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Long noncoding RNA GAS5 attenuates cardiac fibroblast proliferation in atrial fibrillation *via* repressing ALK5

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Abstract. – OBJECTIVE: Recently, long noncoding RNAs (lncRNAs) have caught more attention for their role in the progression of many diseases. Among them, lncRNA GAS5 (Growth Inhibition Specificity 5) was studied in this research to identify how it affects the progression of atrial fibrillation (AF).

PATIENTS AND METHODS: In 40 patients with AF and 30 patients with sinus rhythm (SR), the GAS5 expression of the right atrial appendage (RAA) tissues was detected by the quantitative Real Time-Polymerase Chain Reaction (qRT-PCR). Moreover, the cell proliferation assay was conducted in AC16 cells transfected with GAS5 inhibitor and mimics, respectively. Furthermore, the qRT-PCR was performed to uncover the mechanism.

RESULTS: In the research, the expression of GAS5 in RAA tissues was decreased significantly in AF patients than that in SR ones. Moreover, overexpression of GAS5 inhibited cell growth in AC16 cells, while knockdown of GAS5 promoted cell growth in AC16 cells. In addition, further experiments revealed that ALK5 was a target of GAS5 and its expression in AF tissues negatively correlated to GAS5 expression.

CONCLUSIONS: These results indicate that GAS5 could inhibit cell proliferation of AF *via* suppressing ALK5, which may offer a new vision for interpreting the mechanism of AF development.

Key Words:
Long noncoding RNA, Atrial fibrillation, GAS5, ALK5.

Introduction

Atrial fibrillation (AF) is the most prevalent heart rhythm disease all over the world, especially in the elderly; it accounts for approximately 0.5% of the total population in the world[1,2]. AF exacerbates heart failure and ischemic stroke, which is a crucial contributor to cardiac morbidity and mortality. The main treatments of AF include pharmacological approaches and ablation; however, the therapies currently available lack sufficient efficacy and have considerable potential complications and recurrences, which results in a huge social burden for the patients and even the nations[3]. Thus, it's urgent to find out novel therapies based on the underlying molecular mechanisms of AF which can provide effective treatments.

Non-coding RNAs have been proved[4-6] to play important roles in a variety of biological behaviors including atrial fibrillation. For example, the upregulation of long noncoding RNA (lncRNA) AK055347 speeds up the process of AF by regulating the energy metabolism of cardiomyocytes[7]. Overexpression of MiR122 is found to be associated with cardiomyocyte apoptosis in atrial fibrillation[8]. Through the regulation of AnkB expression, miR34a plays an important role in the electrophysiological remodeling and the progression of AF[9]. High expression of miR-21 promoted the cardiac fibrosis through CADM1/STAT3 pathway and might be a potential therapeutic target[10]. However, the function of lncRNA GAS5 (Growth Inhibition Specificity 5) in AF hasn't been explored so far.

In this study, GAS5 was found decreased in AF patients. Besides, it inhibited the proliferation of cardiomyocytes *in vitro*. What's more, we further discovered the interaction between GAS5 and ALK5 as well as the possible underlying mechanism.

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Patients and Methods

Clinical Samples and Cell Lines

40 AF patients and 30 SR patients who received cardiac surgery at the Affiliated Hospital of Qingdao University were enrolled for the right atrial appendage (RAA) tissues. Before the operation, the written informed consent was achieved. These patients had no other diseases including pulmonary disease, coronary heart disease, diabetes infective endocarditis, diabetes mellitus, hyperthyroidism, hypertension, active rheumatism, or autoimmune disease. Tissues got from the surgery were stored immediately at –80°C. All tissues were analyzed by an experienced pathologist. This study conforms to requirements of the Ethics Committee of the Affiliated Hospital of Qingdao University.

AC16 cell line (American Type Culture Collection) (ATCC; Manassas, VA, USA) was used in this study. Culture medium consisted of penicillin, Roswell Park Memorial Institute-1640 (RPMI-1640) medium (Thermo Fisher Scientific, Waltham, MA, USA), and 10% fetal bovine serum (FBS; Gibco, Rockville, MD, USA). Besides, cells were cultured in a humidified incubator, which contained 5% CO_2 and was set at 37°C.

