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ORIGINAL ARTICLE

Long non-coding RNA PCAT1 promotes cell migration and invasion in human laryngeal cancer by sponging miR-210-3p

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Summary

Purpose: *Laryngeal cancer (LC) is one of the most ordinary head and neck cancers worldwide. In this study, the role of long non-coding RNA (lncRNA) PCAT1 in LC was explored.*

Methods: *PCAT1 expression in 50 paired tissue samples from LC patients was monitored by real-time quantitative polymerase chain reaction (RT-qPCR). Afterwards, function assays were conducted to explore how PCAT1 participated in metastasis of LC in vitro and in vivo. Then, bio-information software and luciferase assay were utilized to predict the possible target microRNA (miR) of PCAT1 in LC.*

Results: *PCAT1 was obviously upregulated in LC tissues compared with adjacent tissues. Knockdown of PCAT1 inhibited the ability of cell migration and invasion in LC. Moreover, knockdown of PCAT1 inhibited tumor formation in vivo. Furthermore, miR-210-3p was sponged by PCAT1 in LC cells.*

Conclusion: *PCAT1 was first identified as a novel oncogene in LC and could promote LC cell migration and invasion by sponging miR-210-3p.*

Key words: *Long non-coding RNA, PCAT1, laryngeal cancer, miR-210-3p*

Introduction

As one of the most ordinary head and neck cancers worldwide, laryngeal cancer (LC) brings a huge health burden to patients and society. The number of LC patients reached 12,260 in the United States in 2013, with 3,630 LC-related deaths in the same year [1]. Therefore, it is urgent to understand the underlying mechanism of tumor progression of LC.

Long non-coding RNAs (lncRNAs) are a cluster of transcripts longer than 200 nucleotides. Recently, lncRNAs have been reported to be associated with tumor progression and regulate complicated networks in tumorigenesis. For instance, lncRNA FAL1 is upregulated in colon cancer tissues and inhibits cell apoptosis *in vitro* [2]. By sponging miR-335, lncRNA MSTO2P facilitates cell proliferation and colony formation in gastric cancer [3]. Moreover, cell proliferation and apoptosis can be regulated by upregulating lncRNA TINCR, which is induced by SP1 in gastric cancer [4]. Knockdown of lncRNA CRNDE-h is reported to be associated with poor prognosis of colorectal cancer, which might be used as a novel serum-based biomarker for diagnosis of colorectal cancer [5]. LncRNA PCAT1 is a newly-discovered oncogene in multiple types of cancers. However, the role of PCAT1 in LC remains unknown.

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Methods

Tissue specimens

Totally, we collected 50 paired LC samples from patients who had undergone surgery at Affiliated Hospital of Putian University. Before surgery, no patients received chemotherapy or radiotherapy. All fresh tissues obtained from the operation were stored immediately at -80°C. Signed written informed consents were obtained from all participants before the study. This study was approved by the Ethics Committee of Affiliated Hospital of Putian University.

Cell culture

Human LC cell lines M4E, Hep-2, M2E, and normal nasopharyngeal epithelial cell line NP69 were purchased from American Type Culture Collection (ATCC) (Manassas, VA, USA). Culture medium consisted of 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA), penicillin, streptomycin, and Dulbecco's Modified Eagle Medium (DMEM; Gibco, Rockville, MD, USA).

Cell transfection

The cDNA oligonucleotides specifically targeting PCAT1 (sh- PCAT1) was synthesized by GenePharma (Shanghai, China) and was inserted into the shRNA expression vector pGPH1/Neo, which was then used for transfection of Hep-2 cells with Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA). After 48 h, RT-qPCR was used to monitor the transfection efficiency.

RNA extraction and real-time quantitative polymerase chain reaction (RT-qPCR)

Extraction of RNA from tissues and cells utilized TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. The total RNA was reverse-transcribed to complementary DNA (cDNAs) through reverse transcription kit (Takara biotechnology Co., Ltd., Dalian, China). Primers were as follows: PCAT1 primers forward 5'-TGAGAAGAGAAATCTATTGGAACC-3', reverse 5'-GGTTTGTC-TCCGCTGCTTTA-3'; GAPDH primers forward 5'-GGGAGCCAAAAGGGTCAT-3' and reverse 5'-GAGTCCTTCCACGATACCAA-3'. Thermal cycles were as follows: 30 sec at 95°C, 5 sec for 40 cycles at 95°C, 35 sec at 60°C.

