MEDICAL SCIENCE MONITOR

SPECIAL REPORTS

e-ISSN 1643-3750
© Med Sci Monit, 2017; 23: 6042-6048
DOI: 10.12659/MSM.907118

Received: 2017.09.13
Accepted: 2017.11.03
Published: 2017.12.21

# Plasma Long Non-Coding RNA (lncRNA) GAS5 is a New Biomarker for Coronary Artery Disease

Authors' Contribution:
Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
Funds Collection G

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**Source of support:** Departmental sources

Our study aimed to investigate the diagnostic value of long non-coding RNA (lncRNA) GAS5 for coronary artery disease (CAD) and to explore the mechanism of the role of GAS5 in CAD.

A total of 30 patients with CAD were selected from January 2015 to January 2017 in The First Hospital of Tianmen. In addition, 30 healthy individuals were selected as a control group, and patients with various other types of cardiovascular diseases were also selected. Expression of GAS5 in plasma of all participants was detected by quantitative real-time PCR. Receiver operating characteristic (ROC) curve analysis was performed to investigate the diagnostic value of GAS5 for CAD. Levels of mammalian target of rapamycin (mTOR) and phospho-mTOR (p-mTOR) in human primary coronary artery endothelial cells (HCAECs) were detected by western blotting.

Compared with normal healthy people, expression level of lncRNA Novlnc6 was significantly reduced in patients with CAD and diabetes mellitus, but not in patients with other types of cardiovascular diseases, such as hypertension, abnormal aortic aneurysm, viral myocarditis. In addition, the expression level of GAS5 was significantly lower in patients with CAD compared to patients with diabetes mellitus. ROC curve analysis showed that GAS5 may serve as a promising biomarker for CAD. GAS5 knockdown and overexpression showed no significant effect on the level of mTOR) in HCAECs. However, GAS5 knockdown significantly increased the level of phospho-mTOR (p-mTOR), and GAS5 overexpression significantly decreased the level of p-mTOR. Treatment with mTOR inhibitor and activator showed no significant effect on expression of GAS5 in HCAECs.

GAS5 plays a role as upstream regulator of the mTOR pathway to participate in the development of CAD. GAS5 was specifically downregulated in patients with CAD, and it may serve as a promising biomarker for CAD.

**MeSH Keywords:** Coronary Artery Disease • Metabolic Networks and Pathways • RNA, Long Noncoding • TOR Serine-Threonine Kinases

**Full-text PDF:** https://www.medscimonit.com/abstract/index/idArt/907118

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## Background

As one of the most common types of severe heart disease, coronary artery disease (CAD) is mainly caused by the damage to the major blood vessels that provide nutrients, blood, and oxygen to the heart [1]. Therefore, CAD is also called ischemic heart disease. CAD is a group of cardiovascular diseases including myocardial infarction, unstable angina, stable angina, and sudden cardiac death [2]. With the development of modern society and the changes in lifestyle, the incidence of CAD is increasing year by year, and CAD has become a main cause of death all over the world [3]. Especially in China, CAD has been reported to be responsible for more than 40% of all deaths [4]. Although various treatments have been developed to treat CAD, drug treatment is still one of the major treatment strategies for CAD. However, the application of drug treatment is still challenged by the adverse side effects caused by the long-term use of drugs and the development of drug tolerance [5]. Thus, the identification of biomarkers for the diagnosis of CAD would definitely improve the early treatment of this disease.

Long non-coding RNA (lncRNA) is a group of non-coding RNA containing more than 200 nucleotides [6]. It has been well accepted that lncRNAs have pivotal functions in nearly all the biological processes of human body [7]. LncRNAs have also been proven to play major roles in the development and progression of a variety of human diseases including different types of cancers, liver diseases and heart diseases [8–10]. As an lncRNA, GAS5 was found to play important roles in the progression of human cancers [11]. A recent study also found that GAS5 can control the activation of cardiac fibroblast indicating the possible involvement of GAS5 in hear diseases [12]. However, the functionality of GAS5 in the development of CAD still has not been reported.