RNA Extraction and Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR)

Total RNA, obtained from samples with TRIzol reagent (Invitrogen, Carlsbad, CA, USA), was reverse-transcribed to complementary Deoxyribose Nucleic Acids (cDNAs) using reverse Transcription Kit (TaKaRa Biotechnology Co., Ltd., Dalian, China). The performance of qRT-PCR was conducted on ABI 7500 system (Applied Biosystems, Foster City, CA, USA). SYBR Green real-time PCR was applied. Following are the primers used for qRT-PCR: GAS5, forwards 5'-CCTATCCCTTTCTCTAAGAA-3' and reverse 5'-ACTTCTGCAAAAACGTGCTG-3'; ALK5, forward 5'-CTTGACATGATTAGCTGGCATGATT-3' and reverse 5'-CCTGTGCAATATGCCGTGTAGA-3'; GAPDH, forward 5'-CCAAAATCAGATGGGGCAATGCTGG-3' and reverse 5'-TGATGGCATGGACTGTGGTCATTCA-3'. The thermal cycle was as follows: 30 sec at 95°C, 5 sec at 95°C for 40 cycles, 35 sec at 60°C.

Cell Transfection

After synthesized, a lentiviral virus targeting GAS5 was cloned into the pLenti-EF1a-EGFP-F2A-Puro vector (Biosettia Inc., San Diego, CA, USA). 293T cells were used for packaging GAS5 lentiviruses (GAS5) and the empty vector (control). Lentiviral small hairpin RNA (shRNA) targeting GAS5 was synthesized and then cloned into the pLenti-EF1a-EGFP-F2A-Puro vector (Biosettia Inc., San Diego, CA, USA). Then, 293T cells were used for the packaging of the viruses, the GAS5 lentiviruses (sh-GAS5) and the empty vector (sh-ctrl).

Cell Counting Kit-8 (CCK-8) Assay

In a 96-well plate, cell lines were seeded 4 × 10^3 cells per well, CCK-8 reagent (Dojindo, Kumamoto, Japan) was respectively added to the wells at 0, 24, 48, and 72 h according to the instructions, and then the plate was incubated for 2 h at 37°C. OD (optical density) value was examined using a microplate reader (Bio-Rad, Hercules, CA, USA).

Colony Formation Assay

After cultured with FBS for 14 days in a six-well plate, all the cells were fixed with methanol and stained with 0.1% crystal violet. Meanwhile, a number of colonies were counted for comparison.

Statistical Analysis

Statistical Product and Service Solutions (SPSS) 17.0 (SPSS, Chicago, IL, USA) was utilized for the statistical analysis. The results were presented as mean ± SD. Chi-square test, Student's *t*-test, and Kaplan-Meier method were selected when appropriate. It was considered statistically significant when $p<0.05$.

Results

GAS5 Level AF and SR Patients

Firstly, qRT-PCR was conducted for detecting GAS5 expression in 40 AF patients' and 30 SR patients' RAA tissues. As a result, GAS5 was significantly downregulated in AF patients compared with SR patients (Figure 1).

GAS5 Inhibited Cell Proliferation of AC16 Cells

AC16 cells were transfected with GAS5 lentiviruses or shRNA. Then, the GAS5 expression was determined by the qRT-PCR (Figure 2A and 2B). Moreover, the results of CCK-8 assay showed that cell proliferation of AC16 cells was

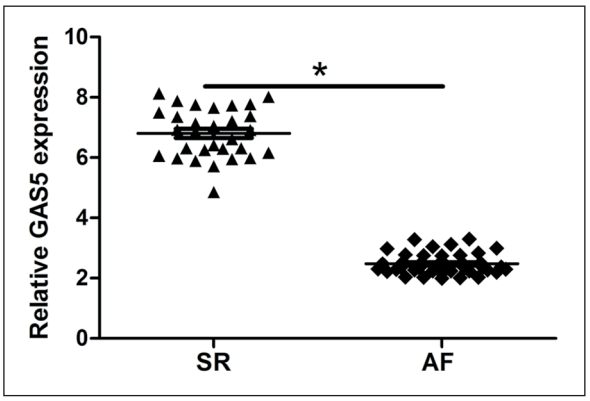

Figure 1. Expression levels of GAS5 in RAA (right atrial appendage) tissues. GAS5 expression was significantly decreased in the AF patients compared with SR patients. Data are presented as the mean ± standard error of the mean. *$p<0.05$.

inhibited after GAS5 was overexpressed (Figure 3A), and cell proliferation of AC16 cells was promoted after GAS5 was knocked down (Figure 3B). Furthermore, the results of colony formation assay showed that colonies of AC16 cells were decreased after GAS5 was overexpressed, and colonies of AC16 cells was increased after GAS5 was knocked down (Figure 3C).

