Aberrantly Expressed IncRNAs in Primary Varicose Great Saphenous Veins

Xiang Li1,2,3*, Xiao-Yan Jiang1,2,3*, Jin Ge4*, Jing Wang1,2, Guo-Jun Chen4, Liang Xu1, Duan-Yang Xie1,2,3, Tian-You Yuan1,2,3, Da-Sheng Zhang1,2, Hong Zhang1,2, Yi-Han Chen1,2,3*

1 Key Laboratory of Arrhythmia of the Ministry of Education of China, East Hospital, Tongji University School of Medicine, Shanghai, China, 2 Institute of Medical Genetics, Tongji University, Shanghai, China, 3 Department of Pathology and Pathophysiology, Tongji University School of Medicine, Shanghai, China, 4 Department of Vascular Surgery, East Hospital, Tongji University School of Medicine, Shanghai, China

Abstract

Long non-coding RNAs (lncRNAs) are key regulatory molecules involved in a variety of biological processes and human diseases. However, the pathological effects of lncRNAs on primary varicose great saphenous veins (GSVs) remain unclear. The purpose of the present study was to identify aberrantly expressed lncRNAs involved in the prevalence of GSV varicosities and predict their potential functions. Using microarray with 33,045 lncRNA and 30,215 mRNA probes, 557 lncRNAs and 980 mRNAs that differed significantly in expression between the varicose great saphenous veins and control veins were identified in six pairs of samples. These lncRNAs were sub-grouped and mRNAs expressed at different levels were clustered into several pathways with six focused on metabolic pathways. Quantitative real-time PCR replication of nine lncRNAs was performed in 32 subjects, validating six lncRNAs (AF119885, AK021444, NR_027830, G36810, NR_027927, uc.345). A coding-non-coding gene co-expression network revealed that four of these six lncRNAs may be correlated with 11 mRNAs and pathway analysis revealed that they may be correlated with another 8 mRNAs associated with metabolic pathways. In conclusion, aberrantly expressed lncRNAs for GSV varicosities were here systematically screened and validated and their functions were predicted. These findings provide novel insight into the physiology of lncRNAs and the pathogenesis of varicose veins for further investigation. These aberrantly expressed lncRNAs may serve as new therapeutic targets for varicose veins. The Human Ethics Committee of Shanghai East Hospital, Tongji University School of Medicine approved the study (NO.: 2011-DF-53).

Introduction

Varicose veins, whose manifestations can vary from leg edema to chronic, disabling venous ulceration, affect around 25% of the adult population and can lead to considerable morbidity and congestion of health service resources [1]. Great saphenous veins (GSVs) or saphenofemoral junction accounts for about 70% of varicosities and predict their potential functions. Many molecules from many different pathways are involved in the pathological processes associated with varicose veins. These molecules include hypoxia-inducible factor 1-alpha (HIF-1alpha) in the hypoxia pathway [7], the Janus kinase/signal transducers and activators of transcription (JAK-STAT) and nuclear factor kappaB (NF-kappaB) in the inflammatory pathway [8], poly ADP ribose polymerase (PARP) and bax in the apoptotic pathway [9], adhesion molecules and cytokines such as intercellular adherence molecule 1 (ICAM-1), interleukin-1 alpha (IL-1alpha), and tumor necrosis factor alpha (TNF-alpha) [10]. However, these molecules account for only some of the tangled mechanisms, and many more remain to be identified. In addition, molecules such as long non-coding RNAs (lncRNAs) and micro-RNAs whose concentrations are correlated with those of their targets at the mRNA level, post-transcriptional level, or protein level must be systematically screened and validated.

lncRNAs are a class of transcripts whose lengths exceed 200 nt [11]. They are found throughout the genome. lncRNAs play key
regulatory roles in regulating transcription in both cis form and antisense form, localization of proteins, and organizational frameworks of sub-cellular structures. lncRNAs also post-transcriptionally control mRNAs by affecting splicing, editing, translation, and degradation. In addition, many lncRNAs are processed into small RNAs or, modulate how other RNAs are processed. It is becoming increasingly clear that lncRNAs function in several different ways and play key roles in many intracellular regulatory processes [12,13]. The abnormal regulation of lncRNAs is involved in several human diseases, such as cancer [14–16], Alzheimer’s disease [17], spinocerebellar ataxia (SCA) [18] and cardiovascular disease [19–25]. However, the relationship between lncRNAs and varicose veins is still unclear.

