Original Paper

Association Between the Deletion Allele of Ins/Del Polymorphism (Rs145204276) in the Promoter Region of GAS5 with the Risk of Atherosclerosis

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Key Words

Atherosclerosis • GAS5 • Rs145204276 • MiR-21 • PDCD4

Abstract

Background/Aims: LncRNA is a growth arrest-specific transcript 5 (GAS5) with tumor suppressor activities in some cancers, but its role in atherosclerosis is unclear. **Methods:** Bioinformatics algorithm analysis was utilized to search the target of GAS5 and miR-21, followed by luciferase assay to confirm these targets. Real-time PCR and western-blot were utilized to verify the connection among GAS5, miR-21 and Programmed cell death 4 (*PDCD4*). MTT assay and flow cytometry analysis were performed to explore the mechanism of GAS5 in the regulation of atherosclerosis. **Results:** GAS5 directly targets miR-21 and functions as a competing endogenous RNA to suppress miR-21 expression. We also observed that rs145204276 polymorphism, including INS/INS and DEL/DEL, on GAS5 promoter increased transcription activity of GAS5, but the presence of rs145204276 DEL/DEL allele significantly promoted the transcription of GAS5 promoter compared with rs145204276 INS/INS allele. *PDCD4* was predicted as a direct target gene of miR-21 with a binding site on *PDCD4* 3'UTR. It was further confirmed by luciferase assay that miR-21 significantly reduced the luciferase activity of wild-type *PDCD4* 3'UTR but not that of mutant *PDCD4* 3'UTR. In addition, high glucose significantly inhibited the growth rate of EC genotyped as DEL/DEL or INS/INS, and apparently promoted the apoptotic rate of either DEL/DEL or INS/INS genotype ECs. Furthermore, the effect of high glucose was stronger in the INS/INS group, while the expression of GAS5 was dramatically upregulated with the presence of GAS5 DEL/DEL, while GAS5 positively regulated *PDCD4* expression via inhibiting miR-21 expression. GAS5 siRNA and miR-21 mimics significantly decreased GAS5 and *PDCD4* expressions, and the inhibitory effects of GAS5 siRNA or miR-21 mimics on GAS5 and *PDCD4* expressions in the INS/INS group was stronger. Moreover, GAS5 siRNA and miR-21 mimics remarkably triggered cells

proliferation and suppressed cell apoptosis, and the inhibition effects of GAS5 siRNA or miR-21 mimics on either cell viability and apoptosis in the INS/INS group was stronger. In this study, we enrolled 1,306 subjects with or without atherosclerosis and found that the INS/DEL or DEL/DEL genotypes significantly decreased the risk of atherosclerosis compared with the ins/ins genotype (adjusted odds ratio: 0.74 and 0.40, respectively). **Conclusion:** In summary, rs145204276 was associated with the risk of atherosclerosis by affecting the proliferation and apoptosis of endothelial cells via regulating the GAS5/miR-21/*PDCD4* signaling pathway.

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Introduction

Atherosclerosis, originally known as carotid stenosis or coronary artery disease (CAD), is a main contributor to stroke and myocardial infarction (MI), which accounted for over 50% of deaths in the world and is among the top contributors to disability and mortality [1, 2]. A critical process for the development of atherosclerosis is endothelial cell (EC) apoptosis [3]. The endothelium may become unable to during inflammation, immunity and lipid homeostasis due to the apoptosis of endothelial cells [4]. In addition, the damage to endothelial cells can destroy the barrier function, impair the integrity of endothelium and enhance lipid deposition, eventually resulting in atherogenesis [5].

Programmed cell death 4 (*PDCD4*) was first identified as a tumor inhibitor and its expression is significantly increased by apoptosis [6]. In addition to tumor cells, cardiac muscle and endothelial cells also express *PDCD4* [7] which is known to play a critical function in cardiovascular cell biology by inhibiting the expression of contractile genes in vascular smooth muscle cells and by triggering apoptosis and suppressing proliferation of many types of cardiovascular cells, such as fibroblasts, cardiac myocytes and vascular smooth muscle cells (endothelial cells) [7-10]. Low *PDCD4* level was related to lower proliferation of ECs [11].

