Original Paper

LncRNA Expression Profile of Human Thoracic Aortic Dissection by High-Throughput Sequencing

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Key Words

Human thoracic aortic dissection (TAD) • Long non-coding RNA (lncRNA) • High throughput sequencing (HTS) • Bioinformatics analyses

Abstract

Background/Aims: In this study, the long non-coding RNA (lncRNA) expression profile in human thoracic aortic dissection (TAD), a highly lethal cardiovascular disease, was investigated.

Methods: Human TAD (n=3) and normal aortic tissues (NA) (n=3) were examined by high-throughput sequencing. Bioinformatics analyses were performed to predict the roles of aberrantly expressed lncRNAs. Quantitative real-time polymerase chain reaction (qRT-PCR) was applied to validate the results. **Results:** A total of 269 lncRNAs (159 up-regulated and 110 down-regulated) and 2,255 mRNAs (1,294 up-regulated and 961 down-regulated) were aberrantly expressed in human TAD (fold-change > 1.5, $P < 0.05$). QRT-PCR results of five dysregulated genes were consistent with HTS data. A lncRNA-mRNA coexpression analysis showed positive correlations between the up-regulated lncRNA (ENSG00000269936) and its adjacent up-regulated mRNA (*MAP2K6*, $R = 0.940$, $P < 0.01$), and between the down-regulated lncRNA_1421 and its down-regulated mRNAs (*FBLN5*, $R = 0.950$, $P < 0.01$; *ACTA2*, $R = 0.96$, $P < 0.01$; *TIMP3*, $R = 0.96$, $P < 0.05$). The lncRNA-miRNA-mRNA network indicated that the up-regulated lncRNA XIST and *p21* had similar sequences targeted by has-miR-17-5p. The results of luciferase assay and fluorescence immuno-cytochemistry were consistent with that. And qRT-PCR results showed that lncRNA XIST and *p21* were expressed at a higher level and has-miR-17-5p was expressed at a lower level in TAD than in NA. The predicted binding motifs of three up-regulated lncRNAs (ENSG00000248508, ENSG00000226530, and EG00000259719) were correlated with up-regulated *RUNX1* ($R = 0.982$, $P < 0.001$; $R = 0.967$, $P < 0.01$; $R = 0.960$, $P < 0.01$, respectively). **Conclusions:** Our study revealed a set of dysregulated lncRNAs and predicted their multiple potential functions in human TAD. These findings suggest that lncRNAs are novel potential therapeutic targets for human TAD.

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Introduction

Acute thoracic aortic dissection (TAD) is one of the most lethal cardiovascular diseases, even when treated with conventional pharmacological approaches, classic surgical procedures, or endovascular intervention. In addition to remedial treatments, the clinical control of traditional risk factors, including hypertension, dyslipidemia, and congenital cardiovascular abnormalities, reduces the incidence of TAD. However, even with effective management or in the absence of risk factors, TAD may still occur. Consequently, effective therapeutic targets are needed to prevent the pathological progression of TAD, including a loss of smooth muscle cells (SMC) and dynamic imbalance of the extracellular matrix (ECM). Despite great progress, the mechanisms underlying TAD are still not fully understood, thereby limiting the identification of potential targets [1, 2].

Non-coding RNAs are rapidly emerging as crucial regulatory molecules in the development of aortic diseases. MicroRNAs (miRs or miRNAs) are associated with both aortic SMC apoptosis and ECM degradation [3-9]. In addition, long non-coding RNAs (lncRNAs) are involved in various biological processes via the dysregulation of target gene expression [10, 11]. They also play important roles in pathophysiological changes of vascular SMC and ECM. Various lncRNAs, such as *ANRIL*, *SENCB*, *lncRNA HIF1-AS1*, and *lncRNA-p21*, are involved in the regulation of human vascular SMC proliferation, contraction, migration, and apoptosis [12-15]. The lncRNA *HAS2-AS1* is involved in ECM remodeling via the regulation of *HAS2* transcription in vascular SMC [16]. Generally, lncRNA research related to human TAD has focused on their functions in individual cells. To date, no study has investigated the lncRNA expression profile in TAD tissues, particularly in human TAD tissues.

In addition, previous studies have mainly used microarray technology, to which cannot be used for whole-transcriptome analyses. Owing to its ability in whole transcriptome analyses, high-throughput sequencing (HTS) technology is more suitable to characterize the genetic basis of diseases compared with microarrays. Therefore, we used HTS to investigate the lncRNA expression profile in human TAD tissues, and further applied a series of bioinformatics analyses to predict the functions of these lncRNAs.

Materials and Methods

Patient sample preparation and ethics statement

The study protocol was approved by the Research Ethics Committees of both Zhongshan Hospital, Sun Yat-sen University and Guangzhou General Hospital of Guangzhou Military Region. Normal aortic (NA) tissues of donors (n=3) and TAD tissues of patients (n=3) were obtained from Zhongshan Hospital, Sun Yat-sen University and Guangzhou General Hospital of Guangzhou Military Region after informed consent was obtained (Table 1). The diagnosis of TAD was confirmed by the computed tomography angiography. All resected ascending aorta tissues following surgery were immediately frozen in liquid nitrogen and stored at -80°C until RNA extraction was performed.

Hematoxylin and eosin staining and immunohistochemistry staining

Samples were fixed by paraformaldehyde and then embedded in paraffin. Then, paraffin-embedded samples were cut into 4 µm-thick sections and stained with Hematoxylin and eosin stain (H&E) and immunohistochemistry with an antibody against SMC α -actin (Sigma, USA), to confirm the formation of aortic dissection. Slides were visualized using an optical microscope (OLYMPUS BX51, Olympus Optical, Tokyo, Japan) under different magnification.