The present study was designed for screening and validation of lncRNAs at the micro-array level and examination of their relationship with the prevalence of varicose veins mRNAs and mRNA pathways associated with the aberrantly expressed lncRNAs, were identified at the whole micro-array level. These mRNAs and pathways may be relevant to the incidence of GSV varicosity.

**Materials and Methods**

**Patients and Tissue Samples**

Thirty-two samples of human primary GSVs were retrieved from 32 patients (14 men, 18 women) who were undergoing GSVs varicose vein excision in Shanghai East Hospital, Tongji University School of Medicine, China. The diagnosis of primary varicose GSVs was based on the clinical signs and duplex ultrasound scanning. All patients were characterized as having primary varicosities, and patients with secondary varicosities were excluded. None of the participants had any history of deep venous thrombosis, superficial thrombophlebitis, post-thrombotic syndrome, Klippel-Trenaunay syndrome, May-Thurner syndrome or any other venous disease. The clinical, etiological, anatomical and pathological elements classification system (CEAP) was used to classify chronic lower-extremity venous disease (CVD) in this case [26,27]. The patients’ clinical signs placed all of them in classes 4–6, 30 of them were in class 4. Preoperative lower-extremity venous duplex ultrasound scanning assessment was performed on all patients, and examinations of both the superficial and the deep venous systems examination were conducted. All patients exhibited reflux in the GSVs. Patient demographics and clinical risk factors are given in Table 1.

Paired tissues were used to evaluate the differences in expression level between varicose veins (VVs) and adjacent normal segments of saphenous veins (NVs). According to visual inspection and pathological examination, VV was the obvious varicose vein, and NV was the adjacent normal vein. NV samples were collected about 3–4 cm from the VV area. The tissues were snap-frozen into liquid nitrogen immediately after resection for later RNA extraction. Six pairs of samples were taken from six patients from 32 patients were used for lncRNAs expression microarray and all 32 samples were used for quantitative real-time PCR (Q-RT PCR) validation. Written informed consent was obtained from all participants. The study was approved by the Human Ethics Committee of Shanghai East Hospital, Tongji University School of Medicine (NO.: 2011-DF-53).

**RNA Extraction and RNA Quantity Control**

Total RNA was extracted from 32 pairs of samples which had been snap frozen using TRIzol reagent (Invitrogen, Carlsbad, CA, U.S.) according to the manufacturer’s protocol. The amount and quality of RNA were measured using NanoDrop ND-1000 and RNA integrity was assessed by standard denaturing agarose gel electrophoresis.

**RNA Labeling and Microarray Hybridization**

Sample labeling and microarray hybridization were performed using a modified version of the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technology). Briefly, rRNA was removed from the total RNA sample, and then the mRNA was purified (mRNA-ONLY™ Eukaryotic mRNA Isolation Kit, Epicentre). Then, each sample was amplified and transcribed into fluorescent cRNA along the entire length of the transcripts without 3’ bias using a random priming method. The labeled cRNAs were purified using an RNeasy Mini Kit (Qiagen). The concentration and specific activity of the labeled cRNAs (pmol Cy3/µg cRNA) were measured using NanoDrop ND-1000. Then 1 µg of each labeled RNA was fragmented by adding 11 µl 10 × Blocking Agent and 2.2 µl of 25 × fragmentation buffer. Then the mixture was heated at 60°C for 30 min. Then 55 µl 2 × GE hybridization buffer was added to dilute the labeled cRNA. Then 100 µl of hybridization solution was dispensed into the gasket slide and placed in the Arraystar Human LncRNA Array v2.0 with 33,045 LncRNAs were collected from the authoritative data sources including RefSeq, UCSC KnownGenes, Ensembl and many related studies. The slides were incubated for 17 h at 65°C in an Agilent Hybridization Oven. The hybridized arrays were washed, fixed, and scanned with using the Agilent DNA Microarray Scanner (part number G2505B). The microarray work was performed by KangChen Bio-tech (Shanghai). The microarray data discussed in this article have been deposited in National Center for Biotechnology Information (NCBI) Gene Expression Omnibus (GEO) and are accessible through (GEO) Series accession number GSE51260 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE51260).