It has been shown that up to 98% of the human genome encodes for noncoding transcripts and it was postulated that variations in organism complexity may be caused by the great variations in noncoding transcripts between lower and higher organisms [12]. A lot of such noncoding transcripts are processed to produce small noncoding RNAs including lncRNAs or miRNAs. As a family of small (18–22 nucleotide, nt) and endogenous noncoding RNA molecules, miRNAs target specific messenger RNAs and hence play a negative regulatory role in gene expression by triggering the translational repression or degradation of their target genes [13, 14]. In contrary, lncRNAs belong to a big family of transcribed RNA molecules (over 200 nt) with little potential to code proteins [15, 16]. Recent studies have focused on the effects of lncRNAs in atherosclerosis [17]. It was shown that antisense noncoding RNA in the *INK4* locus (*ANRIL*) and lncRNA-H19 are associated with the risk of CAD and atherosclerosis [18]. It is also revealed that long intergenic noncoding RNA p21 (lincRNA-p21) is related to atherosclerosis via affecting the proliferation and apoptosis of endothelial cells [19]. Recently, a study has demonstrated that the expression of miR-21 was markedly up-regulated in atherosclerotic plaques [20]. Furthermore, miR-21 had a negative regulatory role in lipid buildup through TLR4-NF- κ B pathway in LPS-activated macrophages, indicating the involvement of miR-21 in atherosclerosis [21].

The gene expression and function could be significantly impacted by single nucleotide polymorphisms (SNP). Growing data have revealed that SNPs in some lncRNAs are associated with chemotherapy response and tumorigenesis [22]. For example, the rs145204276 located in the promoter region of *GAS5* has been reported to be an endogenous competing RNA of miR-21 [23-25]. Also, *PDCD4* is believed to play a critical function in the pathogenesis of atherosclerosis by modulating the apoptosis of vascular endothelial cells [26, 27], while our in-silicon analysis also found that miR-21 virtually targeted *PDCD4*. In this study, we aimed to explore the role of *GAS5*, miR-21, and its target gene *PDCD4*, in the control of apoptosis of endothelial cells. We also investigated the influence of rs145204276 on the risk of atherosclerosis in a Chinese population.

Materials and Methods

Collection of umbilical vein

A total of 1306 subjects were enrolled for our research, which consists of 681 patients diagnosed with atherosclerosis and 624 subjects without atherosclerosis. The demographic and clinicopathological characteristics of the participants, such as gender, age, hypertension, diabetes mellitus, hypercholesterolemia, and smoking are displayed in Table 1. The Ethics Committee of our institute approved the protocol of this study, which was conducted following the Declaration of Helsinki. The umbilical cord vein samples were collected from all subjects after spontaneous delivery, and stored at -80°C for further analysis.

Endothelial cell isolation and culture

The tissue samples were maintained in Hanks' balanced salt solution. Subsequently, a solution containing 4 mg/mL dispase (Hyclone; GE Healthcare Life Sciences, Logan, UT) and 3 mg/mL collagenase type I (Hyclone; GE Healthcare Life Sciences, Logan, UT) was utilized to digest and mince the tissue samples at 37°C for 60 min. A 70-mm strainer (Falcon; Corning Life Sciences, Tewksbury, MA) was utilized to pass the cells to yield single cell suspensions. An alpha modification of Eagle medium (GIBCO/Invitrogen, Carlsbad, CA) containing 10% fetal bovine serum (Sigma-Aldrich, St Louis, MO), 100 mg/mL streptomycin and 100 U/mL penicillin (Sigma-Aldrich, St Louis, MO) and 2 mmol/L glutamine (Sigma-Aldrich, St Louis, MO) was utilized to culture the cells in 48-well plates at 5% CO₂ and 37°C. The growth medium was changed every 2 days till the cells were cloned, which were then transferred into a new dish. After the confluence reached 80%, the experiments were done using passage 2 Cells.

Genotyping

A Gentra DNA Isolation Kit (Thermo Fisher Scientific, Waltham, MA) was utilized to isolate the DNA from blood samples following the manufacturer's instruction. SNP rs145204276 was genotyped for all subjects with atherosclerosis. A BigDye3 terminator cycle sequencing kit (PE Applied Biosystems, Thermo Fisher Scientific, Waltham, MA) was utilized to analyze both forward and reverse sequencing based on the manufacturer's recommendation. An ABI PRISM 3100 Genomic Analyser (PE Applied Biosystems, Thermo Fisher Scientific, Waltham, MA) was utilized to perform the genotyping analysis.

