LncRNA HSPA7 in human atherosclerotic plaques sponges miR-223 and promotes the proinflammatory vascular smooth muscle cell transition

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Although there are many genetic loci in noncoding regions associated with vascular disease, studies on long noncoding RNAs (lncRNAs) discovered from human plaques that affect atherosclerosis have been highly limited. We aimed to identify and functionally validate a lncRNA using human atherosclerotic plaques. Human aortic samples were obtained from patients who underwent aortic surgery, and tissues were classified according to atherosclerotic plaques. RNA was extracted and analyzed for differentially expressed lncRNAs in plaques. Human aortic smooth muscle cells (HASMCs) were stimulated with oxidized low-density lipoprotein (oxLDL) to evaluate the effect of the identified lncRNA on the inflammatory transition of the cells. Among 380 RNAs differentially expressed between the plaque and control tissues, lncRNA HSPA7 was selected and confirmed to show upregulated expression upon oxLDL treatment. HSPA7 knockdown inhibited the migration of HASMCs and the secretion of inflammatory proteins such as IL-1β and IL-6; however, HSPA7 knockdown recovered the oxLDL-induced reduction in the expression of contractile markers. Although miR-223 inhibition promoted the activity of NF-kB and the secretion of inflammatory proteins, HSPA7 knockdown diminished these effects. The effects of miR-223 inhibition and HSPA7 knockdown were also found in THP-1 cell-derived macrophages. The impact of HSPA7 on miR-223 was mediated in an AGO2-dependent manner. HSPA7 is differentially increased in human atheroma and promotes the inflammatory transition of vascular smooth muscle cells by sponging miR-223. For the first time, this study elucidated the molecular mechanism of action of HSPA7, a lncRNA of previously unknown function, in humans.

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INTRODUCTION
The risk of atherosclerotic cardiovascular diseases, such as coronary artery disease, is strongly attributable to genetic factors. According to recent genetic studies, many novel genetic loci are associated with coronary artery disease but have an unknown function. In addition, many of them are reportedly located in noncoding regions of the human genome. For example, the association between the 9p21 locus and myocardial infarction has been replicated by research groups. ANRIL, a noncoding RNA, was found to be located in this locus, and its function in influencing cell proliferation was partly elucidated.

Long noncoding RNAs (lncRNAs) are the longest types of noncoding RNAs and are differentiated from other shorter noncoding RNAs. LncRNAs have very diverse functions and are regarded as attractive therapeutic targets due to their tissue, cell, and disease specificities. As lncRNAs affect distal targets, they stabilize ribonucleoprotein complexes, alter phosphorylation pathways, or act as competing endogenous RNAs. Dozens of lncRNAs have been reported to affect the development of atherosclerosis. Prior studies showed that specific lncRNAs regulate cells in arteries, including endothelial cells, vascular smooth muscle cells (VSMCs), and macrophages. For instance, lncRNA H-19 is known to increase VSMC proliferation, whereas lncRNA-p21 inhibits it. Although some additional lncRNAs were identified to regulate vascular cells, lncRNAs discovered from human vascular cells that affect atherosclerosis have been highly limited. Conversely, microRNAs inhibit target mRNAs usually via degradation or translational blocking. Some microRNAs are involved in atherosclerosis by affecting SMC proliferation or phenotypic changes.

In the current study, we aimed to analyze human atherosclerotic plaques and identify and functionally validate a lncRNA with a previously unknown function. We challenged VSMCs with several stimuli, including oxidized low-density lipoprotein (oxLDL), a hallmark of atherosclerosis, and evaluated the effects and mechanisms of action of the identified lncRNAs.

MATERIALS AND METHODS
Study subjects and aortic tissue extraction
The study protocol (no. 4-2013-0688) was approved by the Institutional Review Board of Severance Hospital, Seoul, Korea. All participants provided

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written informed consent. Aortic samples were obtained from patients who underwent aortic graft replacement surgery for a thoracic aortic aneurysm. Samples were classified by an experienced pathologist according to the presence of atherosclerotic plaques. The characteristics of the participants according to aortic atherosclerotic plaques are presented in Table 1. The pathologist classified the lesions according to the modified classification of the American Heart Association without any knowledge of the specimens.