The Interaction Between ALK5 and GAS5

Our previous study suggested that GAS5 acted as an anti-fibrotic role in atrial fibrillation. Recent studies verified that ALK5 could enhance the progression of AF. To explore the interaction between ALK5 and GAS5, we conducted qRT-PCR and found that ALK5 level of AC16 cells was higher in GAS5 shRNA group compared with that in control group (Figure 4A), and ALK5 level of AC16 cells was lower in GAS5 lentiviruses group compared with that in control group (Figure 4B). We further detected ALK5 expression in RAA tissues and found that ALK5 expression was significantly higher in AF patients than that in SR patients (Figure 4C). The linear correlation analysis revealed that the ALK5 expression negatively correlated to GAS5 expression in ovarian cancer tissues (Figure 4D).

Discussion

AF is one of the most common arrhythmias which bring a huge burden to both the patients and society. During AF progression, cardiac fibrotic remodeling is a vital progress. A number of studies have shown that several noncoding RNAs take part in the cardiac fibrotic remodeling through regulating fibroblast proliferation. For example, by targeting the VEGF-A/MAPK signal pathway, the miRNA-29a suppressed the fibroblasts proliferation and cardiac fibrosis showing that miRNA-29a might play a role in cardiac fibrosis[8]. In addition, miR-378 secreted from cardiomyocytes following mechanical stress played a suppressive role in excessive cardiac fibrosis[9]. While, miR-101a acted as an anti-fibrotic role in cardiac fibrosis by modulating TGF-β signaling pathway[10]. By the miR-135a-TRPM7-collagen pathway, miR-135a played a part in suppressing cardiac fibrosis[11].

LncRNA GAS5 (Growth Inhibition Specificity 5) has been proved to play an important role in many diseases. For example, lncRNA GAS5

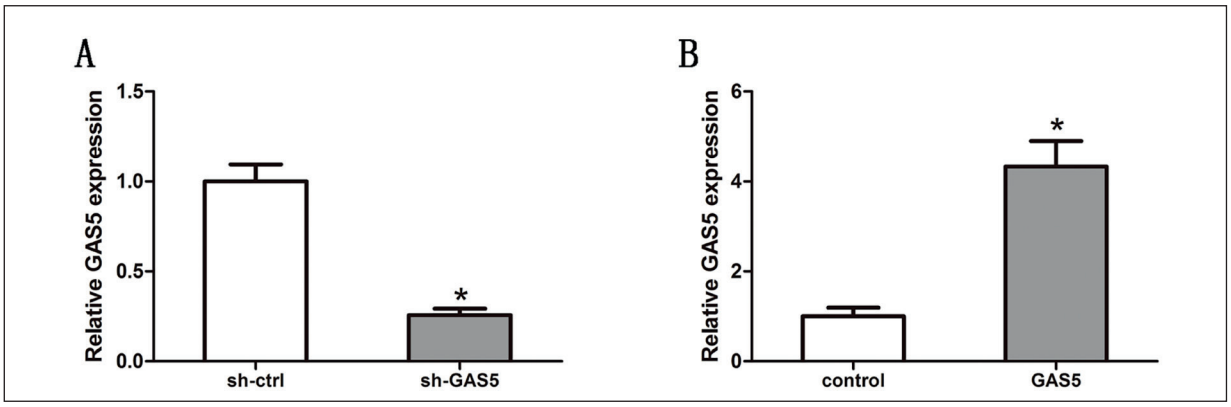

Figure 2. GAS5 decreased AC16 cell proliferation. ***A,*** GAS5 expression in cells transfected with empty vector (sh-ctrl) or GAS5 lentiviral small hairpin RNA (sh-GAS5) was detected by qRT-PCR. ***B,*** GAS5 expression in cells transfected with GAS5 lentiviruses (GAS5) or empty vector (control) was detected by qRT-PCR. GAPDH was used as an internal control. *$p<0.05$, as compared with the control cells.

