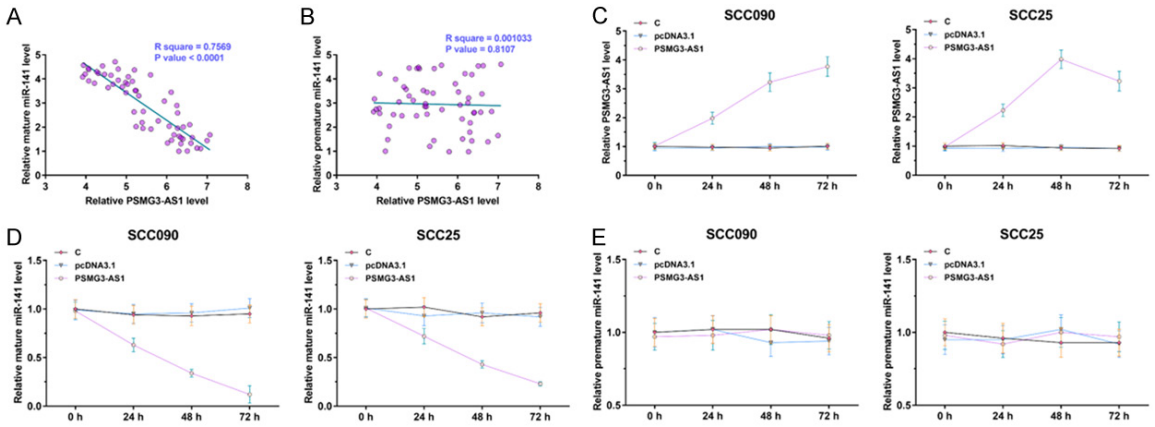

Figure 3. PSMG3-AS1 regulated the expression of mature miR-141. The correlations between PSMG3-AS1 and miR-141 (both mature and premature miRNAs) across OSCC (A) and non-tumor (B) tissues were analyzed by Pearson's correlation coefficient. To explore the interaction between PSMG3-AS1 and miR-141, OSCC cells were overexpressed with PSMG3-AS1 and miR-141 (C), followed by the analysis of the effects of PSMG3-AS1 overexpression on the expression of mature miR-141 (D) and premature miR-141 (E) by RT-qPCRs. *, $P < 0.05$.

were analyzed by Pearson's correlation coefficient. PSMG3-AS1 and mature miR-141 were inversely and significantly correlated across OSCC tissues (**Figure 3A**), while PSMG3-AS1 and premature miR-141 were not correlated (**Figure 3B**). To explore the interaction between PSMG3-AS1 and miR-141, PSMG3-AS1 was overexpressed in OSCC cells (**Figure 3C**, $P < 0.05$). It showed that PSMG3-AS1 decreased the accumulation of mature miR-141 (**Figure 3D**, $P < 0.05$), but not the accumulation of premature miR-141 (**Figure 3E**, $P < 0.05$). Therefore, PSMG3-AS1 may suppress the maturation of miR-141 in OSCC cells.

PSMG3-AS1 inhibited the role of mature miR-141 in suppressing OSCC cell proliferation and in vivo tumor growth

Cell proliferation promotes cancer progression. Therefore, the involvement of PSMG3-AS1 and miR-141 in OSCC cell proliferation was assessed. BrdU assay and animal model experiment were carried out to investigate the function of PSMG3-AS1 and miR-141 in regulating the proliferation and *in vivo* tumor growth of both OSCC cell lines. The results demonstrated the inhibitory effects of miR-141 on the proliferation (**Figure 4A**) and *in vivo* tumor growth