In this study, expression of GAS5 in patients with CAD and various cardiovascular diseases was detected. The diagnostic value of GAS5 for CAD was analyzed and the interaction between GAS5 and mammalian target of rapamycin (mTOR) pathway was also explored.

## Material and Methods

### Patients

A total of 30 patients with CAD treated at The First Hospital of Tianmen were selected from January 2015 to January 2017 for inclusion in this study. CAD patients were diagnosed by coronary angiography according to the guidelines established by American College of Cardiology/American Heart Association [13]. Patients with cancer and other severe diseases, patients with serious infection within six weeks of the start of the study and the patients with active chronic inflammatory disease were excluded. At the same time, a total of 30 healthy individuals were selected to serve as control group. The 30 patients included 12 females and 18 males; the ages ranged from 29 years to 76 years with an average age of 49±11.8 years. The 30 healthy individuals included 14 females and 16 males; the ages ranged from 26 years to 69 years with the average age of 46±10.7 years. In addition, patients with other types of cardiovascular diseases were also included in this study. See Table 1 for the general information of those patients. No significant differences in background information was found among the different groups. The Ethics Committee of our hospital approved this study. All patients signed the informed consent.

### Blood extraction and plasma preparation

Whole blood (100 mL) was extracted from each patient. Blood was transferred to anticoagulant tubes, followed by

**Table 1.** General information of patients used in this study.

| Diseases | Cases | Gender | | Age range | Average age |
|---|---|---|---|---|---|
| | | Male | Female | | |
| Healthy control | 30 | 16 | 14 | 26–69 | 46±10.7 |
| Coronary artery disease | 30 | 18 | 12 | 29–76 | 49±11.8 |
| Diabetes mellitus | 10 | 4 | 6 | 32–61 | 41±9.8 |
| Hypertension | 7 | 3 | 4 | 27–69 | 43±12.3 |
| Abnormal aortic aneurysm | 8 | 4 | 4 | 22–71 | 41±15.4 |
| Viral myocarditis | 10 | 4 | 6 | 33–76 | 51±13.1 |
| Atrial fibrillation | 9 | 4 | 5 | 35–63 | 47±14.4 |
| Valvular disease | 13 | 7 | 6 | 29–71 | 48±15.1 |
| Dilated cardiomyopathy | 7 | 3 | 4 | 31–72 | 45±17.1 |
| Peripheral artery disease | 8 | 4 | 4 | 29–71 | 41±12.0 |





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centrifugation at 3,000 rpm/minute for 10 minutes to collect plasma. Plasma samples were aliquoted into Eppendorf tubes with 500 µL in each tube, and were stored at 4°C before use.

### Cell culture

Human primary coronary artery endothelial cells (HCAECs) were purchased from (ATCC® PCS-100-020™). Cells were cultured according to the instructions provided by ATCC. Serum was not added into the medium in cases of drug treatment. Cells were harvest during logarithmic growth phase for subsequent experiments.

### Establishment of lncRNA GAS5 knockdown and overexpression HCAEC cell lines

LncRNA GAS5 were synthesized by Guilin Pavay Gene Pharmaceutical Co., Ltd (Guangxi, China). The sequences were: 5'-GGACCAGCUUAAUGGUUCUTT-3' (sense) and 5'-AGAACCAUUAAGCUGGUCCTT-3' (antisense). GAS5 overexpression vector was established by inserting an EcoRI-EcoRI fragment containing full length GAS5 into pIRSE2-EGFP (Clontech, Palo Alto, CA, USA). HCAECs were cultured overnight to reach 80% to 90% confluent. Lipofectamine 2000 transfection reagent (11668-019, Invitrogen, Carlsbad, USA) was used for transfection according to the instructions.