**Q-RT-PCR**

Total RNA was extracted from frozen vein specimens using TRIzol reagent (Invitrogen Life Technologies) and then reverse transcribed using a PrimeScript™ RT Reagent Kit (Takara) according to the manufacturer’s instructions. LncRNA expression in VV and paired NV tissues was measured by Q-RT-PCR using Power SYBR® Green PCR Master Mix (Applied Biosystems) on the ABI PRISM® 7900 Sequence Detection System (SDS) instrument. The Q-RT-PCR primers were designed using Primer 3.0 and blasted specifically in NCBI. Then 1 µg of total RNA was converted to cDNA according to the manufacturer’s protocol. PCR was performed in a total reaction volume of 10 µl, including 5 µl SYBR Green PCR Master Mix (2×), 0.4 µl each of PCR forward and reverse primer (10 µM), 0.5 µl of cDNA, and 3.7 µl of double-distilled water. The quantitative real-time PCR reaction was set at an initial denaturation step of 10 min at 95°C followed by 40 cycles of 95°C for 15 s and, 60°C for 1 min. All experiments were performed in triplicate.

**Data Computational Analysis**

For lncRNAs and mRNA microarray screening analysis, Agilent Feature Extraction software (version 10.7.3.1) was used to analyze acquired array images. After quantile normalization of the raw data, lncRNAs and mRNAs were chosen for further data analysis. A volcano plot was used to filter lncRNAs/mRNAs that were differentially expressed between two groups and Hierarchical clustering was performed using the GeneSpring GX v12.0 software package (Agilent Technologies). LncRNAs that were significantly differently expressed between all six pairs of VV and NV tissues were selected for Q-RT-PCR validation (P<0.05, ≥2
fold-change), as were any neighbor mRNAs that were also significantly differently expressed between all six pairs of VV and NV tissues ($P<0.05$, ≥2 fold-change).

For Q-RT-PCR validation analysis, all samples were normalized to GAPDH. The mean value in each triplicate was used to calculate relative lncRNAs concentrations ($D_{\text{Ct}} = \text{Ct mean } \text{lncRNAs} - \text{Ct mean GAPDH}$). Expression fold changes were calculated using $2^{-2D_{\text{Ct}}}$ methods. The differences in the level of lncRNAs expression between VVs and NVs were analyzed using the Student’s t-test and SPSS (Version 16.0, SPSS Inc.) with the value of $P<0.05$ was considered as statistically significant.

Gene Ontology (GO) analysis and pathway analysis were performed to identify differentially expressed mRNA pathways. GO analysis showed significantly up-regulated and down-regulated mRNAs in question to be related to biological processes (BP), cellular components (CC) and molecular functions (MF) ($P<0.05$). The latest version of the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and GO categories derived from Gene Ontology (www.geneontology.org) were used for pathway analysis, which was performed using the standard enrichment computation method [28]. A coding-non-coding gene co-expression network (CNC network) was drawn using Cytoscape with Pearson coefficient ($|r|$) >0.99 [29,30]. The analysis work was performed by KangChen Bio-tech (Shanghai P.R. China).

Results

Profiles of the Differently Expressed lncRNAs and mRNAs

Among the 33,045 lncRNAs and 30,215 coding transcripts probed in the microarray, 12,264 lncRNAs and 14,862 mRNAs were detected in all six pairs of samples. There was an average of 2,426 differentially expressed lncRNAs ($P<0.05$, ≥2 fold-change), as were any neighbor mRNAs that were also significantly differently expressed between all six pairs of VV and NV tissues ($P<0.05$, ≥2 fold-change).

For Q-RT-PCR validation analysis, all samples were normalized to GAPDH. The mean value in each triplicate was used to calculate relative lncRNAs concentrations ($D_{\text{Ct}} = \text{Ct mean } \text{lncRNAs} - \text{Ct mean GAPDH}$). Expression fold changes were calculated using $2^{-2D_{\text{Ct}}}$ methods. The differences in the level of lncRNAs expression between VVs and NVs were analyzed using the Student’s t-test and SPSS (Version 16.0, SPSS Inc.) with the value of $P<0.05$ was considered as statistically significant.