Wound healing assay

Cells transferred into 6-well plates were cultured in DMEM medium overnight. After scratched with a plastic tip, cells were cultured in serum-free DMEM. Wound closure was viewed at 48 h. Each assay was independently repeated in triplicate.

Transwell assay

Twenty-four h after transfection, 2×10^5 cells in 100 µL serum-free DMEM were transfered to top chamber of an 8-µm culture inserts (Corning, Corning, NY, USA) coated with or without 50 µg Matrigel Matrix dilution (BD, Bedford, MA, USA). Twenty percent FBS-DMEM were added to the lower chamber of the culture inserts. After 24 h , these inserts were treated with methanol for 30 min and stained by hematoxylin for 20 min. Leica DMI4000B microscope (Leica Microsystems, Heidelberg, Germany) was utilized for counting migrated and invaded cells in three random fields (×20).

Luciferase assay

The 3'-UTR of PCAT1 was cloned into the pGL3 vector (Promega, Madison, WI, USA) as wild-type (WT) 3'-UTR. Quick-change site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) was used for site-directed mutagenesis of the miR-210-3p binding site in PCAT1 3'-UTR as mutant (MUT) 3'-UTR. Then, they were used for transfection of Hep-2 cells. The luciferase assay was conducted on the dual Luciferase reporter assay system (Promega, Madison, WI, USA).

RNA immunoprecipitation assay (RIP)

Magna RIP RNA-Binding Protein Immunoprecipitation Kit (Millipore, Billerica, MA, USA) was used for RIP assay. Co-precipitated RNAs were detected by RT-qPCR.

Xenograft model

After PCAT1 was silenced in Hep-2 cells, cells were replanted into NOD/SCID mice (6 weeks old).

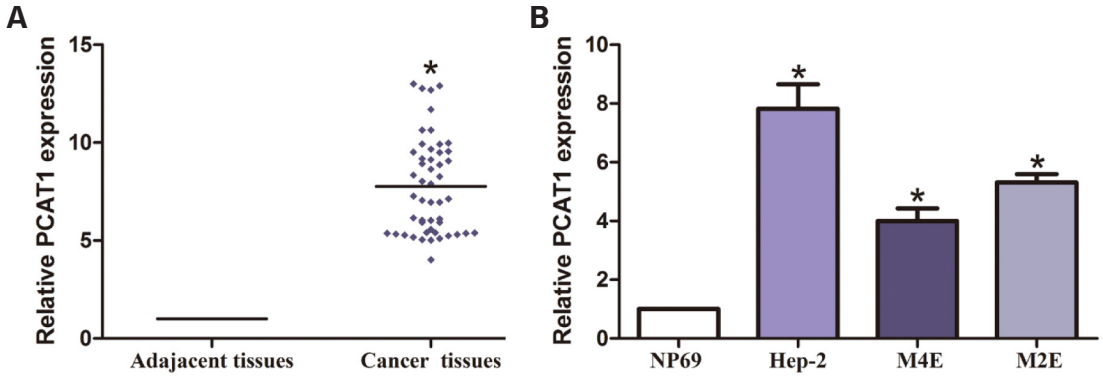

Figure 1. Expression levels of PCAT1 in laryngeal cancer tissues and cell lines. **(A):** PCAT1 expression was significantly upregulated in the laryngeal cancer tissues compared with adjacent tissues. **(B):** Expression levels of PCAT1 were detected in the human laryngeal cancer cell lines and NP69 (normal nasopharyngeal epithelial cell line) by RT-qPCR. Data are presented as mean ± standard error of the mean. *p<0.05.

Tumor volume was calculated every 5 days by the following formula: volume=length×width^2×1/2. Tumors were extracted after 4 weeks. The research was approved by the Animal Ethics Committee of Putian University.