### Quantitative real-time PCR

Total RNA was extracted from plasma and cultured HCAECs using TRIzol reagent (Invitrogen, USA). RNA samples were tested by NanoDrop™ 2000 Spectrophotometers (Thermo Fisher Scientific, USA), and samples with a A260/A280 ratio between 1.8 and 2.0 were used in reverse transcription to synthesize cDNA using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific, USA). SYBR® Green Real-Time PCR Master Mixes (Thermo Fisher Scientific, USA) was used to prepare PCR reaction system. The following primers were used in PCR reactions: 5'-CCATGGATGACTTGCTTGGG-3' (forward) and 5'-TGCATGCTTGCTTGTTGTGG-3' (reverse) for GAS5; 5'-TCGACAGTCAGCCGCATCTTCTTT-3' (forward) and 5'-ACCAAATCCGTTGACTCCGACCTT-3' (reverse) for GAPDH. PCR reaction conditions were: 95°C for one minute, followed by 40 cycles of 95°C for 15 seconds and 65°C for 40 seconds. Data were analyzed using 2-ΔΔCT methods, and relative expression level of GAS5 was normalized to endogenous control GAPDH.

### Western blotting

Conventional method was used to extract total protein from cultured cells, and protein quality was tested by BCA method. Then 10% SDS-PAGE gel electrophoresis was performed to separate protein, followed by transmembrane to PVDF. After blocking in 5% skim milk, membranes were washed and incubated with primary antibodies including rabbit anti-mTOR (1: 2,000, ab2732, Abcam), anti-phospho-mTOR (Ser2448) (1: 1,000, ab84400, Abcam), anti-GAPDH (1: 1,000, ab9845, Abcam) overnight at 4°C. After washing with PBS, anti-rabbit IgG-HRP secondary antibody (1: 1,000, MBS435036, MyBioSource) was added and incubated with the membranes at room temperature for one hour. After washing, signals were detected using ECL method (Sigma-Aldrich, USA). Relative expression levels of mTOR and p-mTOR were calculated using ImageJ according to endogenous control GAPDH.

### Statistical analysis

GraphPad software was used for all statistical analyses. Normal distribution data were recorded by (x±s), and comparisons between two groups were performed by *t*-test. Abnormal distribution data were processed using non-parametric Mann-Whitney U test. A value of $p<0.05$ was considered statistically significant.

## Results

### Expression of lncRNA GAS5 in patients with CAD

The expression of GAS5 in plasma sample of 30 CAD patients and 30 normal healthy individuals was detected by quantitative real-time (qRT)-PCR. As shown in Figure 1A, the expression level of GAS5 was found to be significantly lower in CAD patients than in the control group indicating the involvement of GAS5 in the development of CAD. ROC curve analysis was performed to explore the clinical value of GAS5 in the diagnosis of CAD. As shown in Figure 1B, the area under the ROC curve was 0.9783 ($p<0.0001$) suggesting that GAS5 may serve as a promising biomarker for CAD.

### Expression of lncRNA GAS5 in patients with different types of cardiovascular diseases

It has been reported that the expression of lncRNA GAS5 was significantly downregulated in patients with diabetes mellitus. To investigate the specificity of the expression pattern of GAS5 in CAD, expression of GAS5 was detected in various cardiovascular diseases including diabetes mellitus, hypertension, abnormal aortic aneurysm, viral myocarditis, atrial fibrillation, valvular disease, dilated cardiomyopathy, and peripheral artery disease. As shown in Figure 2, expression level of lncRNA Novlnc6 was significantly lower in patients with CAD and diabetes mellitus than that in normal healthy individuals ($p<0.05$ or $p<0.01$). No significant differences in expression level of GAS5 were found between the control individuals and the patients with other types of cardiovascular diseases ($p>0.05$). In addition, the expression level of GAS5 was also found to be significantly lower in patients with CAD than that in patients with diabetes mellitus ($p<0.05$). Those results suggest