Gene Ontology (GO) analysis and pathway analysis were performed to identify differentially expressed mRNA pathways. GO analysis showed significantly up-regulated and down-regulated mRNAs in question to be related to biological processes (BP), cellular components (CC) and molecular functions (MF) ($P<0.05$). The latest version of the Kyoto Encyclopedia of Genes and Genomes (KEGG) database and GO categories derived from Gene Ontology (www.geneontology.org) were used for pathway analysis, which was performed using the standard enrichment computation method [28]. A coding-non-coding gene co-expression network (CNC network) was drawn using Cytoscape with Pearson coefficient ($|r|$) >0.99 [29,30]. The analysis work was performed by KangChen Bio-tech (Shanghai P.R. China).

Results

Profiles of the Differently Expressed lncRNAs and mRNAs

Among the 33,045 lncRNAs and 30,215 coding transcripts probed in the microarray, 12,264 lncRNAs and 14,862 mRNAs were detected in all six pairs of samples. There was an average of 2,426 differentially expressed lncRNAs (Table 3), BC041954 (fold change = 23.1) was the most significantly up-regulated lncRNA and AK023929 was the most significantly down-regulated (fold change = 4.84). There were more down-regulated lncRNAs than up-regulated lncRNAs.

The GO analytical data of aberrantly expressed mRNAs are shown in Table S3 (for biological processes), Table S4 (for cellular components), and Table S5 (for molecular functions). The top 20 most significantly differentially expressed mRNAs are shown in Table 4, and the top 5 most enriched GO terms, specifically biological processes, cellular components and molecular functions, are shown in Table 5. Pathway analysis showed these 123 significantly differentially expressed mRNAs to be involved in the pathogenesis of GSV varicosities (Table S6). Among them, 26 down-regulated and 2 up-regulated pathways were detected and 7 pathways were found to contain 46 mRNAs (changed ≥2 fold, enrichment score (−log10 $P$-value) value >2, $P<0.01$). Among these pathways, six (glycolysis/gluconeogenesis, fatty acid metabolism, tyrosine metabolism, pyrimidine metabolism, peroxisome and maturity-onset diabetes of the young) were focused on metabolic pathways.

| Table 2. Numbers of LncRNA and mRNA expressed differently between six pairs of VVs and paired NVs tissues. |
|----------------|----------------|----------------|----------------|
| Fold change   | Fold change   | Fold change   |
| 2–4           | 4–8           | >8            |
| total         |               |               |
| LncRNA        |               |               |
| Up-regulation | 300           | 2             | 1              |
| Down-regulation | 249           | 6             | 0              |
| mRNA          |               |               |
| Up-regulation | 236           | 3             | 0              |
| Down-regulation | 708           | 32            | 1              |

Table 1. The clinical information of 32 patients involved in the study.

|                      | Male | Female | Total |
|----------------------|------|--------|-------|
| Patients number of gender | 14   | 18     | 32    |
| Age±SD(years)         | 54.4 ± 13.2 | 54.9 ± 8.9 | 54.7 ± 10.8 |
| Course of CVI(years)  | 11.1 ± 9.4  | 12.3 ± 8.6  | 11.6 ± 8.9  |
| Previous chronic illnesses |      |        |       |
| Hypertension          | 5    | 3      | 8     |
| Parkinson             | 1    | 0      | 1     |
| Diabetes              | 1    | 0      | 1     |
| Limbs of Surgery      |      |        |       |
| left limb             | 5    | 5      | 10    |
| right limb            | 7    | 8      | 15    |
| double limbs          | 2    | 5      | 7     |
| CEAP grade            |      |        |       |
| Class 6               | 1    | 0      | 1     |
| Class 5               | 1    | 0      | 1     |
| Class 4               | 12   | 18     | 30    |
| Specialist physic examination | |        |       |
| Perthes test (—)      | 14   | 18     | 32    |
| Duplex ultrasound scanning |    |        |       |
| Deep venous thrombosis | 0    | 2      | 2     |
| Valve insufficiency of GSV | 14   | 18     | 32    |
| Reflex of GSV         | 14   | 18     | 32    |

Table 2. Numbers of LncRNA and mRNA expressed differently between six pairs of VVs and paired NVs tissues.