Table 1. Clinical characteristics of TAD and NA groups. TAD, thoracic aortic dissection; NA, normal donors without aortic diseases

	TAD (n=3)	NA (n=3)
Age(years)	43.4±6.2	38.6±5.3
Males/females	3/0	3/0
Smoking	2	1
Atherosclerosis	0	0
Hypertension	2	1

Total RNA extraction

Total RNA was extracted from the tissue samples (50-100 mg tissue) using the TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. Then RNA degradation and contamination was detected by 1% agarose gels. RNA integrity and concentration was assessed by the RNA Nano 6000 Assay Kit of the Bioanalyzer 2100 system (Agilent Technologies, CA, USA). The samples were preserved at -80 °C for further analysis.

Library construction, examination, clustering, sequencing, quality control and gene expression analysis

Ribosomal RNA was removed from 3 µg of total RNA (including mRNAs and lncRNAs) by Human Epicentre Ribo-Zero™ Gold Kits (Epicentre, US). Then, the sequencing libraries were generated according to the manufacturer's instructions with varied index label by NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina (NEB, Ipswich, USA). After that, the qualification and quantification of library insert size were assessed respectively using the Agilent Bioanalyzer 2100 system (Agilent Technologies, CA, USA) and Taqman fluorescence probe of AB Step One Plus Real-Time PCR system (Library valid concentration > 10nM). Subsequently, the cluster was generated following the performance of clustering of the index-coded specimen on a cBot cluster generation system through TruSeq PE Cluster Kit v4-cBot-HS (Illumina). At last, all libraries were sequenced through an Illumina HiSeq 4000 platform, and 150 bp paired-end reads were generated.

Sequencing-received raw data was filtered by Perl scripts to ensure the quality of data. And the clean data was obtained by removing all adaptor-polluted reads, low quality tags, empty reads, and reads whose number of N bases accounting for more than 5 % from raw sequences. Then, the obtained clean data was carried out on statistics analyses on its quantity and quality.

DEGseq v1.18.0 was used for differential gene expression analysis between two samples.

Gene expression level and conservation Analysis

HTSeq v0.6.0 was used to count the Reads Count for each gene in each sample. RPKM (Reads Per Kilobase Million Mapped Reads) was calculated to estimate gene expression levels in each sample.

Then both the lncRNA GTF file merged by Cuffmerge v2.2.1 and PhastCons data from UCSC database were obtained. With these two data sets, the exon number, length, JS score and conservation analysis could be performed for each transcript.

Gene ontology enrichment and pathway analyses

A Gene Ontology (GO) analysis was used to examine the functions of aberrantly expressed mRNAs according to the Database for Annotation, Visualization and Integrated Discovery (DAVID; <http://david.abcc.ncifcrf.gov/>). A pathway analysis was used to identify the roles of aberrantly expressed protein-coding genes in biological pathways based on the Kyoto Encyclopedia of Genes and Genomes pathways (KEGG), Biocarta, and Reactome (<http://www.genome.jp/kegg/>). Fisher's exact tests were used for the classification of the GO terms and selection of significant pathways. The threshold for significance was $P < 0.05$.

lncRNA and mRNA coexpression analysis

To determine the functions of lncRNAs in the regulation of target gene expression in cis and in trans, the relationship between the predicted potential target genes of aberrantly expressed lncRNAs and the aberrantly expressed mRNAs was examined. The threshold for significance was defined as $OR \geq 0.8$ and $P < 0.05$. The predicted potential target genes whose loci were within a 10-kb window upstream or downstream of the given aberrantly expressed lncRNA were considered cis-regulated genes. Other genes were identified as a trans-regulated. To create a visual representation, an lncRNA-mRNA regulatory network was constructed using Cytoscape 3.0.

lncRNA-miRNA-mRNA network

To determine the function of aberrantly expressed lncRNAs as ceRNAs in TAD, an aberrantly expressed lncRNA-miRNA regulatory network was established using starBase v2.0 [17]._ENREF_12 Then, the target mRNAs of miRNAs affected by the potential ceRNAs were predicted using miRTarBase.

siRNA transfection

The short interfering RNA (siRNA) was used for inhibiting endogenous RNA expression. Four siRNAs, include Si1, Si2, Si3 and Si4 were designed for the knockdown of XIST which could originally up-regulate

the expression of p21. Si1 which was the most powerfully down-regulating the expression of p21, was named as human XIST RNA interference (siRNA) and chosen for subsequent experiments. The human XIST RNA interference (siRNA) target sequence was 5'-ACCTCTCCTTTCTCTGCCTAC-3', and a scramble form was performed as a control, 5'-UUCUCCGAACGUGUCACGUT-3'. The human p21 RNA interference (siRNA) target sequence was 5'-AAGAAAAGCAGACCUACUCCA-3', and a scramble form was performed as a control, 5'-UUCUCCGAACGUGUCACGUT-3'. The antagomiR-17-5p sequence (anti-miR-17-5p) was 5'-GTACCTGCACTGTAAGCACTTTG-3', and the miR-17-5p-negative control (miR-17-5p-NC) sequence was 5'-CAGAUACGUUACAGUGCAGGUAG-3'. Then, the adenoviruses harbouring those sequences were respectively constructed using the Lipofectamine 2000 (Invitrogen) according to the manufacturer's instruction.

Luciferase Assay

The loss of SMCs is one of the main pathological progressions of TAD. And the SMCs are the intrinsic cells in the aorta wall. In that, we chose it for present study. Human aortic SMCs (Cellbio Bionengineering Co., Ltd, Shanghai, China) were cultured in 96-well plates (5×10³ cells/well) 24 h and cotransfected with the wild-type (WT) or mutated (Mut) XIST reporter vector, and has-miR-17-5p or its mimics. After lysates harvested 24 h, renilla luciferase activities were measured according to the dual-luciferase assay manual (Promega).

Fluorescence Immuno-cytochemistry

Human aortic SMCs were transfected with si-XIST or si-XIST and anti-miR-17-5p (si-XIST + anti-miR-17-5p) or si-XIST, anti-miR-17-5p and si-p21 (si-XIST + anti-miR-17-5p + si-p21) or left untreated for 48h. Then fixed SMCs were incubated respectively with the two primary antibodies, including anti-ki67 (CST, dilution 1:200) and anti-H3P (abcam, dilution 1:100). And Alexa Fluor 488 or Alexa Fluor 594 as the secondary antibodies (BIOSS antibodies, dilution of 1:200) was applied for fluorescent confocal analysis.