MicroRNA and lncRNA RNA isolation and real-time PCR

Trizol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA) was utilized to extract total RNA from endothelial cells and tissue samples in accordance with the supplier's guideline. RNA was quantified based on its OD260 value using an Eppendorf Biophotometer (Hamburg, Germany). The extracted RNA was resuspended in 50 µL RNase free water and then stored at -80°C. A first strand cDNA synthesis kit (TOYOBO, Japan) was utilized to reversely transcribe 2 µg total RNA into cDNA using oligo (dT) primers according to the manufacturer's protocol. The synthesized cDNA was then diluted by 1:20 and kept at -20°C. GAPDH served as the internal control gene during the reverse transcription. Subsequently, a Chromo4 Real-Time Detection System (MJ Research, Cambridge, MA) in conjunction with a SYBR Green qPCR kit (Finnzymes, Finland) was utilized to conduct qPCR in a 20 µL volume containing 1 µL cDNA, 1 µL primers, 1 µL sterile deionized water and 10 µL SYBR Green I mixture. The qPCR reaction was run at 95°C for 90 s, followed by 45 cycles of 95°C for 15 s, 60°C for 20 s and 72°C for 15 s. All reactions were repeated in triplicate and the 2^{-ΔΔCT} method was utilized to calculate the expression of *PDCD4* mRNA, *GAS5* and *miR-21*. Option Monitor software (version 2.03, MJ Research, Cambridge, MA) was utilized to analyze the expression data.

Cell culture and transfection

The alpha modification of Eagle medium (GIBCO/Invitrogen, Carlsbad, CA) was utilized to culture the endothelial cells in 48-well plates at 5% CO₂ and 37°C. A

Table 1. Demographic and clinicopathological characteristics of the participants in this study

Characteristic	Atherosclerosis (-) N=681	Atherosclerosis (+) N=624	P value
Age (year)	63.81 ± 11.48	64.17 ± 9.71	0.543
Gender M/F	412/269	365/259	0.461
Hypertension (%)	412 (60.50)	372 (59.62)	0.744
Diabetes mellitus (%)	118 (17.33)	109 (17.47)	0.950
Hypercholesterolemia (%)	158 (23.20)	144 (23.08)	0.956
Smoking (%)	185 (27.17)	166 (26.60)	0.818

Lipofectamine®RNAiMAX Transfection Reagent (Applied Biosystems, MA) was utilized to transfect the cells with GAS5 siRNA, miR-21 mimics, miR-21 inhibitors or *PDCD4* siRNA (Sunbio, Shanghai, China) following the protocol provided by the manufacturer.

Cell proliferation assay

A Cell Counting Kit-8 (Beyotime, Shanghai, China) was utilized to carry out cell proliferation assay according to the supplier's instruction. In brief, endothelial cells were seeded into 96-well plates at a final density of 2×10^5 cells/well and then transfected with GAS5 siRNA, miR-21 mimics or *PDCD4* siRNA. A SpectraMax® i3x microplate reader (Molecular Devices, Sunnyvale, CA) was utilized to detect cell viability at the absorbance of 450 nm. All tests were carried out three times.

Luciferase assay to identify the interaction between 3'UTR of PDCD4 and miR-21

Commonly cited prediction programs, such as miRBase (<http://www.mirbase.org>), were utilized to predict that *PDCD4* was a target of miR-21. Subsequently, the 3'-UTR of *PDCD4*, which contained the miR-21 binding site, was amplified by PCR. Afterwards, we mutated the seed region of target site of miR-21, 3'-UTR of *PDCD4* and 3'-UTR of mutant *PDCD4*. These mutation products were inserted into a pmirGLO Dual-Luciferase miRNA TargetExpression Vector (Promega, Madison, WI) and then FuGENE-HD transfection reagent (Promega, Madison, WI) was utilized to co-transfect endothelial cells with 25 nM miR-21 mimics (Gene Pharma, Shanghai, China) and 150 ng plasmid (pmirGLO-mut*PDCD4*-3'-UTR or pmirGLO-*PDCD4*-3'-UTR) in accordance with the manufacturer's recommendation. The cells were harvested 24 hours post-transfection. A Dual-Glo Luciferase Assay System (Promega, Madison, WI) was utilized to analyze the luciferase activity, while a Renilla luciferase reporter served as the internal control for transfection. Finally, a GLoMax 20/20 system (Promega, Madison, WI) was utilized to measure the relative luciferase activity. All experiments were run in triplicate.