| Atherosclerosis (n = 2) | Control (n = 4) |
|------------------------|----------------|
| Patient number         | 1   | 2   | 3   | 4   | 5   | 6   |
| Age                    | 79  | 72  | 68  | 48  | 61  | 73  |
| Sex                    | Female | Male | Male | Male | Female | Female |
| Diabetes mellitus      | No  | No  | No  | No  | No  | No  |
| Hypertension           | Yes | Yes | No  | Yes | No  | Yes |
| Smoking                | No  | Yes | Yes | No  | No  | No  |
| Hyperlipidemia*        | No  | Yes | No  | No  | No  | No  |
| Coronary artery disease| No  | Yes | No  | No  | No  | No  |
| Body mass index        | 23.4| 27.6| 23.2| 20.9| 23.9| 20.7|

*Defined as low-density lipoprotein-cholesterol ≥160 mg/dL.

RNA sequencing and analysis of lncRNAs

RNA was extracted from cells using a Ribospin RNA Extraction Kit (GeneAll, Seoul, Korea). RNA concentration and purity were assessed using a NanoDrop ND1000 spectrophotometer. Total RNA sequencing libraries were prepared using a TruSeq RNA sample preparation kit (Illumina, San Diego, CA, USA). The fragmentation step resulted in an RNA-Seq library that included inserts of approximately 100–400 bp. The average insert size in an Illumina TruSeq library was approximately 200 bp. cDNA fragments underwent an end repair process: the addition of a single A base to the 3' end and then ligation of adapters. Finally, the products were purified and enriched with polymerase chain reaction (PCR) to create final double-stranded cDNA libraries. Libraries were quantified using KAPA Library Quantification kits for the Illumina HiSeq 2500 platform according to the protocol guide KK4BSS (KAPA Biosystems, Wilmington, MA, USA). We normalized the RNA sequencing data by quintile normalization as previously described.

The differentially expressed genes between samples with and without atherosclerotic plaques were compared using Cuffdiff. Genes with upregulated and downregulated expression with \( p \text{-values} < 0.05 \) and >two-fold changes were identified.

Cells and other reagents

Human aortic smooth muscle cells (HASMCs) were purchased from Lonza (Basel, Switzerland) and cultured in SMC growth basal medium containing growth factor supplemented with 2% fetal bovine serum and penicillin/ streptomycin at 37 °C. THP-1 cells, a human monocytecell line, were purchased from the Korean Cell Line Bank (Seoul, Korea). The cells were cultured in RPMI-1640 medium containing 10% fetal bovine serum and streptomycin (100 U/mL)–penicillin (100 μg/mL) at 37 °C with 5% CO\(_2\). THP-1 cells were plated in six-well dishes (1 × 10\(^6\) cells/well) and treated with 50 nM phorbol 12-myristate-13-acetate for 24 h for differentiation into macrophages. Lipopolysaccharide (LPS) and angiotensin II (AngII) were purchased from Sigma-Aldrich (St. Louis, MO, USA) for HASMC stimulation. Angiotensin II is a key peptide hormone in the renin-angiotensin-aldosterone system. This molecule exerts adverse cardiovascular effects by increasing vascular tone, fluid retention, thrombosis, and inflammation.

Low-density lipoprotein (LDL) for oxLDL was isolated from the plasma of healthy donors using sequential ultracentrifugation. LDL was dialyzed for 24 h at 4 °C with phosphate-buffered saline and oxidized for 24 h using 5 mM CuSO\(_4\) at 37 °C. Ethylenediaminetetraacetic acid was added to stop the reaction, and thiolbarbituric acid reactive substance assays were used to analyze the oxidation state of LDL before each experiment.

Migration assay

For analysis and comparison of HASMC migration, the cells were added to the upper Transwell chamber (Neuro Probe, Inc., Gaithersburg, MD, USA) in serum-free medium after transfection with siHSPA7 or control siRNA for 24 h. The lower chamber was filled with SMC growth basal medium with fetal bovine serum. Next, LPS (10 ng/mL), oxLDL (50 μg/mL), or AngII (300 nM) was added to the upper chamber and incubated for another 24 h. Thereafter, the cells were stained with a Diff Quik staining kit (Kobe, Japan), and those on the lower surface of the filter were photographed and counted under a fluorescence microscope. All treatments were performed in duplicate wells. The sequences of siHSPA7 are as follows: sense: GUGACUGACAGCACCUGAGCGAUCU; antisense: GCUCUCUGACUGGCUGACGCUCUGACCGUCUAA.