Transcription factor (TF) target prediction

TF regulates gene transcription via binding to sequence motifs of genomic DNA is one of the important regulatory mechanisms of gene expression. Therefore, sequence motifs of DNA from 2.0 kb upstream of the transcription start site of aberrantly expressed lncRNAs were predicted. Accordingly, sequence motifs of aberrantly expressed lncRNAs whose expression levels were most strongly positively correlated (OR>0.90) with the expression levels of aberrantly expressed TFs were mapped to the genome in the Jasp database (<http://jaspar.genereg.net/>). Then, those TFs whose prediction score were greater than 8 were selected. A Pearson's correlation analysis was used to measure the regulatory ability of transcription factors by calculating the correlation between TFs and lncRNAs.

Quantitative real-time polymerase chain reaction

QRT-PCR was performed to validate high-throughput sequencing results using SYBR Green assays. Total RNA was reverse-transcribed using the Reverse Transcription Kit (Takara, Tokyo, Japan). The U6 was used as internal controls for miRNA, and GAPDH was used for lncRNA and mRNA. Fold changes in expression were calculated using 2^{-ΔΔCt} method. All primers used in this study are listed in Table 2.

Statistical analysis

All statistical analyses, including comparisons using the Student's *t*-test, unpaired *t*-test, Fisher's exact test, χ^2 test, and Pearson's correlation, as appropriate, were performed using SPSS 19.0 (SPSS, Chicago, IL, USA). *P*-values of <0.05 were considered statistically significant, and all the statistical analyses were two-sided.

Table 2. Primers used in analysis of genes expression by qRT-PCR

Primer name	Primer FW(5'-3')	Primer RW(5'-3')
NEAT1	GACCTCTCACCTACCCACT	ATGCCCAAACCTAGACCTGCC
XIST	CTGCTGATCATTTGGTGGTGT	CTCTGCTGACCTGCTATCAT
lncRNA_1421	AGGGAGTTTCTGCAACCAGATT	AGCATCTGCTTTTGTGGGAGC
ENSG00000248508	CTAACTCTGCCACACACGGT	GCTCAGACCTGCGAACCTCTT
ENSG00000226530	GGGTGAAGAGTTGGCACACA	CCCAAGAGTTTACAGAAAGCG
ENSG00000259719	ATTGCCCTGGTCACTCAAC	GGATGCCGAGAACTCACACC
has-miR-17-5p	ACTACCTGCACTGTAAGCACTTTG	
U6	CAAAATTCGTGAAGCGTTCCATAT	
ACTB	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCCAGCAGCAT
MYH11	GCAGCTTGGAAATATCGTCT	TGGATCTGGTGAATCTGTC
FBLN5	AGCAACCAATGTGTGGATGTG	TGGCAGTAACCATAGCGACAT
ACTA2	TGTGAAGCAGCTCCAGCTATG	TTGCTCTGTGCTTCTGTCACC
TIMP3	TCTGCAACTCCGACATCG	TTGGTGAAGCTCGGTACAT
RUNX1	CACCTACCACAGAGCCATCA	CTCGGAAAGGACAAGCTCC
GAPDH	AGGTGAAGGTGGAGTCAAC	CGCTCTGGAAGATGGTGTAT

Results

H&E and immunohistochemistry staining of aortic tissues

Normal aortic tissue showed a complete and continuous aortic structure, including intima, tunica media and adventitia (Fig. 1A). Specimens of aortic dissection showed a separation between intima and tunica media with thrombus formation in the dissected location (Fig. 1B). Marked changes of SMC in normal and dissected aortic location were also observed in immunohistochemical staining with an antibody against SMC α -actin (Fig. 1C-D).

Characteristics of lncRNAs and mRNAs

To examine aberrant expression, we performed HTS of lncRNAs and mRNAs in both TAD and NA tissues. The lncRNA and mRNA expression profiles were highly correlated among the 6 samples (Fig. 2A). Moreover, compared with mRNAs, lncRNAs showed lower transcript abundances (Fig. 2B), higher tissue specificity (Fig. 2C), and less conservation (Fig. 2D). The lncRNAs had more than 2 exons and were longer than 200bp (Fig. 2E and F). These properties are consistent with the typical expression features of lncRNAs [18].

Profiles of the aberrantly expressed lncRNAs and mRNAs

The correlations between aberrantly expressed lncRNA and mRNA expression profiles were much higher within groups than that among samples (Fig. 3A), indicating that the results were highly reliable. A total of

Fig. 1. H&E and immunohistochemical staining of aortic tissues. H&E staining of normal aortic tissue (A) and aortic dissection tissue (B). Immunohistochemical staining of SMC in normal aorta (C) and aortic dissection tissue (D) with an antibody against SMC α -actin.

Fig. 2. Comparisons of lncRNA and mRNA characteristics in TAD and normal aortic tissues. (A) The correlations among lncRNA and mRNA expression profiles in six samples. (B) RPKM (reads per kilobase per million) distribution for lncRNAs and mRNAs. (C) JS Score Distribution (Jensen Shannon Score Distribution) for lncRNAs and mRNAs. (D) Conservation score for two individual subtypes of lncRNAs (lncRNA-exon and lncRNA-gene) and mRNAs. (E) Distribution of the number of exons in lncRNAs and mRNAs. (F) Distribution of transcript lengths in lncRNAs and mRNAs. NA represents normal aortic tissue; TAD represents thoracic aortic dissection tissue.