Statistics

In this study, SPSS 20.0 (SPSS, Chicago, IL, USA) was used for statistical analyses. Data analysis was conducted by two-tailed Student's *t*-test. It was considered statistically significant if p value was <0.05.

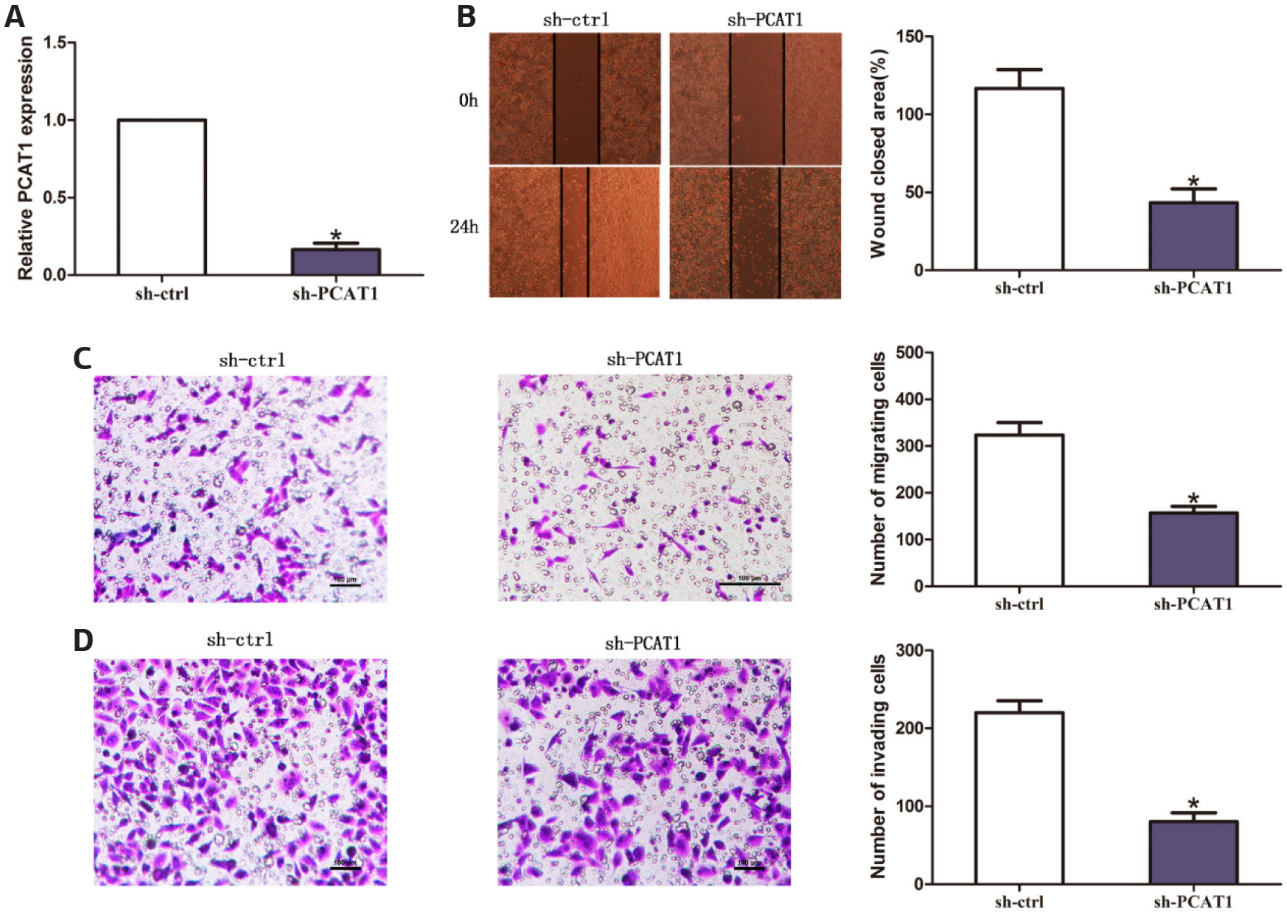

Figure 2. Knockdown of PCAT1 inhibited Hep-2 laryngeal cancer cell migration and invasion. **(A):** PCAT1 expression in Hep-2 laryngeal cancer cells transfected with PCAT1 shRNA (sh-PCAT1) and control vector (sh-ctrl) was detected by RT-qPCR. GAPDH was used as an internal control. **(B):** Wound healing assay showed that knockdown of PCAT1 significantly repressed cell migration in Hep-2 laryngeal cancer cells. **(C):** Transwell assay showed that the number of migrated cells was significantly decreased after knockdown of PCAT1 in Hep-2 laryngeal cancer cells. **(D):** Transwell assay showed that the number of invaded cells was significantly decreased after knockdown of PCAT1 in Hep-2 laryngeal cancer cells. The results represent the average of three independent experiments (mean ± standard error of the mean). *p<0.05, as compared with the control cells.