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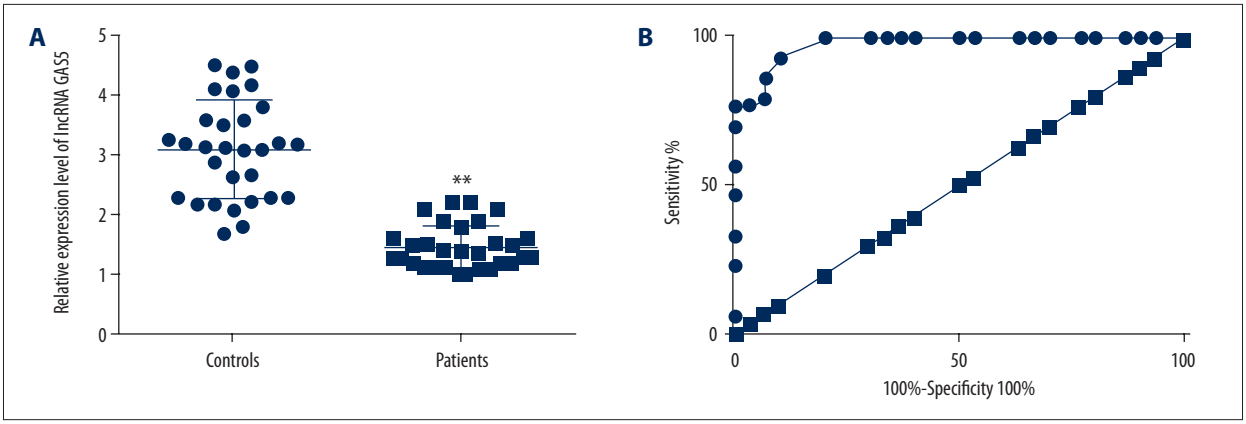


**Figure 1.** Expression of lncRNA GAS5 in patients with CAD and the diagnostic value of lncRNA GAS5 for CAD. (**A**) Relative expression levels of lncRNA GAS5 in patients with CAD. (**B**) ROC curve analysis of the diagnostic value of lncRNA GAS5 for CAD. ** Compared with control group, $p<0.01$.

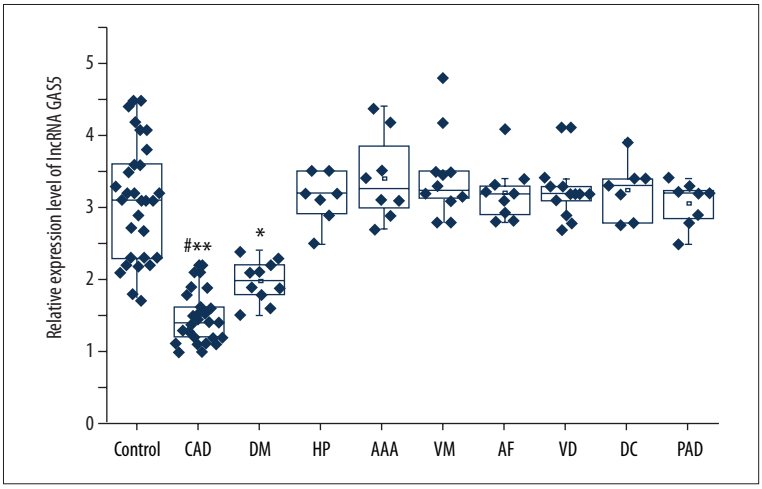


**Figure 2.** Expression of lncRNA GAS5 in patients with different types of cardiovascular diseases. DM – diabetes mellitus; HP – hypertension; AAA – abnormal aortic aneurysm; VM – viral myocarditis; AF – atrial fibrillation; VD – valvular heart disease; DC – dilated cardiomyopathy; PAD – peripheral artery disease. ** Compared with control group, $p<0.01$; * compared with control group, $p<0.05$; # compared with DM patients, $p<0.05$.

that the downregulation of GAS5 can potentially be a specific diagnostic biomarker for CAD.