|                      | Fold change | Fold change | Fold change | total |
|----------------------|-------------|-------------|-------------|-------|
|                      | 2–4         | 4–8         | >8          |       |
| LncRNA               |             |             |             |       |
| Up-regulation        | 300         | 2           | 1           | 302   |
| Down-regulation      | 249         | 6           | 0           | 255   |
| mRNA                 |             |             |             |       |
| Up-regulation        | 236         | 3           | 0           | 239   |
| Down-regulation      | 708         | 32          | 1           | 741   |
Differently Expressed lncRNA Subgroups

Overall, 48 enhancer-like lncRNAs were found to be differentially expressed (Table S7) using with Gencode annotation [31,32]. Their nearby significantly differentially expressed coding RNAs (distance $\leq 300$ kb) are shown in Table S8. The Arraystar Human LncRNA Array v2.0 microarray contains profiling data of all probes in the 4 HOX loci, targeting 407 discrete transcribed regions, lncRNAs and coding transcripts. Among them, 10 lncRNAs and 10 coding transcripts were found to be differentially expressed in the human HOX loci of VVs and those of paired NVs (Table S9). Seventy-eight Rinn’s lincRNAs (Table S10), 14 lincRNAs, and 19 nearby coding RNAs found to be significantly differentially expressed (Table S11) [33,34].

**Table 3.** Top 20 significantly differential expressed LncRNAs from the microarray data.

| LncRNA accession number | Regulation | Fold change | Source               | FDR   | SD   |
|-------------------------|------------|-------------|----------------------|-------|------|
| BC041954                | up         | 23.1        | misc_RNA             | 0.0184| 0.0334|
| chr10:127700955--127703336 | up          | 4.25        | lncRNAdb             | 0.0012| 0.0194|
| AK097695                | up         | 4.18        | RNAdb                | 0.0019| 0.0194|
| ENST00000424739         | up         | 3.97        | Ensembl              | 0.0020| 0.0194|
| ENST00000484962         | up         | 3.77        | Ensembl              | 0.0089| 0.0274|
| BC042185                | up         | 3.77        | RNAdb                | 0.0395| 0.0474|
| ENST00000512300         | up         | 3.62        | Ensembl              | 0.0140| 0.0299|
| ENST00000445497         | up         | 3.57        | Ensembl              | 0.0068| 0.0247|
| ENST00000494923         | up         | 3.47        | Ensembl              | 0.0131| 0.0290|
| ENST00000484961         | up         | 3.33        | Ensembl              | 0.0014| 0.0194|
| ENST00000510464         | up         | 3.19        | Ensembl              | 0.0305| 0.0423|
| ENST00000450430         | up         | 3.18        | Ensembl              | 0.0318| 0.0423|
| nc-HOX89.205-            | up         | 3.16        | HOX cluster          | 0.0068| 0.0247|
| ENST00000437515         | up         | 3.15        | Ensembl              | 0.0082| 0.0266|
| uc.459-                  | up         | 3.15        | UCR                  | 0.0438| 0.0487|
| ENST00000392399         | up         | 3.09        | Ensembl              | 0.0100| 0.0285|
| ENST00000428292         | up         | 3.09        | Ensembl              | 0.0163| 0.0326|
| BC002831                | up         | 3.09        | misc_RNA             | 0.0069| 0.0248|
| chr7:117584264--117586960+ | up          | 3.08        | lincRNA              | 0.0082| 0.0266|
| AM492791                | up         | 3.03        | misc_RNA             | 0.0346| 0.0441|
| AK023929                | down       | 4.84        | NRED                 | 0.0045| 0.0218|
| AK1268867               | down       | 4.57        | misc_RNA             | 0.0092| 0.0278|
| uc02brm.2               | down       | 4.39        | UCSC_knowngene       | 0.0039| 0.0200|
| ENST00000427890         | down       | 4.28        | Ensembl              | 0.0223| 0.0368|
| AK126761                | down       | 4.11        | misc_RNA             | 0.0462| 0.0487|
| ENST00000429435         | down       | 4.03        | Ensembl              | 0.0124| 0.0290|
| uc.28+                  | down       | 3.89        | UCR                  | 0.0029| 0.0197|
| AK297077                | down       | 3.83        | lincRNA              | 0.0107| 0.0286|
| BX648634                | down       | 3.82        | misc_RNA             | 0.0462| 0.0487|
| NR_026570               | down       | 3.79        | RefSeq_NR            | 0.0060| 0.0246|
| NR_015407               | down       | 3.62        | RefSeq_NR            | 0.0385| 0.0466|
| AK022120                | down       | 3.57        | NRED                 | 0.0093| 0.0278|
| CR624187                | down       | 3.57        | RNAdb                | 0.0485| 0.0494|
| ENST00000474978         | down       | 3.53        | Ensembl              | 0.0278| 0.0410|
| NR_027830               | down       | 3.48        | RefSeq_NR            | 0.0005| 0.0194|
| uc03jev.1               | down       | 3.46        | UCSC_knowngene       | 0.0107| 0.0286|
| ENST00000420004         | down       | 3.43        | Ensembl              | 0.0041| 0.0205|
| ENST00000423135         | down       | 3.38        | Ensembl              | 0.0025| 0.0194|
| AK091713                | down       | 3.37        | NRED                 | 0.0058| 0.0246|
| ENST00000455804         | down       | 3.34        | Ensembl              | 0.0017| 0.0194|