Enzyme-linked immunosorbent assay (ELISA)

Cell culture supernatants were collected, and the amount of secreted IL-1β or IL-6 was quantified using ELISAs (R&D Systems, Minneapolis, MN, USA) according to the manufacturer’s protocol. Ninety-six-well plates were coated with 1 mg/well capture antibody. The coated plates were washed twice with phosphate-buffered saline containing 0.05% Tween-20. Following exposure to the medium, the assay plates were exposed sequentially to each of the biotin-conjugated secondary antibodies. The plates were read at an absorbance of 450 nm. Target proteins were analyzed according to the manufacturer’s specification. Appropriate specificity controls were included, and all samples were run in duplicate.

Quantitative real-time PCR (RT-qPCR)

RNA was extracted from cells using a Ribospin RNA Extraction Kit (GeneAll, Seoul, Korea). The integrity of the extracted RNA was analyzed using a NanoDrop and quantified using spectrophotometric absorbance at 260 nm. Next, 1 μg of RNA was used for cDNA synthesis using an iScript™ cDNA Synthesis kit (Bio-Rad, Hercules, CA, USA). RT-qPCR was performed with a SYBR Green dye system on a LightCycler 480 real-time PCR machine (Roche, Basel, Switzerland) using a standard protocol. LightCycler software was used to analyze gene expression based on cycle threshold values normalized to β-actin expression. β-actin was used, as it is a commonly used housekeeper for normalization in RT-qPCR. Amplified gene expression was assessed by melting curve analysis, and no reverse transcriptase or template controls were included. Analyses were performed in duplicates. RT-qPCR was used to verify lncRNAs detected by RNA sequencing and SMC phenotype marker genes. PCR primers are as follows: F: CATTCAAGTGATAGTTGCG; R: CAGGCAGCAGATTCCATATA. The CN1 and TAL1 genes encode calponin-1 and SM22α, respectively. These are markers of the contractile phenotype of SMCs. Inflammation is known to switch the vascular SMC phenotype from contractile to synthetic.

RNA-binding protein immunoprecipitation (RIP) assay

The RIP assay was conducted using an EZ-Magna RIP kit (Merck Millipore, Burlington, MA, USA) according to the manufacturer’s instructions. HASMCs were harvested and lysed in RIP lysis buffer. Cell extracts were then incubated with RIP buffer containing magnetic beads conjugated to anti-AGO2 antibody and IgG (Merck Millipore). Immunoprecipitated RNA was isolated, and qPCR analysis was performed to detect HSPA7 and miR-223. The \( 2^{-\Delta\Delta Ct} \) method was used for quantification of RNAs, and U6 and β-actin were used as controls for miR-223 and HSPA7, respectively.

Table 1. Characteristics of study participants according to the presence of aortic atherosclerotic plaques.

| Characteristic         | Atherosclerosis (n = 2) | Control (n = 4) |
|------------------------|------------------------|----------------|
| Age                    | 79  | 72  | 68  | 48  | 61  | 73  |
| Sex                    | Female | Male | Male | Male | Female | Female |
| Body mass index        | 23.4| 27.6| 23.2| 20.9| 23.9| 20.7|

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**RESULTS**

Identification of IncRNAs associated with human atherosclerosis

RNA sequencing was performed to identify IncRNAs associated with atherosclerotic plaques. A total of 380 RNAs were found to be differentially expressed between plaques and controls (Fig. 1a). Hierarchical clustering and multidimensional scaling were conducted based on fragments per kilobase of transcripts per million mapped reads values (fold change > 2; p < 0.05), and the gene expression pattern in plaques was distinct from that of the controls. Multidimensional scaling visualized differences in gene expression between the two groups (Fig. 1b). Figure 1c presents high-ranking IncRNAs with up- or downregulated expression in plaques. qPCR to confirm the differential expression of IncRNAs showed four with significantly up- or downregulated expression.

Among them, two IncRNAs with upregulated expression, HSPA7 and LOC102724297, and one with downregulated expression, LINC00982, were selected for the validation experiment (Fig. 1d).

When HASMCs were incubated with LPS, oxLDL, or AngII, only HSPA7 showed upregulated expression, whereas the expression of the other two IncRNAs was not substantially changed (Fig. 1e).