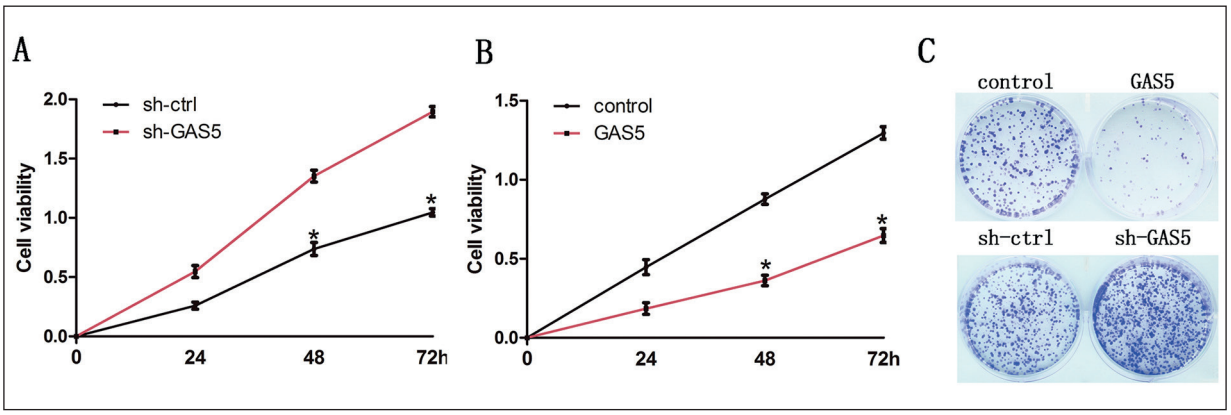

Figure 3. GAS5 suppressed AC16 cell proliferation. ***A,*** CCK-8 assay showed that knockdown of GAS5 significantly promoted cell proliferation in AC16 cells. ***B,*** CCK-8 assay showed that the overexpression of GAS5 significantly decreased the cell proliferation in AC16 cells. ***C,*** Colony formation assay showed that the overexpression of GAS5 significantly decreased the colonies of AC16 cells, and the knockdown of GAS5 significantly increased the colonies of AC16 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.