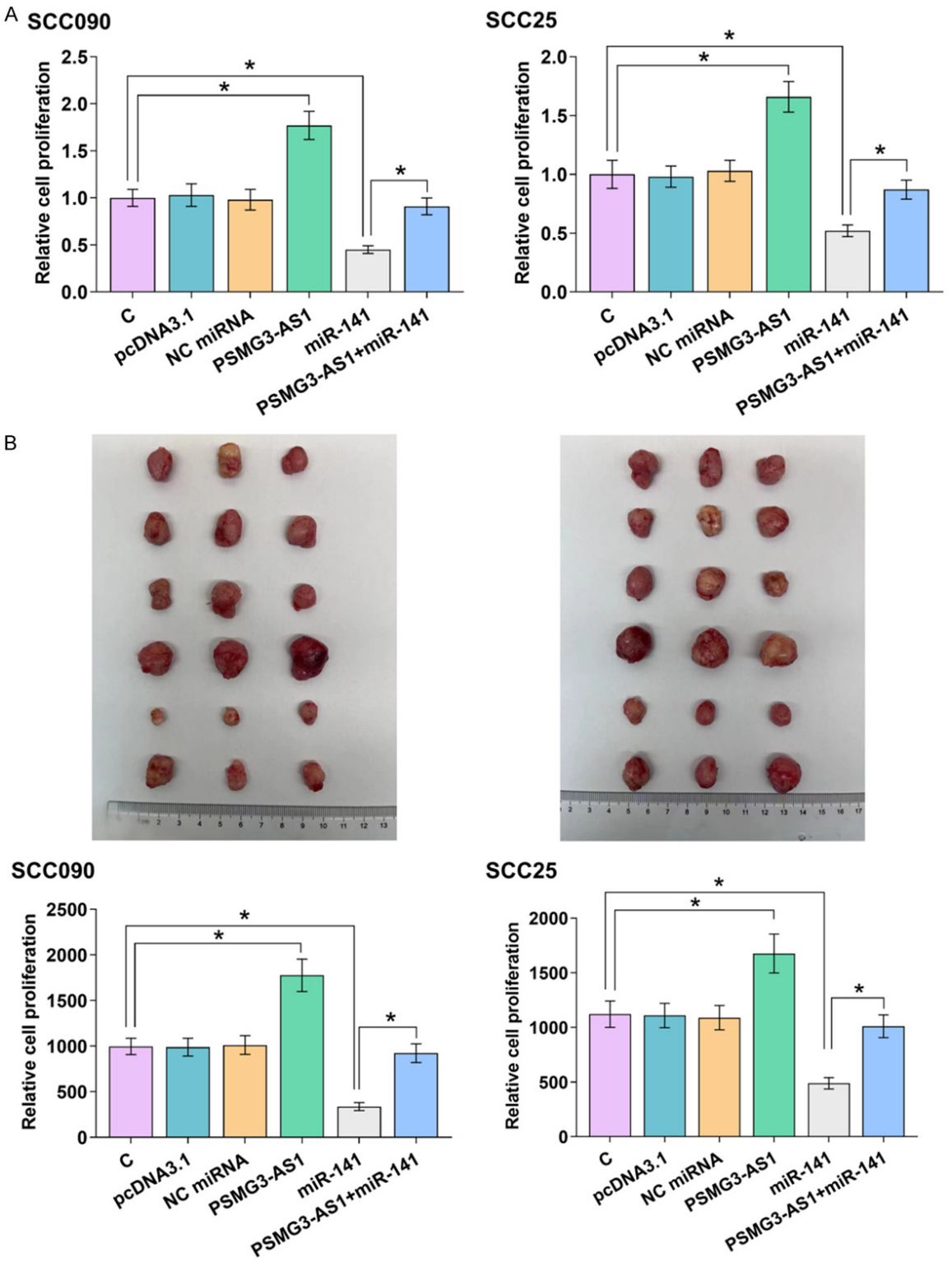

Figure 4. PSMG3-AS1 overexpressed inhibited the role of mature miR-141 in suppressing OSCC cell proliferation and tumor growth. BrdU assay was carried out to analyze the function of PSMG3-AS1 and miR-141 in regulating the proliferation (A) and *in vivo* tumor growth (B) of both OSCC cell lines. *, $P < 0.05$.

(**Figure 4B**) of OSCC cells. Overexpression of PSMG3-AS1 increased OSCC cell proliferation and tumor growth and suppressed the inhibitory role of miR-141 in cell proliferation and

tumor growth (**Figure 4**, $P < 0.05$). Therefore, PSMG3-AS1 may promote OSCC cell proliferation and tumor growth through miR-141.

Discussion

The present study explored the interaction between PSMG3-AS1 and miR-141 in OSCC. PSMG3-AS1 plays an important role in various physiological processes of cancer cells [23, 24]. In our study, we observed that PSMG3-AS1 was highly upregulated in OSCC tissues. Overexpression of PSMG3-AS1 accelerated SCC090 and SCC25 cell proliferation, suggesting that PSMG3-AS1 may play an important role in the occurrence and development of OSCC. Our findings are consistent with the upregulation of PSMG3-AS1 in lung cancer, cervical cancers, hepatocellular carcinoma, and glioblastomas [10, 11, 24, 25].

In OSCC tissues, the expression levels of miR-141 were significantly lower than that of paracancerous tissues. MiR-141 has been characterized as a tumor suppressor in many cancers [26, 27]. For instance, miR-141 targets TGF-β in laryngeal cancer to suppress epithelial-mesenchymal transition [26]. In prostate cancer, miR-141 is downregulated, and overexpression of miR-141 reduces prostate cancer cell stemness and cell metastasis by targeting several pro-metastasis genes [27]. In previous studies, the results identified a significant downregulation of miR-141 in OSCC compared with that in normal tissues [18, 19]. Therefore, miR-141 may be a potential target for the treatment of OSCC. We also observed a negative correlation between the expression of PSMG3-AS1 and mir-141 in OSCC. We speculated that PSMG3-AS1 plays an essential role in OSCC by targeting mir-141. However, we did not observe any interaction between them. Meanwhile, IntaRNA 2.0 predicted that PSMG3AS1 and premature miR-141 may form multiple base pairs. A previous study reported that lncRNA RANBP1 reduced the expression levels of miRNA by inhibiting the movement of pre-miRNAs out of the nucleus, thereby inhibiting miRNA maturation [28]. In effect, the maturation of miR-141 was reported to be inhibited by lnc-PFAR through the same mechanism [29]. In our study, we found a similar mechanism of action. This provides a reference for us to further explore the mechanism of PSMG3-AS1.

LncRNAs are emerging novel targets for OSCC and many other cancers. Despite the efforts made on the elucidation of the roles of lncRNAs in OSCC [30-33], more future studies are still needed.

Conclusion

In summary, PSMG3-AS1 interacts with premature miR-141 and reduces the expression levels of mature miR-141 and further promoting the proliferation of OSCC. Our results revealed the biological role and mechanism of PSMG3-AS1 and miR-141 in OSCC, suggesting that OSCC can be used as a diagnostic molecule and a potential therapeutic target in OSCC.

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The present study was approved by the Ethics Committee of the Second Affiliated Hospital of Nanchang University. The research was carried out following the World Medical Association Declaration of Helsinki. All patients and healthy volunteers provided written informed consent before their inclusion in the study. The study was conducted in strict accordance with the National Institutes of Health Laboratory Animal Care and Use Guidelines.

Disclosure of conflict of interest

None.

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