Luciferase assay to determine the transcription ability of GAS5 promoters with different genotypes

Luciferase assays were performed to determine whether the polymorphism rs145204276 affects the expression of GAS5. Briefly, PCR was utilized to amplify full promoter fragment from DNA isolated from the blood samples of the participants. The produced GAS5 promoter-luciferase reporter plasmids carried either rs145204276 INS or DEL allele. In the next step, sequencing was performed to confirm the rs145204276 alleles of the reporters constructs. Subsequently, endonuclease (NEB, BioLabs Inc., CA) was utilized to digest the psiCHECK2 vector (Promega, MD) and the amplified fragments, respectively. Afterwards, the PCR products were cloned into a psiCHECK2 (Promega, MD) vector. Endothelial cells were incubated in 48-well plates and co-transfected with 50 nM miR-21 mimics in conjunction with 50 ng psiCHECK2 vectors using Lipofectamine 2000 (Life Technologies, Carlsbad, CA). The cells were harvested 24 hours post-transfection and a Dual-Glo Luciferase Assay System (Promega, Madison, WI) was utilized to measure the luciferase activity in transfected cells. At the same time, Renilla luciferase reporter served as the internal control to normalize the transfection of firefly luciferase. All experiments were run in triplicate.

Luciferase assay to determine the interaction between miR-21 and GAS5

The oligo of miR-21 was purchased from GeneChem (Shanghai, China) and inserted into multiple restrictive sites of a pGL3 vector. The mutations in the sequence were introduced using a site-directed mutagenesis kit. The endothelial cells (2×10^6) were plated in 100-mm cellculture dishes and allowed to reach 60–80% confluence after 24 h of culturing. Subsequently, endothelial cells were co-transfected with 3 mg of DNA of the reporter constructs pGL3-miR-21-WT or pGL3-miR-21-Mut in conjunction with pCDNA-GAS5, or an empty vector. The cells were harvested 24 hours post-transfection and analyzed using the Dual-Glo luciferase assay as mentioned earlier.

Western blot analysis

Cold PBS was utilized to wash the cells twice, and RIPA (radio immunoprecipitation) was utilized to lyse the cells at 4°C. Subsequently, the lysates were subjected to centrifuge for 5 min at 14,000 g. A BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL) was utilized to measure the total protein concentration. Subsequently, proteins in the samples were resolved using 10% SDS-PAGE and then transferred onto a polyvinylidenedifluoride transfer membrane (Millipore, Bedford, MA). In the next step, 0.1% Tween-20

(TBST) containing 5% nonfat milk was used to block the membrane, followed by incubation with specific primary anti-*PDCD4* antibodies (1: 5, 000 dilution, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and anti- β -actin antibodies (1: 10, 000 dilution, ProMab Biotechnologies, Richmond, CA). After a 12-hour incubation, the membrane was rinsed with PBS and incubated with horseradish peroxidase (HRP) conjugated secondary antibodies (1:12000 dilution, Bioworld Technology, Inc., Minneapolis, MN) for another 30 minutes at room temperature. Finally, enhanced chemiluminescence (ECL) was utilized to visualize the protein bands, whose intensities were analyzed by ImageJ software (<http://rsb.info.nih.gov/ij>).

Cell apoptosis analysis

Cellular apoptosis was detected using an Annexin-V-FITC/PI double staining assay. In brief, endothelial cells were seeded into 96-well plates at a final density of 2×10^5 cells/well and transfected with GAS5 siRNA, miR-21 mimics or *PDCD4* siRNA. After 48 hours incubation, the cell were harvested, washed with cold PBS and resuspended in an Annexin-V binding buffer at a final density of 1×10^6 cells/mL. Subsequently, the apoptotic profile of the cells was and analyzed with flow cytometry.

Statistical analysis

All results were shown as the means $\pm$ SD (standard deviation). Student's t test was performed to analyze the differences between two groups, while a P value of less than 0.05 was considered statistically significant.

