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Long non-coding RNA PCAT-1 promotes cardiac fibroblast proliferation *via* upregulating TGF-β1

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Abstract. – OBJECTIVE: Recently, the vital functions of long non-coding RNAs (lncRNAs) in many diseases have been explored. This study aims to identify the function of lncRNA PCAT-1 in the development of atrial fibrillation (AF).

PATIENTS AND METHODS: Real Time-quantitative Polymerase Chain Reaction (RT-qPCR) was performed to detect PCAT-1 expression in right atrial appendage (RAA) tissues of AF patients and 35 patients with sinus rhythm (SR). Besides, cell proliferation assay was conducted in AC16 cells with PCAT-1 knockdown. Molecular mechanism of PCAT-1 in influencing the progression of AF was finally investigated.

RESULTS: PCAT-1 expression was higher in RAA tissues of AF patients than those in SR patients. Moreover, knockdown of PCAT-1 inhibited proliferation in AC16 cells. Transforming growth factor-β1 (TGF-β1) was a target of PCAT-1 and its expression in AF was positively correlated to PCAT-1 expression.

CONCLUSIONS: PCAT-1 could promote cell proliferation of AF via promoting TGF-β1, which may provide a new theory for AF development.

Keywords:
Long non-coding RNA, Atrial fibrillation, PCAT-1, TGF-β1.

Introduction

Atrial fibrillation (AF), with the incidence of 1%, is the most prevalent heart rhythm disease worldwide[1,2]. Heart failure and ischemic stroke are the most serious outcomes of AF, leading to cardiac morbidity and mortality. Although pharmacological approaches and ablation are available for AF patients, it brings a huge burden for affected people and society due to poor efficacy and potential complications[3]. Therefore, the underlying molecular mechanisms of AF is urgently needed to be explained and effective therapy is required.

Long non-coding RNAs (lncRNA) have been proved to play important roles in a variety of biological behaviors, including atrial fibrillation. For example, lncRNA NEAT1 is abnormally expressed in Huntington's disease[4]. NEAT1 participates in the inflammatory process of human lupus *via* regulating the mitogen-activated protein kinase (MAPK) pathway[5]. LncRNA HOTAIR facilities the development of Parkinson's disease by targeting LRRK2[6]. Moreover, MG53 regulates AF development by targeting transforming growth factor-β1 (TGF-β1)[7]. The metabolic changes in kidney diseases are associated with lncRNA TUG1[8]. However, the function of lncRNA PCAT-1 in AF has not been explored so far.

In this work, PCAT-1 was upregulated in AF patients. Besides, it promoted the proliferation of cardiomyocytes *in vitro*. The interaction between PCAT-1 and TGF-β1 was identified, which was believed that PCAT-1 influenced AF progression by targeting TGF-β1.

Patients and Methods

Clinical Samples

A total of 51 AF patients and 35 SR patients with valvular heart diseases who received cardiac surgery at the Sir Run Run Shaw Hospital were enrolled. RAA tissues of them were surgically

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resected. Before the operation, informed consent was achieved. These patients had no other diseases, including pulmonary disease, coronary heart disease, diabetes infective endocarditis, mellitus, hyperthyroidism, hypertension, active rheumatism, or autoimmune disease. Tissue samples were immediately stored at -80°C. All tissues were analyzed by an experienced pathologist. This study was approved by the Ethics Committee of Sir Run Run Shaw Hospital.

Cell Lines

AC16 cells (American Type Culture Collection; Manassas, VA, USA) were cultured in Roswell Park Memorial Institute-1640 medium (RPMI-1640; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Life Technologies, Gaithersburg, MD, USA) and 1% penicillin. Cells were maintained in a 5% CO_2 humidified incubator at 37°C.

RNA Extraction and Real Time-Quantitative Polymerase Chain Reaction (RT-qPCR)

Total RNA extracted from tissues using the TRIzol reagent (Invitrogen, Carlsbad, USA) was reversely transcribed to c mentary deoxyribose nucleic acids (cD s) using the Reverse Transcription Kit (Tak Biotechnology Co., Ltd., Dalian, China). R Time-quantitative Polymerase Reactio (RT-qPCR) was conducted the BI 7500 system (Applied Biosyste Foster ty, CA, USA) using SYBR Gre llowi the primers used for RT PC d 5′-TGAGAAGAG ATCTA GAACC-3′, reverse 5′-GG TCTCCGC TTTA-3′; glyceraldehyd phate de ogenase (GAPDH), fo ard 5 AAATCAGATGG-GGCAA TGG-3′ and rse 5′-TGATGG-CATG TGTGGTCATT . The thermal cycl s as f ws: 30 sec at 95°C, 5 sec at 95 d 35 at 60°C, for 40 cycles.