### Effects of lncRNA GAS5 knockdown and overexpression on mTOR pathway

It has been reported that mTOR signaling pathway plays pivotal roles in the development of CAD, and lncRNA GAS5 can interact with mTOR to achieve its biological functions. In this study, GAS5 knockdown and overexpression HCAEC cell lines were constructed to investigate the effects of GAS5 on mTOR. As showed in Figure 3A, the expression level of GAS3 was significantly reduced in cells transfected with GAS5 siRNA, and was significantly increased in cells transfected with GAS5 overexpression vector. However, no significant differences in expression level of GAS5 were found between negative controls and control, indicating successfully established GAS5 knockdown and overexpression cell lines. As shown in Figure 3B–3D, the level of p-mTOR was significantly increased in the GAS5 knockdown cell line, and was significantly decreased in the GAS5 overexpression cell line ($p<0.05$). However, no significantly differences in expression levels of mTOR were found among the different cell lines. These data suggest that GAS5 can negatively regulate the phosphorylation of mTOR but has no significant effects on it expression.

### Effects of mTOR activator MHY1485 (Ser2448) and mTOR inhibitor Ku-63794 (Ser2448) on expression of lncRNA GAS5

To evaluate the interaction between GAS5 and mTOR, mTOR activator MHY1485 (Ser2448) and mTOR inhibitor Ku-63794 (Ser2448) were used to treat HCAECs and the effects on expression level of GAS5 were detected by qRT-PCR. As shown in Figure 4A, MHY1485 increased the level of p-mTOR, and Ku-63794 decreased the level of p-mTOR. As shown in Figure 4B, no significant differences in the expression levels of GAS5 were found among HCAECs with different treatment. Thus, the activation and inactivation of mTOR pathway showed no significant effects on GAS5 expression and GAS5 should be an upstream regulator of mTOR pathway.





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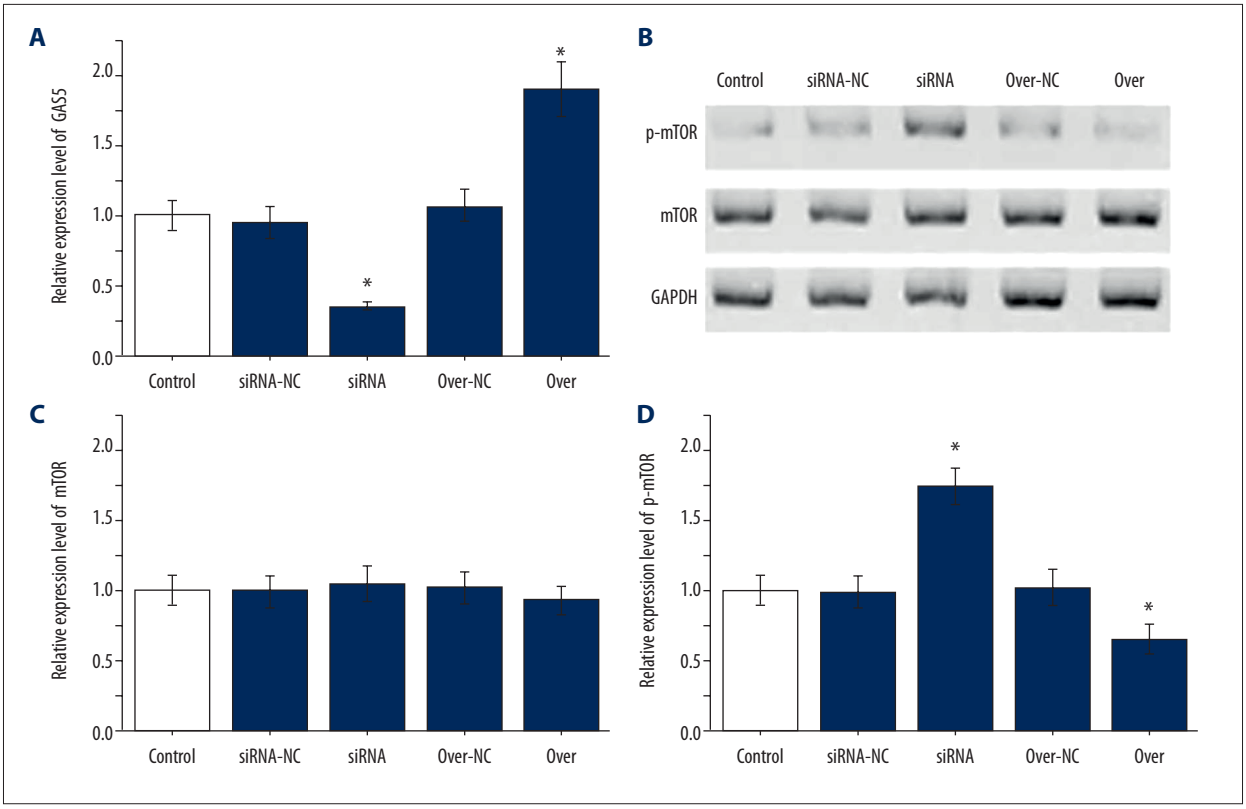