Results

Demographic, clinicopathological and genotypic parameters of the participants recruited in this study

A total of 1, 306 subjects were enrolled for our research, which consisted of 681 atherosclerosis patients and 624 healthy subjects. The demographic and clinicopathological characteristics of the participants, such as gender, age, hypertension, diabetes mellitus, hypercholesterolemia, and smoking history, are displayed in Table 1. Student t-test was used to analyze the demographic and clinicopathological characteristics of participants between these two groups, and it was found that all above clinicopathological characteristics of participants displayed no obvious differences between the two groups. The distribution of genotypes in both case and control groups followed the HWE ($P > 0.05$). Furthermore, a logistic regression test was used to analyze the genotypes, and it was found that the ins/del or del/del genotypes were significantly associated with a decreased risk of atherosclerosis compared with the INS/INS genotypes (values of adjusted odds ratio: 0.74 and 0.40, respectively). Similarly, we found that the DEL allele was significantly associated with a reduced risk of atherosclerosis compared with the INS allele (adjusted odds ratio: 0.57), as shown in Table 2.

Fig. 1. A: MiR-21 contained a putative binding site of GAS5. B: GAS5 substantially decreased the luciferase activity of wild-type miR-21.

Table 2. Association between genotype and allele of rs145204276 and risk of atherosclerosis

Genotype	Atherosclerosis (-) N=681	Atherosclerosis (+) N=624	Odds ratio and P value
Rs145204276			
Genotype			
ins/ins	325 (47.7)	235 (37.7)	
ins/del	305 (44.8)	296 (47.4)	0.74 (0.59-0.94) P=0.0128
del/del	51 (7.5)	93 (14.9)	0.40 (0.27-0.58) P<0.001
Allele			
ins	955 (70.1)	358 (57.3)	
del	407 (29.9)	266 (42.7)	0.57 (0.47-0.70) P<0.001

GAS5 regulates miR-21

LncRNAs were generally considered as molecular sponges or competing endogenous RNAs in the regulation of muscle concentration. Previously, LncRNA GAS5 and miR-21 have been shown to be significantly associated with atherosclerosis. Therefore, an in-silicon analysis was performed in this study to investigate whether GAS5 served as molecular sponges or competing endogenous RNAs to mediate miR-21 expression. As shown in Fig. 1A, miR-21 contains a putative binding site for GAS5 (Fig. 1A). Subsequently, the luciferase constructs containing wild or mutant type miR-21 were used to validate whether GAS5 contains a functional target for miR-21. As shown in Fig. 2B, overexpression of GAS5 decreased luciferase activity of wild-type miR-21 but not that of mutant miR-21, suggesting that GAS5 served as a competing endogenous RNA to mediate miR-21 expression.

Rs145204276 influenced transcriptional activity of GAS5

Because rs145204276 is located at the transcriptional start site of GAS5, and because different genotypes of rs145204276 were shown to affect the expression level of GAS5, the effect of rs145204276 on the transcriptional activity of GAS5 promoter region was explored using luciferase assays. As shown in Fig. 2A, the rs145204276 polymorphism INS and DEL alleles were cloned into the vector upstream of the luciferase reporter gene. As shown in Fig. 2B, the luciferase activity of cells was apparently increased after the cells were transfected with rs145204276 INS or DEL allele. Moreover, the presence of rs145204276 DEL allele significantly increased the transcriptional activity of GAS5 promoter compared with that containing the INS allele.

PDCD4 was a virtual target gene of miR-21

Bioinformatics algorithm analysis was performed using TargetScan to identify that *PDCD4* was a direct target gene of miR-21, with a binding site located on the 3'UTR of *PDCD4* (Fig. 3A). Subsequently, luciferase assays were conducted using constructs containing wild-type or mutant 3'UTR of *PDCD4* and the results showed that miR-21 directly targeted *PDCD4*, as the luciferase activity of cells

Fig. 2. A: Rs145204276 polymorphism INS/INS allele and DEL/DEL allele were located on the promoter of GAS5, and were inserted into a location upstream of the luciferase reporter gene. B: Transfection with rs145204276 insert or deletion allele increased the luciferase activity of GAS5, while the effect of deletion allele was even stronger.