Cell Tra ction

tivira rpin RNA (shRNA) targeting AT-1 w nthesized and then cloned into th EF1a-EGFP-F2A-Puro vector (Bioset-ne.; iego, CA, USA). Cells were trans-cted using Lipofectamine 2000 (Invitrogen, sbad, CA, USA).

Cell Counting Kit-8 Assay (CCK-8)

In a 96-well plate, cells were seeded with 4 × 10^3 cells per well. Cell Counting Kit-8 reagent (CCK-8; Dojindo, Kumamoto, Japan) was respectively added in each well at 0, 24, 48, and 72 h according to the instructions. After 2-h incubation at 37°C, the optical density (OD) val s examined using a microplate reader Hercules, CA, USA).

Colony Formation Assay

After cell culture in a six- l plate days, cells were fixed with met l and stai with 0.1% crystal violet. At la e num r of co was counted for com n.

Ethynyl Deox dine) Incorporation Assay

Cell pro of transfec lls was detected *vi* Ed (Roche, Mannheim, Germany). Representati mages were obtained thr e Zeiss Ax t Photomicroscope (C Zeiss, Oberkochen, Germany).

tistical Ana is

istical P ct and Service Solutions (SP , Chicago, IL, USA) was utilized fo cal analysis. The results were presented as mean ± standard deviation (S.D.). Chi-test and Student's *t*-test were selected for ata yses when appropriate. It was considered statistically significant when $p<0.05$.

Results

PCAT-1 Level AF and SR Patients

RT-qPCR was conducted for detecting PCAT-1 expression in RAA tissues extracted from 51 AF patients and 35 SR patients. As a result, PCAT-1 was significantly upregulated in AF patients compared with those of SR patients (Figure 1).

Knockdown of PCAT-1 Inhibited Proliferation of AC16 Cells

Transfection efficacy of PCAT-1 shRNA was verified in AC16 cells (Figure 2A). Subsequently, the results of the CCK-8 assay showed that the proliferation of AC16 cells was inhibited after PCAT-1 knockdown (Figure 2B). Furthermore, the results of colony formation assay showed that the colony number was significantly reduced in AC16 cells with PCAT-1 knockdown (Figure 2C). EdU assay also showed a decreased number of EdU-positive cells after transfection of PCAT-1 shRNA (Figure 3A and 3B).

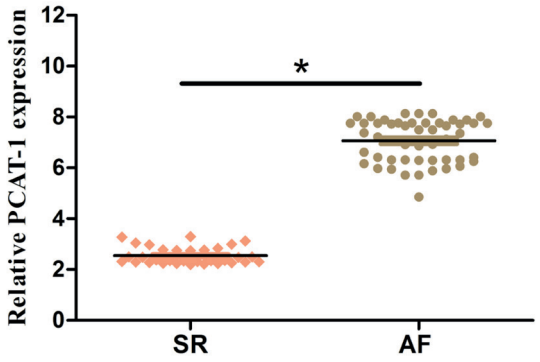

Figure 1. Expression levels of PCAT-1 in RAA tissues. PCAT-1 expression was remarkably downregulated in the AF patients compared with that of SR patients. Data are presented as the mean ± standard error of the mean. *p<0.05.

The Interaction Between TGF-β1 and PCAT-1

Our previous work suggested PCAT-1 acted as an anti-fibrotic role in atrial fibrillation. Recent studies verified that TGF-β1 could inhibit the progression of AF. To explore the interaction between TGF-β1 and PCAT-1, we examined the TGF-β1 level in AC16 cells transfected with PCAT-1 siRNA, which was remarkably downregu- ure 4A). We further detected TGF-β expressio in RAA tissues and found that TG expression was markedly higher in AF patie that in SR patients (Figure 4B). Th linear lation analysis revealed a positi orrelation TGF-β1 expression and T-1 ex ession tissues (Figure 4C).