Fig. 3. Aberrantly expressed genes in TAD and normal aortic tissue samples. (A) The correlations between aberrantly expressed lncRNA and mRNA expression profiles for 6 samples. (B) Volcano plot and hierarchically clustered heatmap illustrating differentially expressed lncRNAs between TAD and normal aortic tissue samples. Up-regulated lncRNAs are denoted in red and down-regulated in green. (C) Volcano plot and hierarchically clustered heatmap illustrating differentially expressed mRNAs between TAD and normal aortic tissue samples. ($P \leq 0.05$ and fold change ≥ 2.0). Up-regulated mRNAs are denoted in red and down-regulated in green. (D) and (E) qRT-PCR validation for five aberrantly expressed lncRNAs. (D) qRT-PCR was used to compare the relative expression levels of three aberrantly expressed lncRNAs (NEAT1, XIST and CARMN) and two aberrantly expressed mRNAs (MYH11 and ACTB). GAPDH was amplified as an internal control. (E) Histogram of the expression levels of five aberrantly expressed genes in TAD and NA. NA represents normal aortic tissue; TAD represents thoracic aortic dissection tissue. * $P < 0.05$ vs. NA. ** $P < 0.01$ vs. NA.

269 lncRNAs (159 up-regulated and 110 down-regulated) and 2,255 mRNAs (1,294 up-regulated and 961 down-regulated) were significantly aberrantly expressed in TAD tissues compared with those in NA tissues (fold-change > 1.5 , $P < 0.05$). The top 10 dys-regulated (up-regulated and down-regulated) unknown lncRNAs, known lncRNAs and mRNAs were respectively shown in Table 3, 4 and 5. A hierarchically clustered heat map and volcano plots illustrate differentially expressed lncRNA (Fig. 3B) and mRNA (Fig. 3C) between TAD and NA tissues.

Three aberrantly expressed lncRNAs (*NEAT1*, *XIST* and *CARMN*) and two aberrantly expressed mRNAs (*MYH11* and *ACTB*) were selected for qRT-PCR analyses (Fig. 3D and E). Based on the qRT-PCR results, three genes (*CARMN*, *MYH11*, and *ACTB*) exhibited lower expression and two genes (*NEAT1* and *XIST*) exhibited higher expression in TAD than in NA tissues. The qRT-PCR results were generally consistent with those of HTS. Additionally, several established biomarkers of aortic disorders were detected, including two up-regulated genes and eight down-regulated genes (Table 6) [19]. To some extent, these findings provided additional validation of our results.

GO and KEGG enrichment analyses of aberrantly expressed mRNAs

Both GO and KEGG enrichment analyses were used to determine the functions of aberrantly expressed mRNAs. A GO enrichment analysis showed that the up-regulated

Table 3. Top 10 up-regulated and down-regulated unknown lncRNAs (TAD vs.NA)

Gene	chromosomal position	TAD normalize	NA normalize	logFC	P-value	Up/Down	nearest mRNA
Inc_1935	Chromosome 12: 50,286,244-50,289,231+	433.9894	0.01	15.40537232	1.72E-12	up	LIMA1
Inc_2758	Chromosome X: 119,466,034-119,468,262+	348.8934	0.01	15.09049885	1.07E-11	up	SLC25A5
Inc_817	Chromosome 5: 149,406,879-149,428,678+	224.7363	0.01	14.45594533	1.61E-09	up	IL17B
Inc_2433	Chromosome 17: 14,210,488-14,217,922+	192.3721	0.01	14.23161223	2.18E-09	up	COX10
Inc_2276	Chromosome 15: 81,953,303-81,995,666+	156.5486	0.01	13.93432257	2.24E-08	up	MEX3B
Inc_1826	Chromosome 11: 71,807,591-71,818,238+	133.305	0.01	13.70244308	9.10E-08	up	ZNF705E
Inc_316	Chromosome 2: 19,026,641-19,028,487+	114.4818	0.01	13.48283096	2.63E-07	up	OSR1
Inc_2100	Chromosome 14: 21,200,461-21,206,900+	108.257	0.01	13.40217234	2.94E-07	up	HNRNPC
Inc_2430	Chromosome 17: 13,940,571-13,932,990+	81.9696	0.01	13.00087332	1.57E-06	up	COX10
Inc_971	Chromosome 6: 137,823,673-137,825,894+	58.68159	0.01	12.51869224	8.24E-06	up	TNFAIP3
Inc_2665	Chromosome 20: 44,655,451-44,696,096+	0.01	38.04268	-11.89340335	7.70E-05	down	ADA
Inc_2667	Chromosome 20: 45,447,232-45,435,971+	0.01	26.89786	-11.39327584	0.000349671	down	PIGT
Inc_2703	Chromosome 22: 11,868,214-11,974,044+	0.01	29.07035	-11.5053329	0.000295451	down	OR11H1
Inc_1145	Chromosome 7: 153,411,234-153,413,963+	0.01	296.2307	-14.85443354	1.95E-11	down	DPP6
Inc_2468	Chromosome 17: 39,243,963-39,250,818+	0.01	51.78437	-12.33830104	1.19E-05	down	FBXL20
Inc_950	Chromosome 6: 113,868,013-113,873,351+	0.01	18.61126	-10.86196032	0.001385051	down	MARCKS
Inc_1019	Chromosome 6: 2,621,919-2,634,603+	0.01	182.0942	-14.1523976	9.72E-10	down	MYLK4
Inc_1162	Chromosome 7: 25,593,351-25,663,053+	0.01	30.96043	-11.59620998	0.000125116	down	NPVF
Inc_839	Chromosome 5: 173,707,614-173,746,209+	0.01	47.27378	-12.20682446	1.23E-05	down	CPEB4
Inc_1415	Chromosome 9: 103,140,523-103,325,034+	0.01	1335.465	-17.02698268	4.46E-16	down	CYL2

Table 5. Top 10 up-regulated and down-regulated mRNAs (TAD vs.NA)