Fig. 3. A: *PDCD4* was identified as a candidate target gene of miR-21, with the 'seed sequence' located in the 3'UTR of *PDCD4*. B: MiR-21 dramatically down-regulated the luciferase activity of wild-type *PDCD4*, but not that of mutant *PDCD4*.

Fig. 4. A: The viability of cells genotyped as INS/INS was decreased following the treatment with high glucose. B: The viability of cells genotyped as DEL/DEL was decreased following the treatment with high glucose. C: The apoptosis of cells genotyped as INS/INS was increased after the treatment with high glucose. D: The apoptosis of cells genotyped as DEL/DEL was increased after the treatment with high glucose.

transfected with wild *PDCD4* was evidently down-regulated but the luciferase activity of cells transfected with mutant *PDCD4* remained unchanged as that of the scramble control (Fig. 3B), indicating that miR-21 directly and negatively regulated *PDCD4* expression.

High glucose affected the proliferation and apoptosis of endothelial cells (ECs)

High glucose is considered as a key factor that can induce atherosclerosis via regulating the viability and apoptosis of ECs. Therefore, the effect of SNP DEL/DEL and INS/INS on the viability and apoptosis of HUVECs was examined in the presence of high glucose. As shown in Fig. 4, high glucose significantly inhibited the growth rate of both genotypes of HUVECs (DEL/DEL or INS/INS, as shown in Fig. 4A and 4B). However, the inhibitory effect of glucose in HUVECs (INS/INS) was stronger than that in HUVECs (DEL/DEL). In addition, the presence of high glucose significantly elicited the apoptosis of both genotypes of HUVECs (Fig. 4C and 4D).

Rs145204276 polymorphism in the promoter region of *GAS5* regulated the expression of miR-21

PDCD4 has been shown to act as a target gene of miR-21 (Fig. 3), which in turn was directly targeted by *GAS5* (Fig. 1). Therefore, we wanted to investigate whether the SNP rs145204276 (DEL/DEL or INS/INS) affects *PDCD4* expression. As shown in Fig. 5A, *GAS5* was highly expressed in cells carrying the DEL/DEL genotype compared with that in cells carrying the INS/INS group. In contrast, the expression of miR-21 was higher in the INS/INS group than that in the DEL/DEL group (Fig. 5B). Interestingly, both levels of *PDCD4* mRNA (Fig. 5C) and protein (Fig. 5D) were increased in the DEL/DEL group in comparison with

Fig. 5. Rs145204276 polymorphism affected the expression of miR-21 and *PDCD4*. A: *GAS5* expression was much higher in the DEL/DEL cells. B: miR-21 expression was much lower in the DEL/DEL cells. C: *PDCD4* mRNA level was much higher in the DEL/DEL cells. D: *PDCD4* protein level was much higher in the DEL/DEL cells.

that in the INS/INS group, suggesting that the DEL allele of the rs145204276 polymorphism in GAS5 dramatically up-regulated the expression of both GAS5 and *PDCD4*.

Alternation of GAS5 influenced the expression of PDCD4 as well as the proliferation and apoptosis of ECs

To further explore the role of GAS5 in the control of atherosclerosis by affecting the proliferation and apoptosis of ECs, we transfected two types of EC cells, i.e., genotyped as INS/INS and DEL/DEL, respectively, with GAS5 siRNA or miR-21 mimics. As shown in Fig. 6 and 7, GAS5 siRNA and miR-21 mimics significantly decreased the level of GAS5 in cells genotyped as INS/INS

(Fig. 6A) and DEL/DEL (Fig. 7A). In addition, the inhibitory effects of GAS5 siRNA or miR-21 mimics on GAS5 expression were stronger in the INS/INS group than those in the DEL/DEL group. Compared with the NC group, GAS5 siRNA and miR-21 mimics remarkably decreased the expression of both *PDCD4* mRNA and protein in cells genotyped as INS/INS (Fig. 6B) and DEL/DEL (Fig. 7B). Taken together, the inhibitory effects of GAS5 siRNA or miR-21 mimics on *PDCD4* expression was stronger in the INS/INS cells than those in the DEL/DEL cells. Furthermore, MTT assay and flow cytometry analysis were performed to detect the effect of GAS5 on the proliferation and apoptosis of HUVECs. As shown in Fig. 6C and 7C, GAS5 siRNA and miR-21 mimics remarkably increased cells growth in cells genotyped as INS/INS (Fig. 6C) and DEL/DEL (Fig. 7C), while attenuating the apoptotic rate of cells genotyped as INS/INS (Fig. 6D) and DEL/DEL (Fig. 7D). In addition, and the inhibitory effects of GAS5 siRNA or miR-21 mimics on cell viability and apoptosis were stronger in the INS/INS cells than those in the DEL/DEL cells. In summary, GAS5 attenuated the proliferation of HUVECs by targeting *PDCD4* and accelerating apoptosis, and the effect of GAS5 was stronger in the INS/INS cells.