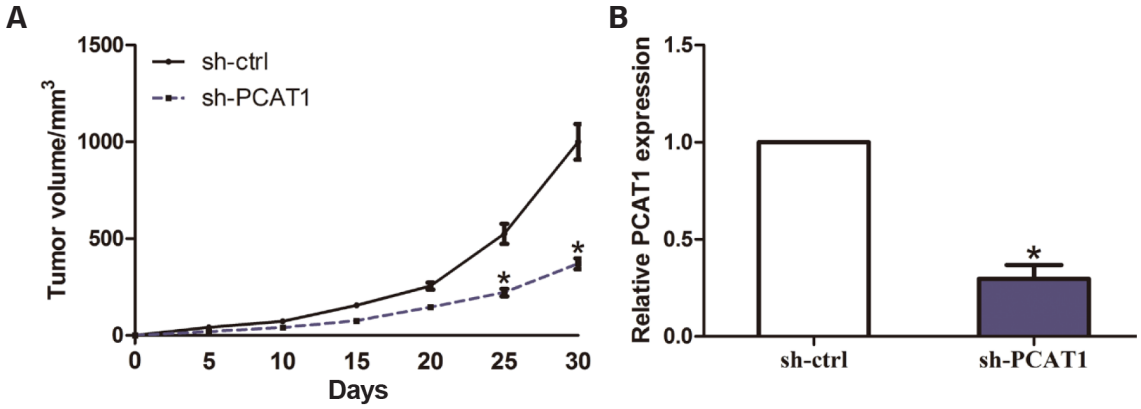

Figure 3. Knockdown of PCAT1 inhibited tumor formation *in vivo*. **(A):** Tumor volume was monitored respectively in sh-ctrl or sh-PCAT1 group. **(B):** The relative expressions of PCAT1 in tumors were examined by RT-qPCR. Data are presented as the mean ± SD of three independent experiments. *p<0.05.

Results

PCAT1 expression level in laryngeal cancer tissues and cells

RT-qPCR was used to detect PCAT1 expression in tissue samples from 50 patients with LC and 3 LC cell lines. The results showed that PCAT1 was significantly upregulated in tumor tissue samples compared with adjacent tissues (Figure 1A). The expression level of PCAT1 in LC cells was higher than that of NP69 (Figure 1B).

Knockdown of PCAT1 suppressed cell migration and invasion of laryngeal cancer cells

In this study, Hep-2 LC cell line was chosen for the knockdown of PCAT1. Then, PCAT1 expression was detected by RT-qPCR (Figure 2A). Wound healing assay showed that knockdown of PCAT1