Gene	Gene symbol	TAD normalize	NA normalize	logFC	P-value	Up/Down
ENSG00000163735	CXCL5	233.602334	0.348672	9.387966939	6.14E-24	up
ENSG00000120337	TNFSF18	37.686702	0.348672	6.756039697	2.95E-07	up
ENSG00000125740	FNOS	8843.173421	102.8456	6.426012545	1.99E-11	up
ENSG00000164326	CARTPT	4383.868420	51.62358	6.408030513	2.44E-06	up
ENSG00000164265	SCOR3A2	22.601086	0.320167	6.141421082	2.88E-05	up
ENSG00000105048	TNNT1	15.909210	0.325334	5.611796358	0.000629251	up
ENSG00000109321	AREG	410.222133	8.692247007	5.560532352	6.23E-22	up
ENSG00000163734	CXCL3	508.135970	13.92110862	5.189868584	2.86E-22	up
ENSG00000170345	FOS	41743.043758	1366.7133	4.93275326	7.83E-10	up
ENSG00000185897	FFAR3	20.383130	0.674006456	4.918469382	0.000290004	up
ENSG00000162772	CXCL14	0.324473	2201.1	-12.72784055	2.33E-49	down
ENSG00000080971	AIBPQ	0.973418	3031.047	-11.60469059	2.16E-05	down
ENSG00000107518	ACR1	0.334318	6394.166	-10.90177016	3.72E-48	down
ENSG00000162711	CADM3	0.324473	292.0862	-9.814081932	2.91E-29	down
ENSG00000124813	RPL109	0.983152	458.0547	-8.863889207	3.53E-37	down
ENSG00000151575	LICAM	0.658680	290.0313	-8.78241576	4.01E-27	down
ENSG00000125648	PI16	0.334207	125.8585	-8.556844938	1.92E-16	down
ENSG00000112984	ITIL1	2.320536	742.3876	-8.321570901	2.25E-11	down
ENSG00000135917	HOMAS	8.611952	2713.315	-8.295506099	1.46E-23	down
ENSG00000207744	RELN	0.658680	196.037	-8.217333174	1.80E-21	down

mRNAs were mainly associated with the following functions: inflammatory response, immune response, signal transduction, cell-cell signaling, T cell proliferation involved in immune response, negative regulation of inflammatory response, cellular response to lipopolysaccharide, heterotypic cell-cell adhesion, leukocyte migration, chemotaxis. Down-regulated mRNAs were associated with the following functions: angiogenesis, regulation of blood pressure, leukocyte migration, platelet degranulation, uterus development, response to hypoxia, glossopharyngeal nerve morphogenesis, planar cell polarity pathway involved in axis elongation, cell adhesion, and positive regulation of gene expression (Fig. 4A).

A KEGG enrichment analysis indicated that the up-regulated mRNAs were chiefly involved in Rheumatoid arthritis, leishmaniasis, intestinal immune network for IgA production, systemic lupus erythematosus, pertussis, cytokine-cytokine receptor interaction, TNF signaling pathway, hematopoietic cell lineage, staphylococcus aureus infection, osteoclast differentiation. The down-regulated mRNAs were involved in PPAR signaling pathway, proteoglycans in cancer, vascular smooth muscle contraction, bacterial invasion of epithelial cells, cGMP-PKG signaling pathway, fatty acid elongation, signaling pathways regulating pluripotency of stem cells, propanoate metabolism, adrenergic signaling in cardiomyocytes,

Table 4. Top 10 up-regulated and down-regulated known lncRNAs (TAD vs.NA)

Gene	chromosomal position	TAD normalize	NA normalize	logFC	P-value	Up/Down	Nearest mRNA
ENSG00000229807	Chromosome X: 73,020,051-73,852,753+	6330.346	27.63586	7.839598743	8.58E-07	up	CHIC1
ENSG00000230838	Chromosome 2: 215,718,043-215,719,424+	361.7735	7.766359	5.541704646	1.97E-18	up	MREG
ENSG00000260823	Chromosome 16: 56,609,501-56,611,375+	26.59032	1.034146	4.684389886	3.04E-05	up	MT2A
ENSG00000236882	Chromosome 5: 95,852,232-95,860,133+	174.2612	8.760695	4.314062588	0.000102164	up	ELL2
ENSG00000241163	Chromosome 3: 72,035,300-72,279,503+	72.20052	4.131444	4.127291184	1.31E-07	up	KYBP
ENSG00000272021	Chromosome 5: 95,849,309-95,849,855+	47.80642	2.923231	4.031568536	3.12E-06	up	GLRX
ENSG00000269927	Chromosome 14: 71,141,125-71,143,253+	40.20674	2.846981	3.819932785	2.33E-05	up	PONX1
ENSG00000231817	Chromosome 13: 46,455,135-46,486,506+	31.94922	2.381508	3.745833117	0.000286799	up	KIAA0226L
ENSG00000260135	Chromosome 16: 55,426,797-55,462,297+	35.78173	3.67111	3.284934794	0.000192417	up	MMP2
ENSG00000280018	Chromosome 21: 6,986,450-6,997,765+	30.89401	3.545312	3.123342413	0.000262283	up	U2AF1L5
ENSG00000267107	Chromosome 19: 41,454,169-41,500,649+	7.106935	115.2637	-4.01956677	7.33E-05	down	PCAT19
ENSG00000267603	Chromosome 17: 70,051,277-70,068,095+	0.01	13.02435	-10.34699596	0.000231985	down	KCN16
ENSG00000261685	Chromosome 16: 50,645,809-50,649,249+	229.7083	1645.072	-2.840276529	5.90E-08	down	NKD1
ENSG00000179136	Chromosome 17: 12,549,782-12,542,854+	136.1124	579.9515	-2.091133945	0.000178449	down	MYOC
ENSG00000259129	Chromosome 14: 47,764,954-47,795,092+	0.30847	14.45235	-5.550029836	0.000315329	down	MDGA2
ENSG00000226197	Chromosome 9: 13,446,491-13,487,511+	3.107876	61.21916	-4.299902347	8.72E-06	down	MPDZ
ENSG00000163364	Chromosome 2: 176,637,931-176,637,931+	2.866995	30.53217	-3.412718981	0.000127288	down	MTX2
ENSG00000240405	Chromosome 3: 69,999,577-70,015,318+	28.96542	693.4513	-4.581390953	1.76E-14	down	MTTF
ENSG00000234665	Chromosome 1: 62,856,999-62,898,087+	7.150474	54.02638	-2.917553238	0.000144046	down	KGFLP1
ENSG00000215808	Chromosome 1: 238,480,384-238,486,023+	1.274606	64.41237	-6.565921404	2.03E-09	down	ZP4