Fig. 6. The effect of GAS5 and miR-21 on the proliferation and apoptosis of cells genotyped as INS/INS. A: GAS5 level in cells transfected with GAS5 siRNA and miR-21 mimics was significantly reduced. B: *PDCD4* level in cells transfected with GAS5 siRNA and miR-21 mimics was significantly reduced. C: Transfecting the cells with GAS5 siRNA and miR-21 mimics significantly increased cell viability. D: Transfecting the cells with GAS5 siRNA and miR-21 mimics significantly inhibited cell apoptosis.

Discussion

In this study, we enrolled a total of 1,306 subjects, which included 681 atherosclerosis patients and 624 healthy subjects. Logistic regression test was used to analyze the genotypes of rs145204276 polymorphism in these patients and the results showed that the INS/DEL or DEL/DEL genotypes were significantly associated with the decreased risk of atherosclerosis compared with that of the ins/ins genotype (values of adjusted odds ratio: 0.74 and 0.40, respectively). Similarly, we found that the DEL allele was significantly associated with a reduced risk of atherosclerosis compared with the INS allele (adjusted odds ratio: 0.57). It has also been revealed that

the expression of three lncRNAs, i.e., Zfas1, SNHG6 and GAS5, was substantially elevated in the plaque of atherosclerosis patients [28, 29]. Interestingly, an earlier study has suggested that non-coding RNAs Zfas1 and GAS5 acted as tumor inhibitors, while the down-regulation of lncRNA GAS5 was found in prostate cancer, non-small cell lung cancer, breast cancer and renal cell carcinoma [30-33]. Furthermore, an association between the rs145204276 polymorphism in the promoter region of GAS5 and the risk of HCC has also been identified. Moreover, rs145204276 may be functionally involved in oncogenesis via modulating the methylation status of a CpG island situated in the GAS5 promoter region, thus influencing the activity and expression of GAS5 [25]. In this study, we conducted luciferase reporter assays using vectors carrying rs145204276 polymorphism INS and DEL alleles to determine the effect of rs145204276 on the transcriptional activity of GAS5. Our results showed that in the presence of rs145204276 DEL allele, the transcription of GAS5 promoter was significantly promoted compared with that of the rs145204276 INS allele.

lncRNAs bind to miRNAs and act as endogenous “sponges” to modulate the functions of miRNAs [34, 35]. However, the mechanisms underlying the trans-regulatory functions of lncRNA/miRNA remain elusive. Furthermore, lncRNA CASC2, a tumor inhibitor, exhibits

Fig. 7. The effect of GAS5 and miR-21 on the proliferation and apoptosis of cells genotyped as DEL/DEL A: GAS5 level in cells transfected with GAS5 siRNA and miR-21 mimics was significantly reduced. B: PDCD4 level in cells transfected with GAS5 siRNA and miR-21 mimics was significantly reduced C: Transfecting the cells with GAS5 siRNA and miR-21 mimics significantly increased cell viability. D: Transfecting the cells with GAS5 siRNA and miR-21 mimics significantly inhibited cell apoptosis.

an anti-cancer function partly via the inhibition of miR-21 activity [36]. In addition, GAS5 inhibits miR-21 expression via a posttranscriptional regulatory pathway involving the RISC complex, while GAS5 contains a region of complementarity with miR-21 [37]. It was also revealed that miR-21 modulates the expression of *PDCD4* mRNA and affects its degradation, resulting in reduced expression of *PDCD4* proteins [38]. Furthermore, the elevation of miR-21 expression and the loss of *PDCD4* activity have been identified in a wide range of cancers [38]. Intriguingly, the synthesis of *PDCD4* in these cancer cells is decreased, although there is an elevated level of both cytoplasmic miR-21 and HuR in primary Glioblastoma cells [39]. In this study, using both in-silicon analysis and luciferase assays, we confirmed the regulatory relationship between miR-21 and GAS5, as well as the regulatory association between miR-21 and *PDCD4* in ECs.