Source: different database each lncRNA was included; LncRNA accession number: the reference ID of lncRNA in each database. doi:10.1371/journal.pone.0086156.t003
Validation of Aberrantly Expressed Candidate lncRNAs

LncRNAs significantly differently expressed between all six pairs of VV and NV tissues with their neighbor mRNAs significantly differently expressed between all six pairs of VV and NV tissues were selected for Q-RT-PCR validation, and 22 lncRNAs were validated. Nine of the 22 lncRNAs whose Q-RT-PCR primers were suitably designed (using Primer 3.0 and blasted specifically in NCBI). Primers are shown in Table S12. These lncRNAs were then replicated with Q-RT-PCR. Of these nine lncRNAs, seven showed the same trends of up- and down-regulation as the microarray data and six were statistically significant (P<0.05) (Figure 1), supporting a strong consistency between the Q-RT-PCR results and the microarray data.

Prediction of the Functions of the Validated IncRNAs

To study the relationship between the lncRNAs and mRNAs more visually, the validated six significantly differently expressed lncRNAs were used to establish coding-non-coding gene (CNC) networks. These CNC networks were used to search for correlations between the lncRNAs and mRNAs and to determine the potential function of the lncRNAs. This would increase understanding of lncRNAs biological networks and of the complex pathogenesis of GSV varicosities. Overall 11 significantly aberrantly expressed mRNAs were found to be correlated with four validated lncRNAs. They were used to construct four separate networks with lncRNAs in the center node of the hub. The mRNAs CHAT and TMEM38B were found to be related to lncRNA AF119885; the mRNAs CCNO, EPC2, FAM13C and SHOC2 were found to be related to lncRNA G36810; the mRNAs EMX1 and SMC3 were found to be related to lncRNA NR_027927; and the mRNAs ATXN7, HOXC4, and RTCD1 were found to be related to lncRNA uc.345. These separate networks are shown in Figure 2 as a whole. Further information (standard deviation (SD), and false discovery rate (FDR)) regarding these 11 mRNAs is shown in Table 6.

The potential functional effects of the six validated candidate lncRNAs were predicted through their correlations with the aforementioned six GSV-varicosities-related metabolic pathways (glycolysis/gluconeogenesis, fatty acid metabolism, tyrosine metabolism, pyrimidine metabolism, peroxisome and maturity onset diabetes of the young) with a criteria of co-expression gene pairs with |r|>0.9 and P<0.01. Here, eight mRNAs were found to be correlated with specific lncRNAs uc.345 with NME7; G36810 with POLR3F, IDH1, and ALDH2; AF119885 with ENTPD1, SLC25A17, and PDHA1; NR_027927 with NEUROG3). Table 7 shows the mRNA pathways and pathway IDs.

Discussion

In the present study, the profiles of aberrantly expressed lncRNAs, mRNAs, and lncRNA-related mRNAs and pathways in primary varicose GSVs were evaluated. Six potential pathogenic lncRNAs were identified by Q-RT-PCR. The potential functional effects of these lncRNAs were predicted. This is the first systemic screening and validation of the GSV varicosities related lncRNAs in the genome-wide RNAs expression level.