Table 6. Information of known aortic dissection biomarker genes in TAD

Gene	name	P-value	logFC
up-regulation			
IL6R	ENSG00000160712	0.006168	2.715744
MAP4K4	ENSG00000071054	0.001657	1.702181
down-regulation			
TIMP1	ENSG00000130758	0.007265	0.660726
FBLN5	ENSG00000140092	0.039146	0.526762
MYH11	ENSG00000133392	0.021778	0.33909
TIMP3	ENSG00000100234	0.010745	0.249099
ACTA2	ENSG00000107796	0.028532	0.222923
ACTN2	ENSG00000077522	0.048531	0.211353
P4HA1	ENSG00000122884	0.005896	2.324997
ITGBA	ENSG00000132470	0.019307	1.703597

Fig. 4. GO and KEGG enrichment analyses for all aberrantly expressed mRNAs. (A) GO enrichment analysis for all aberrantly expressed mRNAs in TAD (Top 10). (B) KEGG enrichment analysis for all aberrantly expressed mRNAs in TAD (Top 10).

Fig. 5. The aberrantly expressed lncRNAs regulated the target genes in cis and trans. (A) GO enrichment analysis for the aberrantly expressed mRNAs which were predicted to be regulated by the aberrantly expressed lncRNAs in cis. (B) The up-regulated lncRNA (ENSG00000269936) was significantly correlated with the expression of its up-regulated mRNA (mitogen-activated protein kinase kinase 6, MAP2K6). (C) GO enrichment analysis for aberrantly expressed mRNAs which were predicted to be regulated by the aberrantly expressed lncRNAs in trans. (D) Co-expression lncRNA-mRNA network shows the potential relationship between aberrantly expressed mRNAs and lncRNAs in trans. (E) and (F) qRT-PCR analysis shows the relative expression levels of one lncRNA (lnc_1421) and three aberrantly expressed mRNAs (FBLN5, ACTA2, and TIMP3) in TAD and NA. GAPDH was amplified as an internal control. (G), (H), and (I) Scatter plots show the correlations between the expression of FBLN5 and lnc_1421, ACTA2, and lnc_1421, as well as TIMP3 and lnc_1421. NA represents normal aortic tissue; TAD represents thoracic aortic dissection tissue. * $P < 0.05$ vs. NA. ** $P < 0.01$ vs. NA.

Fig. 6. Co-expression lncRNA-miRNA-mRNA network. (A) The regulatory network of lncRNA XIST and 202 potential targeted miRNAs. (B) The chart showed that 29 out of 202 predicted miRNAs were reported significantly aberrantly expressed in TAD. (C) The network of lncRNA XIST and those 29 miRNAs. (D)

1053 mRNAs was predicted to be targeted by those 29 miRNAs. Among them, 66 mRNAs were significantly aberrantly expressed in TAD in our data. (E) GO and KEGG enrichment analyses for those 66 aberrantly expressed mRNAs which were predicted to be regulated by co-expression lncRNA xist-miRNA-mRNA network. (F) Histogram of lncRNA XIST, has-miR-17-5p, and p21 relative expression levels in TAD and NA assessed by qRT-PCR. NA represents normal aortic tissue; TAD represents thoracic aortic dissection tissue. **P<0.01 vs. NA.

and hippo signaling pathway (Fig. 4B). These results suggested that those up-regulated mRNAs may directly promote the TAD pathological process, whereas down-regulated mRNAs probably suppress vascular smooth muscle contraction, which may indirectly participate in the TAD pathological process.

Prediction of functional effects of aberrantly expressed lncRNAs on target mRNAs in cis and trans

A GO enrichment analysis was also applied to examine the regulatory functions of aberrantly expressed lncRNAs on aberrantly expressed mRNAs in cis and trans. The aberrantly expressed mRNAs targeted by aberrantly expressed lncRNAs in cis were involved in multiple biological processes, such as regulation of MAP kinase activity, activation of MAPK activity, positive regulation of catalytic activity, positive regulation of MAP kinase activity, positive regulation of molecular function, mesenchymal cell differentiation, mesenchymal cell development, mesenchymal development, protein amino acid phosphorylation, and positive regulation of cell proliferation (Fig. 5A). For example, the expression level of up-regulated lncRNA (ENSG00000269936) was potential correlated with the expression of its adjacent mRNA (mitogen-activated protein kinase kinase 6, MAP2K6) ($R=0.940$, $P<0.01$) (Fig. 5B).

Aberrantly expressed mRNAs that were targeted by aberrantly expressed lncRNAs in trans were associated with various biological processes, such as Muscle contraction, cell junction assembly, substrate adhesion-dependent cell spreading, protein autophosphorylation, cell adhesion, positive regulation of cell migration, skeletal system development, positive regulation of cell proliferation, carbohydrate metabolic process, and positive regulation of

Fig. 7. LncRNA XIST regulated the expression of P21 by binding to has-miR-17-5p in vitro. (A) SMCs were co-transfected with either siRNAs of XIST or scramble form. Two days after transfection, the cells were then collected for qR-PCR analysis to evaluate relative expression of p21. (B) LncRNA XIST and its predicted miRNA(has-miR-17-5p) target. (C) Luciferase reporter analysis of

either wild-type or mutant miR-17-5p activity. LncRNA XIST was co-transfected with the wild-type or mutant vector. (D) Representative ki67 fluorescence immuno-cytochemistry images of proliferation of SMCs transfected with si-XIST or si-XIST and anti-miR-17-5p (si-XIST + anti-miR17-5p) or si-XIST, anti-miR-17-5p and si-p21 (si-XIST + anti-miR17-5p + si-p21) or left untreated (NC) for 48h. Blue, nuclei; red, Ki67. (E) Quantification of ki67-stained SMCs. (F) Representative H3P fluorescence immuno-cytochemistry images of proliferation of different genes transfected SMCs. Blue, nuclei; red, H3P. (G) Quantification analysis of proliferating H3P-stained SMCs. The presented values are the mean $\pm$ standard deviation (SD) of three independent preparations. * $P < 0.05$, ** $P < 0.01$.