It is believed that the apoptosis of ECs leads to the dysfunction or impairment of intact endothelial monolayer, which in turn results in inflammatory reactions, monocyte adhesion, lipid accumulation, and atherosclerotic lesions [4]. It has also been shown that ECs play important roles in the occurrence of atherosclerosis and the apoptosis of ECs may lead to reduced ability of endothelium to protect against inflammation, to enhance immunity and to maintain lipid homeostasis [40]. In this study, we found that high glucose significantly suppressed the proliferation and induced the apoptosis of ECs genotyped as either DEL/DEL or INS/INS, and the effect of high glucose was stronger in INS/INS cells.

PDCD4 was first discovered in tumor cells and blood, where the expression of *PDCD4* was down-regulated but increased upon apoptosis [41]. The human *PDCD4* gene is situated on chromosome 10q24. Analysis of the relevant protein sequence in this region has shown that *PDCD4* consists of 469 amino acids, which form two conserved α -helical MA-3 domains and two basic domains at the COOH terminus and NH₂ terminus, respectively. A recent study has also investigated the biological roles of *PDCD4* and its underlying molecular mechanisms [41]. It was shown that *PDCD4* could regulate the functions of molecules including Sp1/Sp3, urokinase-receptor (u-PAR), JNK/c-Jun/activator protein 1 (AP-1), carbonic anhydrase II, ornithine decarboxylase, Cdk4 and p21Waf1/Cip1 [41]. Therefore, it is hypothesized that *PDCD4* plays a regulatory role in tumor cell proliferation, invasion and apoptosis [6]. In general, *PDCD4* acts as a potent tumor inhibitor [6]. For example, *PDCD4* was found to suppress neoplastic growth and transformation, and hence is regarded as a potential antineoplastic target for novel cancer therapies [42]. In addition, *PDCD4* is believed to induce apoptosis through the stimulation of the caspase-3 pathway [43]. It has also been shown that the activity of caspase-3 plays a crucial role in inducing PH dyshomeostasis and in promoting endothelial apoptosis [26]. In this study, we investigated whether the alternation of GAS5 influenced the expression of *PDCD4* as well as the proliferation and apoptosis of EC cells. We found that GAS5 attenuated the proliferation of HUVECs by targeting *PDCD4* and accelerating the apoptosis of HUVECs. Interestingly, *PDCD4* also acts as a pro-inflammatory protein and its expression is elevated in foam cells derived from ox-LDL-stimulated macrophages and in mice suffering from atherosclerosis, further demonstrating the implication of *PDCD4* in the occurrence of atherosclerosis [44]. Additionally, the MAPK pathway plays an important role in inflammatory disorders, such as atherosclerosis [45]. It has been shown that the MAPK signaling could activate p38 and JNK, and hence regulates the activity of pro- and anti-inflammatory cytokines (IL-10 and TNF- α) in macrophages [46]. Furthermore, Jiang et al. observed that, in *PDCD4* deficient mice, the function of IL-10 was alleviated by the suppression of the p38/ERK-c-Maf pathway [47].

In spite of its important findings, the present study suffers from a certain limitations: First, the sample size in this study was relatively small due to the limited time and funding, thus may constrain the credibility of relevant statistical tests. Secondly, the tissue samples of veins rather than arteries were used in this study. Thus, the results reported here may be inconsistent with those reported by others. Nevertheless, further studies with larger sample sizes and populations with different genetic backgrounds, coupled with animal model studies will lead to a clearer understanding of the molecular interactions involved in this field and a more robust conclusion.

Conclusion

Taken together, our findings suggested that rs145204276 was associated with the risk of atherosclerosis by affecting the methylation status of the GAS5 promoter and its transcriptional activity. Thus, rs145204276 could serve as a potential therapeutic target for the treatment of atherosclerosis. Results from this study demonstrated that lncRNAs (GAS5) might be involved in the atherosclerosis via targeting both miR-and *PDCD4*. In summary, our findings provide new insight into the pathogenic mechanism and the role of GAS5 in atherosclerosis.

Disclosure Statement

The authors claim to have no conflict of interests.

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