**Figure 3.** Levels of lncRNA GAS5, mTOR and p-mTOR in HCAEC with different treatments. (**A**) Expression levels of lncRNA GAS5 in HCAEC with different treatments; (**B**) Levels of mTOR in HCAEC with different treatments and levels of p-mTOR in HCAEC with different treatments. (**C**) Normalized level of mTOR. (**D**) Normalized level of p-mTOR. * Compared with control group, $p<0.05$.

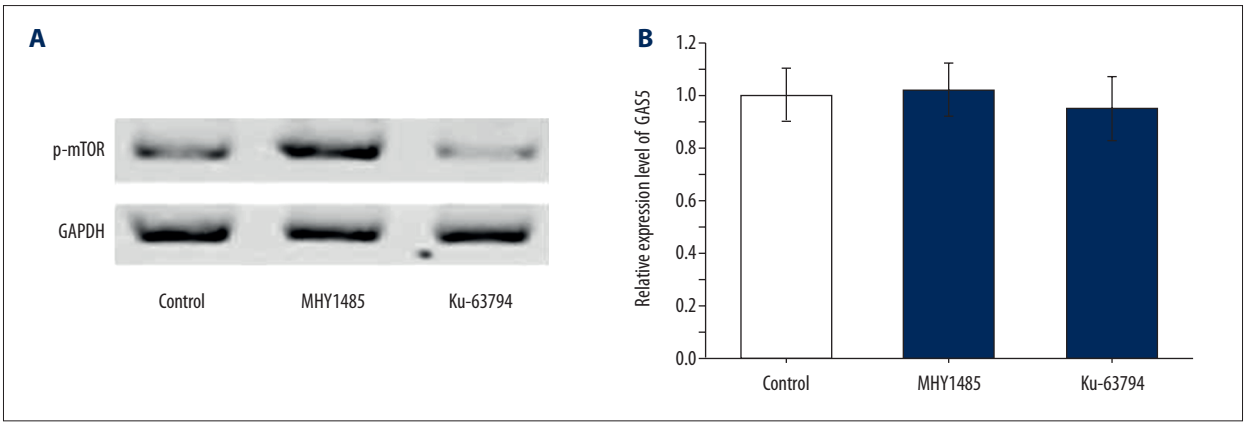


**Figure 4.** mTOR activator and inhibitor showed no significant effects on the expression of GAS5. (**A**) Western blot shows the effects of mTOR activator and inhibitor on the levels of p-mTOR in HCAECs; (**B**) qRT-PCR results show the effects of mTOR activator and inhibitor on the expression level of GAS5 in HCAECs.