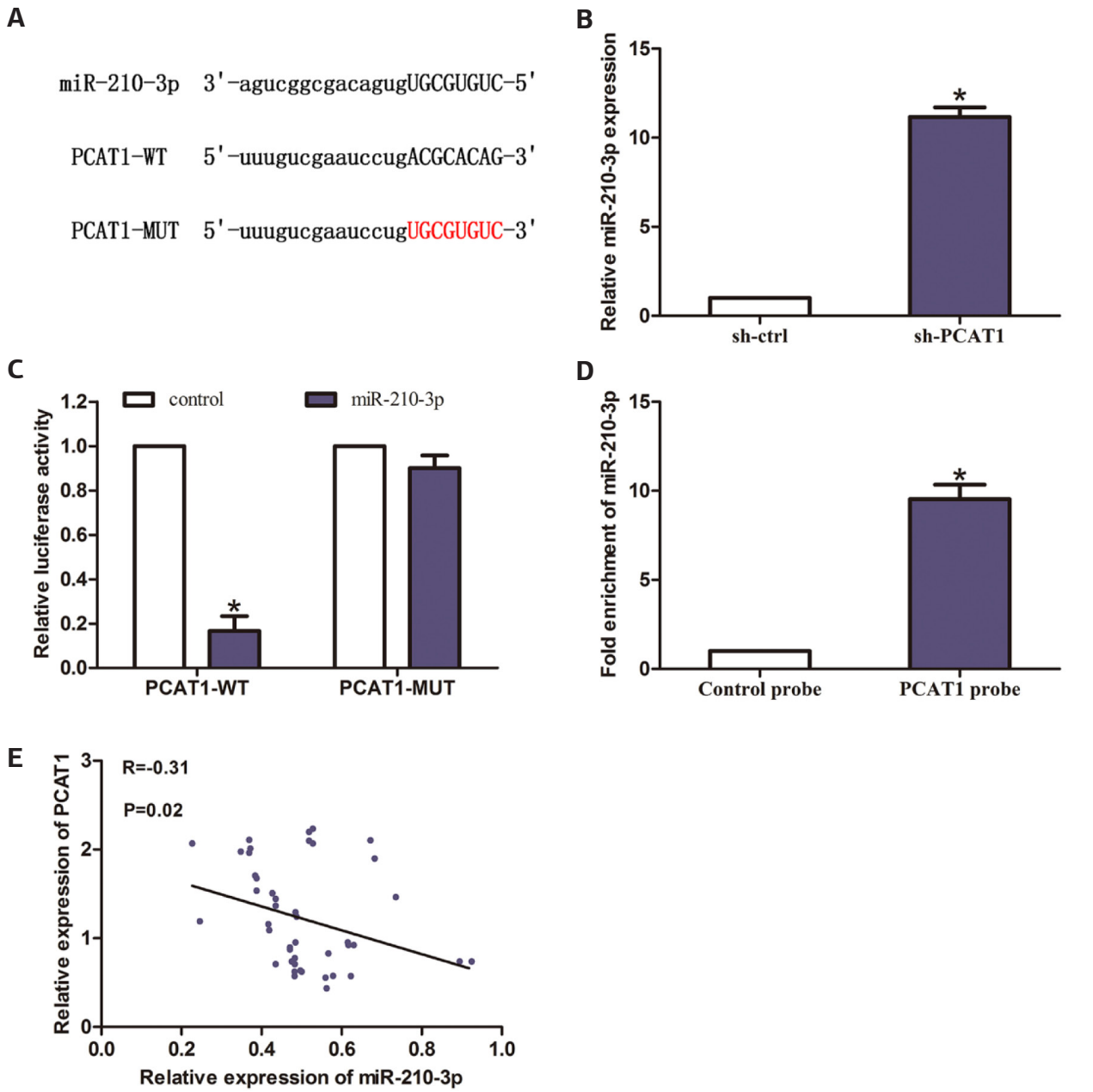

Figure 4. The interaction between PCAT1 and miR-210-3p in laryngeal cancer. **(A):** The binding sites of miR-210-3p on PCAT1. **(B):** The miR-210-3p expression was increased in sh-PCAT1 group compared with sh-ctrl group. **(C):** Co-transfection of miR-210-3p and PCAT1-WT strongly decreased the luciferase activity, while co-transfection of miR-control and PCAT1-WT did not change the luciferase activity, and co-transfection of miR-210-3p and PCAT1-MUT did not change the luciferase activity either. **(D):** RIP assay results demonstrated that miR-210-3p could be remarkably enriched in the PCAT1 group compared with control group. **(E):** The negative correlation between the expression level of miR-210-3p and PCAT-1 in laryngeal cancer tissues. The results represent the average of three independent experiments Data are presented as mean ± standard error of the mean. *p<0.05.

repressed cell migration of Hep-2 cells (Figure 2B). Transwell assay also revealed that the number of migrated and invaded cells was remarkably reduced after PCAT1 was knocked down in Hep-2 cells (Figure 2C and Figure 2D).