Discussi

Recent many s have demonstrated that cardiac fibrotic rem g is vital progress of AF ing this pro enhancement of fi- b st proliferation is a k y event. It is reported non-coding RNAs are capable of regulating

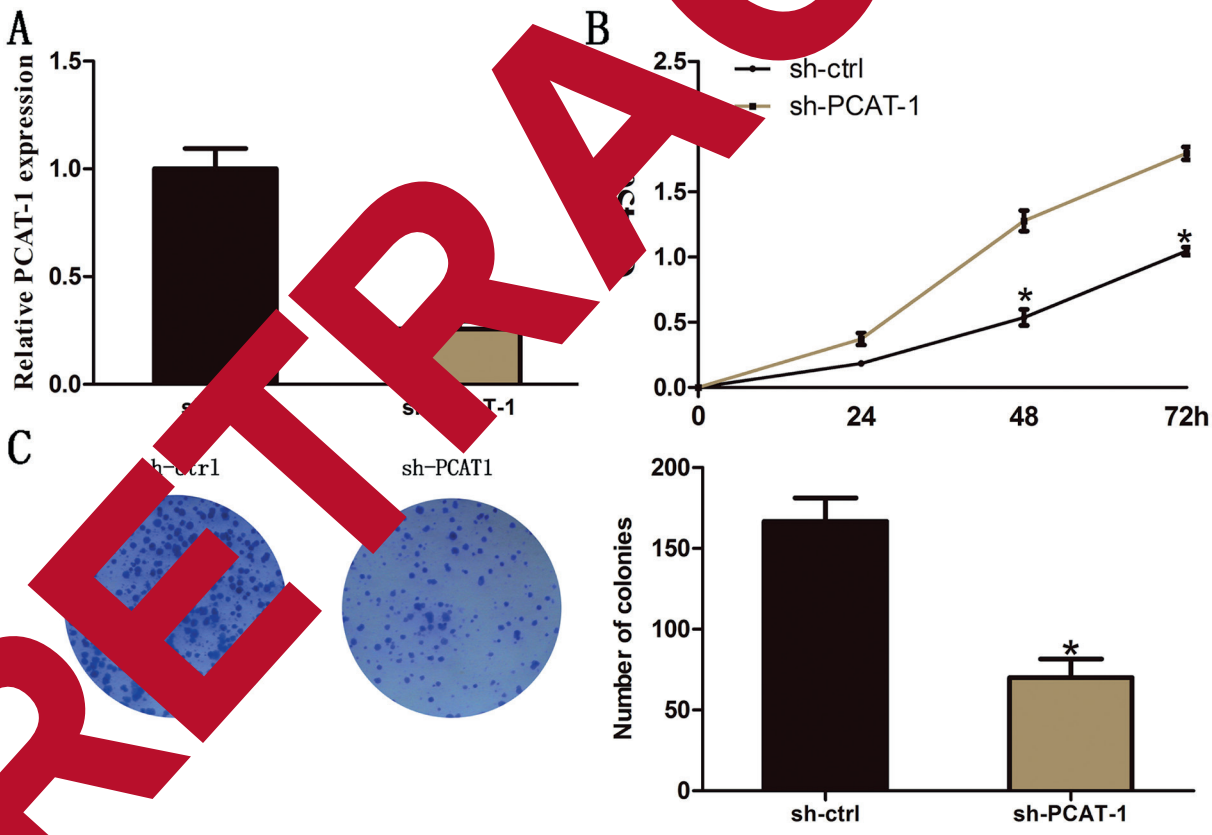

Figure 2. CCK-8 assay and colony formation assay showed that knockdown of PCAT-1 decreased AC16 cell proliferation. **A,** PCAT-1 expression in cells transfected with empty vector (sh-ctrl) or PCAT-1 lentiviral small hairpin RNA (sh-PCAT-1) was detected by RT-qPCR. **B,** CCK-8 assay showed that knockdown of PCAT-1 significantly inhibited cell proliferation in AC16 cells. **C,** Colony formation assay showed that knockdown of PCAT-1 markedly decreased colony number formed in AC16 cells (magnification: 40×). *p<0.05 compared with the control cells.