Knockdown of HSPA7 attenuates migration and inflammatory changes in HASMCs

To examine the effect of HSPA7 knockdown on HASMC migration, we transfected the cells with siHSPA7 or control siRNA for 24 h and plated them in the upper Transwell chamber with or without LPS, oxLDL, or AngII. After another 24 h, analysis of the cells in the lower chamber revealed that migration of HASMCs promoted by oxLDL was significantly inhibited after siHSPA7 treatment (Fig. 2a, b). After transfection with siHSPA7 or control siRNA, HASMCs were treated with or without oxLDL for 24 h. ELISAs and qPCR showed that the oxLDL-promoted secretion and expression of IL-1β and IL-6 were suppressed by siHSPA7. The effect on IL-6 was not notable (Fig. 2c).

Immunofluorescence staining showed that the expression of markers of the contractile SMC phenotype, SM22a, and calponin1, was decreased upon oxLDL treatment, and this change was reversed by siHSPA7. The expression of CD68, a marker of macrophage-like cells, was upregulated upon oxLDL treatment, whereas this change was partly inhibited by siHSPA7 (Fig. 2d).

The effects of HSPA7 on phenotype markers, particularly CNN1 and CD68, were validated using qPCR (Fig. 2e).

**DISCUSSION**

Major findings of the current study include the following: (1) the expression of HSPA7, a lncRNA, was differentially upregulated in human atherosclerotic plaques and induced by oxLDL; (2) HSPA7 knockdown inhibited the migration of HASMCs and secretion and expression of inflammatory mediators, whereas it recovered the expression of contractile markers of the cells reduced upon oxLDL treatment. (3) Although miR-223 inhibition promoted the activity or secretion of inflammatory proteins in HASMCs, HSPA7 knockdown diminished these effects. (4) The impact of HSPA7 on miR-223 was mediated in an AGO2-dependent manner. Notably, this study identified HSPA7 in human atheroma and elucidated its function, although previous studies have not revealed the expression or function of HSPA7 in vascular tissue. Our study not only reported the association of this lncRNA with atherosclerosis but also elucidated its biological effects on target vascular cells and the molecular mechanism of action. Furthermore, although the relationship between miR-223 and inflammation and vascular disease has been suggested, the current study first showed that miR-223 sponging is involved in the HSPA7 pathway.

Several heat-inducible HSP70 genes have been reported in humans, and HSPA7, also called HSP70B, is one of them. Similar to HSP6, this gene is located on chromosome 1. Diverse expression levels of other HSPA genes have been shown in various tissues, including blood, testis, and nerves. Although HSP70B mRNA is expressed in response to heat shock, this gene was found to encode no functional protein a decade ago. Studies on this gene have been extremely limited since. Here, for the first time, we identified the differential expression of HSPA7 in human atheroma and validated its effect on VSMCs.

In our study, HSPA7 affected VSMC status by sponging miR-223. miR-223 was reported to suppress the NLRP3 inflammasome in control siRNA and then treated with 50 μg/ml oxLDL for 24 h. IL-1β and IL-6 secretion increased upon miR-223 inhibitor treatment. The enhanced secretion of chemokines, particularly IL-6, was diminished with the addition of siHSPA7. These findings were verified using qPCR (Fig. 3c). After the same treatment of HASMCs, luciferase activity was measured after 48 h. qPCR showed that upregulated expression of FOXO1, a transcriptional activator of NF-κB, by the miR-223 inhibitor was diminished after siHSPA7 treatment. Although luciferase-reported NF-κB activity was elevated upon miR-223 inhibition, this parameter was attenuated upon HSPA7 knockdown (Fig. 3d). After the same treatment of THP-1 cell-derived macrophages, the secretion of IL-1β was increased with the miR-223 inhibitor, whereas it was decreased with siHSPA7. Although IL-6 exhibited a similar tendency to IL-1β, it was not significant. qPCR demonstrated up- and downregulation of the levels of IL18, IL6, and FOXO1 according to each treatment (Fig. 3e). In immunofluorescence staining, SM22a and calponin1 expression were not changed upon miR-223 inhibitor treatment, whereas the expression of these markers increased after the addition of siHSPA7 (Fig. 3f). These findings were verified using qPCR, particularly that of TAGLN (Fig. 3g).
Fig. 1  HSPA7 expression is upregulated in human atherosclerotic plaques and induced by atherogenic stimuli. a Heatmap of genes differentially expressed in human atherosclerotic plaques compared to control tissues. b Hierarchical clustering and multidimensional scaling of data from plaques and controls. c List of high-ranking lncRNAs with up- or downregulated expression in plaques. d qPCR was performed to confirm lncRNAs with up- or downregulated expression using plaque and control tissues and four lncRNAs with significant differences. e Among the three top-ranked genes, only HSPA7 showed a significant elevation upon proatherogenic stimuli, particularly oxLDL. Experiments were conducted in technical duplicates, and data are presented based on three independent replicates. *: p < 0.05. C control; oxLDL oxidized low-density lipoprotein.
myeloid cells, and miR-223 dysregulation contributes to the pathogenesis of inflammatory diseases\(^\text{18}\). In addition, when miR-223 expression is upregulated in endothelial cells, inflammatory pathways are inhibited\(^\text{21}\). Furthermore, miR-223 knockdown in mice increased atherosclerotic lesions, which indicates the protective role of miR-223 in vascular disease\(^\text{22}\). A recent study conducted in patients with Kawasaki disease showed that miR-223 protects against VSMC dedifferentiation\(^\text{29}\). These findings also support that changes in miR-223 expression may be involved in the development of atherosclerotic vascular disease. Because we demonstrated that miR-223 was a target of HSPA7 in atherosclerotic lesions, miR-223 inhibition by HSPA7 might contribute to the progression of atherosclerosis. Moreover, our findings on miR-223 and VSMCs suggested that miR-223 might be an appropriate target for cardiovascular prevention or a candidate disease marker. Most importantly, our study identified that HSPA7 is a regulator of miR-223 in the atherogenic vascular environment. Although regulation of FOXO1 by miR-223 that is affected by HSPA7 was shown in our data, the relationship between miR-223 and FOXO1 was previously reported. In more than one cancer cell line, overexpression of miR-223 downregulated FOXO1 expression and suppressed tumor cell proliferation\(^\text{30}\).