PCAT1 knockdown inhibited tumor formation in vivo

Then, the ability of PCAT1 in tumor formation was detected *in vivo*. The tumor size in the sh-PCAT1 group was smaller compared with sh-CTRL group (Figure 3A). Moreover, the expression level of PCAT1 in tumor tissues was detected by RT-qPCR. The results showed that PCAT1 was lower expressed in sh-PCAT1 group than in sh-CTRL group (Figure 3B).

The interaction between PCAT1and miR-210-3p in laryngeal cancer

The miRs that contained complementary base with PCAT1 are found by conducting DIANA LncBASE Predicted v.2 (http://carolina.imis.athena-innovation.gr/diana_tools/web/index.php?r=lncbasev2%2Findex-predicted). As a tumor suppressor, miR-210-3p was used to detect miRs which were interacted with PCAT1 (Figure 4A). RT-qPCR assay showed that the expression of miR-210-3p was higher in sh-PCAT1 group than that in sh-CTRL group (Figure 4B). Moreover, the result of luciferase assay showed that the luciferase activity was significantly inhibited *via* co-transfection of PCAT1-WT and miR-210-3p (Figure 4C). RIP assay results revealed that miR-210-3p was enriched in the PCAT1 group when compared with the control group (Figure 4D). Furthermore, miR-210-3p expression level was negatively correlated with PCAT1 expression in LC tissues (Figure 4E). The above results indicated that PCAT1 might work as a miR-210-3p sponge.

Discussion

LC is one of the most general malignancies in the world. Although many therapeutic methods were available for LC in the past few decades, the prognosis remains poor [6,7]. LncRNAs have been indicated to play a variety of roles in the regulation of potential activity and splicing event *via* small RNA regulatory pathways [7].

LncRNA prostate cancer-associated transcript 1 (PCAT1), located in 8q24.21, was first discovered in prostate cancer [8]. Moreover, it plays a fundamental role in prostate cancer and promotes tumor proliferation and metastasis [9]. Recently, researchers have found that PCAT1 also acts as an oncogene in other cancers. For example, multidrug resistance was suppressed after PCAT1 is knocked down in colorectal cancer [10]. PCAT-1 promotes the growth and invasion ability of cervical cancer *in vitro* [11]. In this study, PCAT1 was first identified as a novel oncogene in LC. Our study showed that PCAT1 was upregulated in LC tissues. Furthermore, after PCAT1 was knocked down in LC cells, cell migration and cell invasion of LC were significantly inhibited. Meanwhile, knockdown of PCAT1 inhibited tumor formation *in vivo*. In this study, PCAT1 was first identified as a novel oncogene in LC.

Previous studies have revealed that PCAT-1 contains binding sites for miRs and may act as a sponge for miRs. For instance, PCAT-1 induces CRKL signaling pathway *via* targeting miR-215 in hepatocellular carcinoma [12]. PCAT-1 contributes to tumorigenesis by regulating FSCN1 *via* miR-145-5p in prostate cancer [9]. We used DIANA LncBASE Predicted v.2 to predict the potential miRs containing complementary base of PCAT1. Then, miR-210-3p was selected from those potential miRs due to its tumor suppressor role in many tumors, including LC [13-16].

In this study, we found out that miR-210-3p could directly bind to PCAT1 through luciferase assay. miR-210-3p was significantly enriched by PCAT1 RIP assay. In addition, miR-210-3p expression could be upregulated by knockdown of PCAT1. On the other hand, miR-210-3p expression level was negatively correlated with PCAT1 expression in LC tissues.

In conclusion, in the present study, PCAT1 was remarkably upregulated in LC patients. PCAT1 could facilitate cell migration and invasion of LC *in vitro* and promote tumor formation *in vivo* through sponging miR-210-3p, suggesting that PCAT1/miR-210-3p axis could guide the therapy for LC as a candidate target.

Conflict of interests

The authors declare no conflict of interests.

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