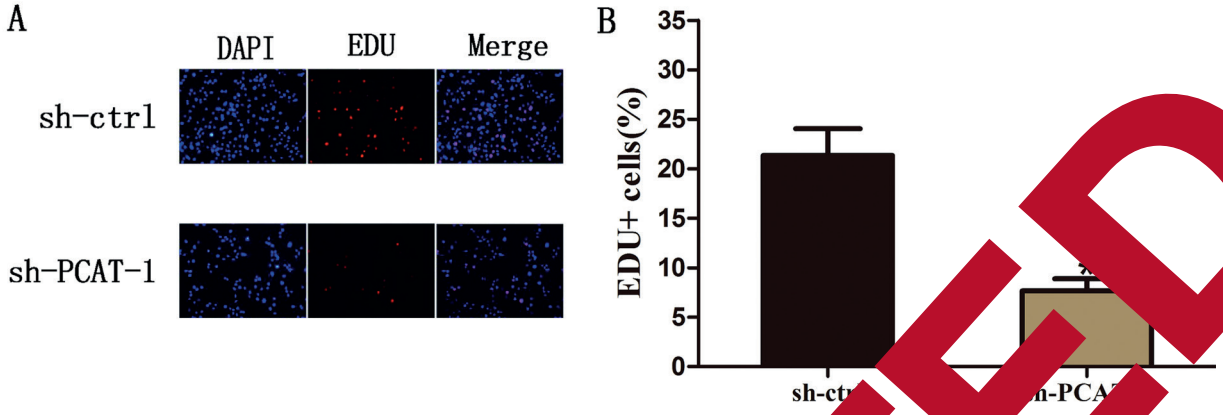

Figure 3. EdU assay showed that knockdown of PCAT-1 suppressed AC16 cell p[illegible]ration. A, [illegible]sent[illegible] images of EdU-labeled cells in the sh-PCAT-1 group and control group (magnification: 40[illegible]. EdU assay sho[illegible]e number of EdU-positive cells was remarkably reduced via knockdown of PCAT-1 in AC16 [illegible]05 compared w[illegible]e control cells.