Here, we additionally showed the molecular mechanism of how HSPA7 exerts its effects. However, to date, detailed action mechanisms have been elucidated for just a few IncRNAs in VSMCs\(^\text{34}\). Lnc-Ang362 is regulated when VSMCs stimulated with angiotensin II act as hosts for the transcript of miR-221 and miR-222, and it is also involved in VSMC proliferation\(^\text{31}\). LncRNA-p21 reportedly inhibits VSMC proliferation and induces apoptosis, while its effect is associated with transcriptional activation of p53\(^\text{3}\). SMILR (smooth muscle-induced IncRNA enhances replication) also regulates VSMC proliferation with the acceleration of the cell cycle by interacting with CENPF (centromere protein F) mRNA\(^\text{32}\). In contrast, it is only incompletely understood how other IncRNAs affect VSMCs, such as ANRIL, SENCR, HAS-A51, and H19\(^\text{35}\).

In previous studies, IncRNAs involved in atherosclerosis have been discovered in mouse cells, as in the case of Mexis\(^\text{36}\). In addition, they were identified via genetic analysis even though the studies were performed in humans, similar to the case of ANRIL\(^\text{37}\). Otherwise, these IncRNAs were found in nonplaque human cells, as in the case of SENCR\(^\text{35}\). Studies identifying IncRNAs in human atherosclerotic plaque have been extremely rare, except for one study by Hung et al. that found PELATON using human carotid plaques\(^\text{36}\). The strength of our study is that we discovered HSPA7 in human plaques and elucidated the relevant biological function.
Our study has potential limitations. We validated that *HSPA7* promoted the inflammatory transition of VSMCs by sponging miR-223. However, we cannot conclude that sponging miR-223 is the only mechanism of action for *HSPA7*, and additional studies may help elucidate the mechanism further. LncRNAs are poorly conserved across species compared to microRNAs, and mice do not have an *HSPA7* homolog. Therefore, it was difficult to use a mouse model for the additional verification of the effect of *HSPA7*. 
However, as mentioned, its discovery in human plaque and simultaneous elucidation of its mechanism of action have made our results scientifically meaningful.

In conclusion, HSPA7, a lncRNA identified in human atherosclerotic plaques, plays a role in the inflammatory transition of VSMCs stimulated by oxLDL. This process is mediated by miR-223 sponging by HSPA7. Notably, our study newly identified a lncRNA of previously unknown function in human plaques and elucidated its molecular mechanism of action. The current study suggests that HSPA7 can be a potential therapeutic target for atherosclerotic vascular disease.

DATA AVAILABILITY
The data underlying this article are available on reasonable request to the corresponding author.

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