TGFBR signaling pathway (Fig. 5C). Moreover, the lncRNA-mRNA network analysis showed the relationships between aberrantly expressed lncRNAs and mRNAs in trans (Fig. 5D). Based on qRT-PCR results, *lncRNA_1421* (ENST00000453455.1), *FBLN5*, *ACTA2*, and *TIMP3* exhibited lower expression in TAD than in NA tissues (Fig. 5E and F). For example, the expression of down-regulated *lncRNA_1421* was significantly correlated with the expression of three down-regulated mRNAs (*FBLN5*, $R = 0.950$, $P < 0.01$; *ACTA2*, $R = 0.96$, $P < 0.01$; *TIMP3*, $R = 0.96$, $P < 0.05$) (Fig. 5G, H and I).

LncRNA-miRNA-mRNA network

LncRNA and mRNA have similar sequences that can be bound by a same miRNA. When bound by miRNA, up-regulated lncRNAs serve as competing endogenous RNAs (ceRNA), that prevents miRNA from binding to natural mRNA targets and increasing the mRNA expression at the post-transcriptional level [20-22]. The lncRNA-miRNA network indicated the relationships between aberrantly expressed lncRNA XIST and its 202 potential target miRNAs (Fig. 6A). 29 out of those miRNAs have been reported significantly aberrantly expressed in TAD (Fig. 6B and C) [8].

Furthermore, those miRNAs was predicted to target 1053 mRNAs. 66 mRNAs were significantly aberrantly expressed in TAD in our data (Fig. 6D). A GO enrichment analysis showed that the aberrantly expressed mRNAs were involved in multiple biological processes, such as positive regulation of cell proliferation, gene expression, transcription from RNA polymerase promoter, MAPK cascade, transcription from RNA polymerase II promoter and apoptotic process, phosphatidylinositol-mediated signaling, somatic stem cell population

Fig. 8. RUNX1 motif prediction of aberrantly expressed lncRNAs. (A) RUNX1 motif prediction of aberrantly expressed lncRNAs from JASPAR. Upper panel: RUNX1 binding site close to the predicted motifs; lower panel: typical upstream sequences of aberrantly expressed lncRNAs containing the motifs. (B) Genome plots showing the predicted motif location on chr21 for RUNX1 binding in each sample. (C) The histogram of three lncRNAs (SRP14-AS1, RP11-348F1.2, and RP11-930O11.1) relative expression levels in TAD and NA assessed by qRT-PCR. The chromosomal positions of SRP14-AS1, RP11-348F1.2, and RP11-930O11.1 were Chromosome 15: 40,039,311-40,067,290, Chromosome X: 51,396,511-51,465,661, and Chromosome 14: 56,310,880-56,345,001, respectively. NA represents normal aortic tissue; TAD represents thoracic aortic dissection tissue. *P<0.05 vs. NA.

maintenance, and embryonic cranial skeleton morphogenesis (Fig. 6E). A KEGG enrichment analysis indicated that the aberrantly expressed mRNAs were involved in the pathways in cancer, PI3K-Akt signaling pathway, MicroRNAs in cancer, MAPK signaling pathway, proteoglycans in cancer, focal adhesion, HIF-1 signaling pathway, glioma, melanoma, and AMPK signaling pathway (Fig. 6E).

Additionally, qRT-PCR results showed that *P21* and lncRNA *XIST* exhibited higher expression and that has-miR-17-5p exhibited lower expression in TAD than in normal aortic tissues (Fig. 6F). These qRT-PCR results validated the lncRNA-miRNA-mRNA co-expression relationships.

LncRNA XIST regulated the expression of P21 by binding to has-miR-17-5p in vitro

To explore whether lncRNA XIST could regulated the expression of *P21*, an siRNA technology was employed to knockdown lncRNA XIST in SMCs. The results showed that the knockdown of lncRNA XIST significantly reduced the expression of *P21* (Fig. 7A).

Through the miRtarBase, lncRNA XIST was predicted to bind to has-miR-17-5p (Fig. 7B). Then luciferase assay system was employed to determine whether LncRNA XIST could directly target the has-miR-17-5p. The luciferase reporter revealed that the level of has-miR-17-5p was suppressed by the wild-type lncRNA XIST, whereas it was not influenced by the mutant lncRNA XIST (Fig. 7C).

In addition, Ki67 and H3P immuno-cytochemistry staining and quantitative analysis was performed to investigate proliferation of SMCs transfected with si-XIST or si-XIST and anti-miR-17-5p (si-XIST + anti-mir17-5p) or si-XIST, anti-miR-17-5p and si-p21 (si-XIST + anti-mir17-5p + si-p21) or left untreated for 48h. The results showed that si-XIST and si-XIST + anti-mir17-5p + si-p21 can significantly enhance the relative expression level of Ki-67 and H3P, whereas si-XIST + anti-mir17-5p can not affect relative expression level of either Ki-67 or H3P (Fig. 7D, E, F and G), indicating that lncRNA XIST could regulates *p21* by binding to has-miR-17-5p, consequently affected the proliferation of SMCs *in vitro*.

Prediction of Transcription factors motif for aberrantly expressed lncRNAs

Transcription factors (TFs) are crucial components that can bind to the promoters of lncRNAs, regulating lncRNA expression, and consequently affecting disease pathogenesis.