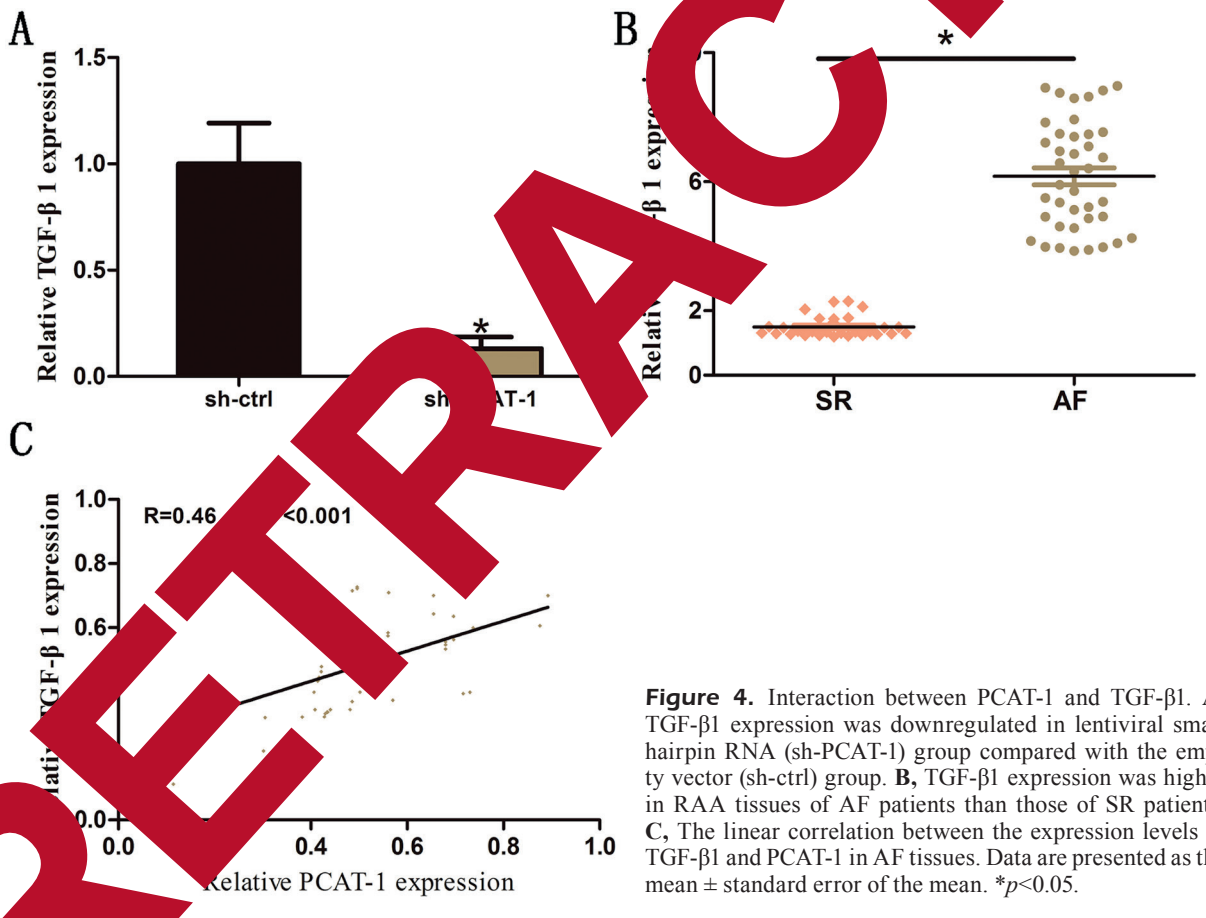

Figure 4. Interaction between PCAT-1 and TGF-β1. **A,** TGF-β1 expression was downregulated in lentiviral small hairpin RNA (sh-PCAT-1) group compared with the empty vector (sh-ctrl) group. **B,** TGF-β1 expression was higher in RAA tissues of AF patients than those of SR patients. **C,** The linear correlation between the expression levels of TGF-β1 and PCAT-1 in AF tissues. Data are presented as the mean ± standard error of the mean. *p<0.05.

[illegible]athological progression of AF. For example, l[illegible]A AK055347 is upregulated in AF patients and induces progression of AF *in vitro*[9]. The serum level of miR-26a is upregulated in postoperative AF patients[10]. MiR-34a participates in the progress of atrial fibrillation by regulating AnkyrinB[11].

LncRNA prostate cancer associated transcript 1 (PCAT1), located on 8q24.21, was initially discovered in prostate cancer[12]. Later, several stud-

ies[13-16] revealed that PCAT-1 is a vital regulator in other diseases. In the present work, PCAT-1 was found to be upregulated in RAA tissues of AF patients compared with those of SR patients. Furthermore, PCAT-1 knockdown suppressed cell growth in AC16 cells. These data indicated that PCAT-1 induced cardiac fibrotic remodeling and further promoted AF progression by promoting fibroblast proliferation.

Recent works revealed that non-coding RNAs participate in cardiac diseases by mediating target genes, among which TGF-β1 plays an important role in fibroblast proliferation. For instance, circulating TGF-β1 is upregulated in paroxysmal AF patients undergoing catheter ablation[17]. TGF-β1 is involved in the development of postoperative AF by interacting with MAPKs and TRAF6[18]. High expression of miR-21 promotes cardiac fibrosis through the CADM1/STAT3 pathway, which might be a potential therapeutic target[19].

Our study firstly revealed that TGF-β1 was upregulated in AF patients compared with that of SR patients. Furthermore, TGF-β1 expression was positively regulated by PCAT-1. All the results above suggested that PCAT-1 might pr[illegible] fibroblast proliferation *via* targeting TGF-[illegible]

Conclusions

Above data identified tha[illegible]AT[illegible]ould induce cardiac fibrotic remo[illegible]g by u[illegible]gulating TGF-β1, thereafter regul[illegible]the de[illegible]of AF. These findings s[illegible]est[illegible]CAT-1[illegible]y contribute to the th[illegible]py for [illegible]a candidate target.

Conflict o[illegible]terests

The Aut[illegible]eclare that they hav[illegible]onflict of interests.

Fund[illegible]owled[illegible]ents

[illegible]ional [illegible]l Sci[illegible] Foundation of China (No. [illegible]46); N[illegible]C[illegible] Foundation of Zhejiang Province (Pr[illegible] No. L[illegible]007); Medical Health Science and Tec[illegible]ogy of Zhej[illegible]ng Province (Project No. 2016146503); Y[illegible]al Research Projects (2018YJ05).

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