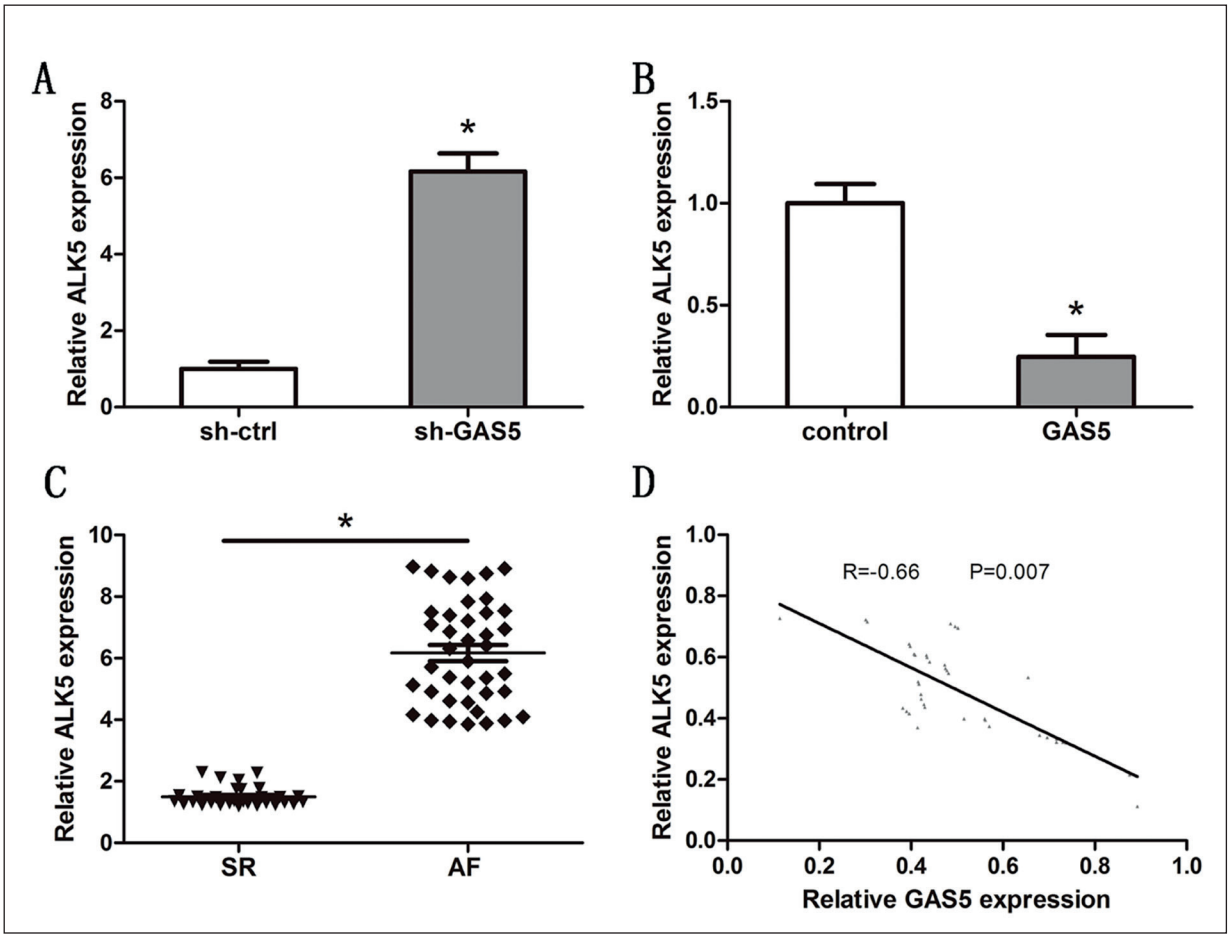

Figure 4. Interaction between GAS5 and ALK5. ***A,*** ALK5 expression was increased in lentiviral small hairpin RNA (sh-GAS5) group compared with empty vector (sh-ctrl) group. ***B,*** ALK5 expression was decreased in GAS5 lentiviruses (GAS5) group compared with empty vector (control) group. ***C,*** ALK5 expression of RAA tissues was lower in AF patients than that in SR patients. ***D,*** The linear correlation between the expression level of ALK5 and GAS5 in AF tissues. The results represent the average of three independent experiments Data are presented as the mean ± standard error of the mean. *$p<0.05$.

is considered to be a new vascular remodeling regulator induced by hypertension. Knockdown of GAS5 aggravates hypertension-induced microvascular dysfunction resulting in retinal neovascularization and capillary leakage[11]. In the present study, GAS5 was found downregulated in RAA tissues of AF patients compared with SR patients. Furthermore, after GAS5 expression was upregulated, cell growth in AC16 cells was found to be inhibited. Moreover, knockdown of GAS5 could promote AC16 cell growth. These data indicate that GAS5 repressed cardiac fibrotic remodeling and further attenuated AF progression through inhibiting fibroblast proliferation.

Latest studies reveal that noncoding RNAs function in cardiac diseases by targeting related genes, among which Alk5 (activin-like kinase 5) has been recently investigated for its important role in fibroblast proliferation. Alk5 (activin-like kinase 5) plays an important role in the development of the smooth muscle cells which surround coronary arteries and control homeostasis of the myocardial vascular plexus during the cardiogenesis[12]. It has been reported[13] that ALK5 serves as an important part in maintaining the integrity of the heart valve, and inhibition of ALK5 leads to physeal dysplasia in rats. While in the kidney disease model, the repression of ALK5 is a crucial therapy treatment[14]. In addition, ALK5 controls the lung myofibroblast and regulates the growth of lipofibroblast call during lung development[15]. ALK5 targeted by miR-27b plays an anti-fibrotic role in the left atrium which may be a novel therapeutic target for the treatment of the atrial fibrillation[16].

Our study revealed that ALK5 was downregulated in AF patients compared with SR patients. Furthermore, ALK5 expression could be downregulated with overexpression of GAS5 and ALK5 expression could be upregulated with knockdown of GAS5. All the above-results suggest that GAS5 might inhibit myocardiocytes' proliferation *via* targeting ALK5.

Conclusions

GAS5 could reverse cardiac fibrotic remodeling through repressing ALK5. These findings suggest that GAS5 may contribute to therapy for AF as a candidate target.

Conflict of Interest

The Authors declare that they have no conflict of interests.

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