[23]. Four aberrantly expressed TFs, *Runx1*, *srebf2*, *phtf2*, and *Runx2*, were found in the set of aberrantly expressed mRNAs. *Runx1*, which was up-regulated in our analysis, is reported to be related to TAD [24, 25]. The predicted binding motifs of three corresponding up-regulated lncRNAs, including ENSG00000248508 (SRP14-AS1), ENSG00000226530 (RP11-348F1.2), and ENSGN00000259719 (RP11-930O11.1), were positively correlated with up-regulated *RUNX1* ($R=0.982$, $P<0.001$; $R=0.967$, $P<0.01$; $R=0.960$, $P<0.01$, respectively) (Fig. 8A). Genome plots showed the predicted motif location on chr21 for *RUNX1* binding in each sample (Fig. 8B). Moreover, qRT-PCR results showed that three lncRNAs (SRP14-AS1, RP11-348F1.2, and RP11-930O11.1) were expressed at higher levels in TAD than in NA tissues (Fig. 8C). The qRT-PCR results were consistent with the HTS results.

Discussion

Using HTS technique, we systematically demonstrated not only that both lncRNAs and mRNAs were aberrantly expressed in human TAD tissues compared with those in NA tissues, but also that these lncRNAs may be largely involved in SMC loss and ECM degradation. Importantly, we found that ENSG00000269936, lnc_1421, and the lncRNA XIST could alter the expression levels of *MAP2K6*, three other genes (*FBLN5*, *ACTA2*, and *TIMP3*), and one mRNA (*p21*) by functioning as cis-regulators, trans-regulators, and ceRNA, respectively. Furthermore, we discovered that three lncRNAs (ENSG00000248508, ENSG00000226530, and ENSG00000259719) were up-regulated by *RUNX1*.

By filtering the aberrantly expressed genes located near the aberrantly expressed lncRNAs, we found that several lncRNAs might regulate the transcription of chromosomally adjacent genes in cis. For instance, we found that up-regulated ENSG00000269936 might target protein-coding gene *MAP2K6* in cis. *MAP2K6* is a member of the MAPK signaling pathway, which is the most highly upregulated pathway in our data. Previous studies have shown that the p38 MAPK signaling pathway is a crucial mechanism in the angiotensin II-mediated up-regulation of MMP-2, pathological mechanical stretch-promoted high expression of MMP-2, and monocyte chemoattractant protein-1-stimulated over-expression of MMP-9 in human aortic smooth muscle cells [26-28]. Moreover, the p38 MAPK pathway is an important mechanism for increased cyclooxygenase-2 (COX-2) expression in rat aortic fibroblasts treated with angiotensin II [29]. Accordingly, ENSG00000269936 may have regulatory functions in TAD pathogenesis by targeting its adjacent gene, *MAP2K6*, in cis.

We also discovered that some lncRNAs might regulate the expression of their target genes in trans. For example, down-regulated lncRNA_1421, may target genes in trans, including *ACTA2*, *FBLN5*, and *TIMP3*. lncRNA_1421 is a 2666-bp lncRNA located on chromosome 9, while *ACTA2*, *FBLN5*, and *TIMP3*, which are associated with TAD, are located on chromosomes 10q23.24, 14, and 22, respectively. *ACTA2* encodes the aortic smooth muscle actin, and mutations in this gene are associated with the formation of human non-syndromic aortic dissection [30]. In addition, *FBLN5* encodes extracellular matrix protein. *FBLN5*-deficient mice show severe vascular remodeling by inhibiting the assembly of mature elastic fibers [31]. Moreover, *TIMP3* encodes inhibitors of matrix metalloproteinases, which lead to extracellular matrix degradation [32]. Consequently, lncRNA_1421 might have regulatory functions in TAD by simultaneously regulating the expression of *ACTA2*, *FBLN5*, and *TIMP3* in trans.

Moreover, lncRNAs could act as ceRNAs by sponging miRNAs and subsequently increase the expression of target mRNAs. Based on the results of the lncRNA-miR-mRNA network and *in vitro* experiments, we found that up-regulated lncRNA XIST could increase the expression of *p21* by acting as a miR-17-5p sponge. Previous studies have demonstrated that miR-17-5p is involved in the expression of several genes, such as *p21*, *PKD2*, and *SOD2*, which are associated with aortic diseases [33-35]. Among them, *p21* was the only mRNA that was aberrantly expressed in TAD in our study. *p21* plays an important role in transcriptional regulation after DNA damage and apoptosis [36, 37]. And miR-17-5p could also downregulate

the expression of p21 in nasopharyngeal carcinoma cell lines and tissues [38]. It's well known that XIST is not expressed in normal males. However, our data actually indicated the expression of XIST in TAD was significantly higher than that in normal aortic tissues in males. XIST is expressed in male TAD however not in normal male aortic tissues, which maybe part mechanism of TAD. Thus, the lncRNA XIST might have regulatory functions in TAD by acting as a ceRNA via sponging miR-17-5p, thereby increasing the expression of p21.

Furthermore, we found that several aberrantly expressed lncRNAs might be initially up-regulated by TFs binding to DNA motifs of their transcription start site. For example, *RUNX1*, an aberrantly expressed mRNA, might significantly up-regulate the expression of lncRNAs, such as ENSG00000248508, ENSG00000226530, and EG00000259719. *RUNX1* plays an important role in the transcriptional regulation of gene expression and the development of immune system [39]. In human abdominal aortic aneurysms, significantly increased *RUNX1* up-regulates MMP-9 expression and contributes to the immune response [24, 25]. Hence, we inferred that, to a certain extent, the aberrantly expressed lncRNAs were up-regulated by *RUNX1* and had regulatory functions in TAD by participating in *RUNX1*-mediated regulation.

In conclusion, the dysregulated lncRNAs in human TAD tissues were capable of regulating the expression of target genes via several potential mechanisms, including serving as cis-acting factors, trans-acting factors and ceRNAs, and finally lead to the pathological processes of TAD. These findings indicate that the dysregulated lncRNAs may be a novel therapeutic target to prevent the pathological progression of TAD. However, additional work is needed to verify the functions of these dysregulated lncRNAs in the pathogenesis of TAD. And future studies should be endeavored to make clear whether and how XIST expresses in female TAD, and whether XIST is working in the development of female TAD.

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Disclosure Statement

No conflict of interests exists.

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