## Discussion

The treatment of CAD is still challengeable due to the limited effective treatment methods available and the side effects caused by the long-term use of drugs. Therefore, early diagnosis and early treatment is the key to improve treatment outcomes. Biomarkers that can be used to reflect the physical condition and high-risk of certain diseases have been widely used in the diagnosis of various human diseases, including CAD [15]. A recent study of CAD showed that the sensitivity of plasma lncRNA coroMarker in the diagnosis of CAD can reach 78.05% and the specificity can be as high as 91.89%, indicating that this lncRNA can potentially serve as a biomarker for the diagnosis of CAD [14]. MicroRNA (miRNA) is also a





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group of non-coding RNAs. Different from lncRNA, the length of miRNA is usually around 22 nt. In another recent study, Ren et al. reported that miR-17/92a cluster, miR-106b/25 cluster, miR-126*, miR-21/590-5p family, and miR-451 all have important functions in the development of CAD and their unique expression patterns in CAD make them promising biomarkers for CAD [15]. Beside RNA, protein biomarkers have also been identified. Expression levels of TLR3 and TLR4 proteins were found to be closely correlated with the severity of CAD [16]. Therefore, the progression of CAD can potentially be reflected by the expression level of those two proteins.

The functionality of GAS5 has mainly been studied in cancer. In the study of cervical cancer, Cao et al. reported that GAS5 was usually downregulated in tumor tissues compared with adjacent healthy tissues, and the decrease in the expression level of GAS5 was closely related to the poor prognosis of patients [17]. In a study of stomach cancer, GAS5 was reported to be able to regulate the expression of p21 by binding to YBX1, and thus enhance G1 tumor cell arrest and inhibit the development of cancer [18]. There have been fewer studies on the function of GAS5 in the development of heart diseases. In a recent study, Tao et al. reported that GAS5 could regulate the expression of miR-21 and PTEN to suppress cardiac fibrosis, suggesting that GAS5 may play a protection role in heart diseases [12]. In our study, the expression of GAS5 was found to be specifically reduced in patients with CAD compared with healthy controls and patients with other types of cardiovascular diseases. In addition, GAS5 was found to be able to accurately predict the occurrence of CAD. Those results suggested that the expression of GAS5 was downregulated by CAD, and the decrease in the expression level of GAS5 might be a promising biomarker for the diagnosis of CAD.

The mTOR pathway plays indispensable roles in the development of CAD. In a recent study, Gao et al. reported that the activation of mTOR signaling pathway could initiate the monocyte pro-inflammatory response in patients with CAD, to promote the progression of this disease [19]. The activation of mTOR signaling pathway has also been treated as an indicator of various human diseases including CAD, and the inhibition of this pathway has been proven to be a promising strategy to improve CAD [20]. Previous studies have shown that GAS5 can interact with mTOR signaling pathway to achieve its biological functions [21]. In our study, GAS5 knockdown and overexpression showed no significant effects on the level of mTOR in HCAECs. However, the downregulation of GAS5 significantly increased the level of p-mTOR, and GAS5 overexpression significantly decreased the level of p-mTOR, indicating that GAS5 can inhibit the activation of mTOR but has no significant effects on the expression level of total mTOR. In addition, mTOR inhibitor and activator showed no significant effect on expression of GAS5 in HCAECs. These data suggest that GAS5 can play a role as an upstream regulator of mTOR signaling pathway in HCAECs.

## Conclusions

In conclusion, lncRNA GAS5 was specifically downregulated in patients with CAD but not in patients with other types of cardiovascular diseases. The decrease in the expression level of GAS5 was found to be a promising biomarker for CAD. LncRNA GAS5 can serve as an upstream regulator of mTOR signaling pathway to negatively regulate its activation. Our study was limited by the small sample size. Future studies with larger sample sizes are needed to further verify the conclusions in this study.

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