The aberrantly expressed lncRNA patterns were classified into different subgroups with different analytical methods. Forty-eight enhancer-like lncRNAs were found to be differentially expressed using GENCODE annotation, 10 discrete transcribed regions were targeted by 4 HOX loci, and 78 Rinn’s lincRNAs were identified using chromatin-state maps. Enhancer-like lncRNAs were associated with decreased expression of their neighboring genes through loss-of-function effects [31]. A total of 407 discrete transcribed regions including exons (101), introns (75), and intergenic transcripts (231) were identified in the 4 HOX loci [35]. Rinn’s lincRNAs profiles showed strong purifying selection, clear evolutionary conservation, and possible functions in many

Table 4. Top 20 significantly differential expressed mRNAs from the microarray data.

| mRNA gene symbol | NCBI accession | Regulation | Fold change | FDR | SD |
|------------------|----------------|------------|-------------|-----|----|
| POSTN            | NM_00135936    | up         | 5.99        | 9.50E-03 | 1.92 |
| PRND             | NM_012409      | up         | 4.65        | 9.20E-03 | 1.84 |
| POSTN            | NM_00135935    | up         | 4.41        | 8.30E-03 | 1.77 |
| PPARGc1B         | NM_133263      | up         | 4.05        | 4.55E-02 | 1.71 |
| FAM64A           | NM_019013      | up         | 3.77        | 2.34E-02 | 1.41 |
| PODXL            | NM_001018111   | up         | 3.49        | 1.04E-02 | 1.37 |
| ALPL             | NM_000478      | up         | 3.47        | 1.02E-02 | 1.18 |
| PRSS2            | NM_002770      | up         | 3.46        | 1.24E-02 | 1.59 |
| KRT17            | NM_000422      | up         | 3.33        | 8.30E-03 | 1.42 |
| POSTN            | NM_001135934   | up         | 3.31        | 7.70E-03 | 1.22 |
| ADAMTS514        | NM_080722      | up         | 3.31        | 2.13E-02 | 1.50 |
| C9orf140         | NM_178448      | up         | 3.28        | 2.88E-02 | 1.58 |
| PCSK9            | NM_174936      | up         | 3.25        | 7.70E-03 | 1.23 |
| RBP4             | NM_006744      | up         | 3.17        | 7.70E-03 | 1.10 |
| LRRc15           | NM_001335057   | up         | 3.16        | 2.48E-02 | 1.48 |
| GIN52            | NM_016095      | up         | 3.09        | 1.96E-02 | 1.45 |
| TK1              | NM_003258      | up         | 3.08        | 1.18E-02 | 1.12 |
| MAGEL2           | NM_019066      | up         | 3.08        | 7.70E-03 | 1.20 |
| NECAB2           | NM_019065      | up         | 3.04        | 9.00E-03 | 1.06 |
| SLC22A14         | NM_004803      | up         | 3.03        | 2.56E-02 | 1.27 |
| MYOC             | NM_000261      | up         | 8.46        | 1.08E-02 | 1.92 |
| CUL3             | NM_003591      | down       | 6.81        | 1.27E-02 | 1.81 |
| CNT3             | NM_001839      | down       | 6.52        | 1.37E-02 | 2.65 |
| 7-Sep            | NM_001011553   | down       | 6.19        | 7.70E-03 | 1.69 |
| SRPS4            | NM_003316      | down       | 5.89        | 7.70E-03 | 1.54 |
| ADH1A            | NM_000667      | down       | 5.87        | 8.30E-03 | 1.46 |
| ADH1B            | NM_000668      | down       | 5.62        | 1.02E-02 | 1.50 |
| KDM3B            | NM_016604      | down       | 5.55        | 9.50E-03 | 1.58 |
| IDH1             | NM_005896      | down       | 5.08        | 7.70E-03 | 1.51 |
| HNBP3            | NM_00108257    | down       | 5.08        | 1.67E-02 | 2.35 |
| DHX40            | NM_024612      | down       | 5.00        | 7.70E-03 | 1.33 |
| M1S12            | NM_024039      | down       | 4.85        | 8.30E-03 | 1.32 |
| CXCL1            | NM_001511      | down       | 4.74        | 8.40E-03 | 1.54 |
| SEC63            | NM_007214      | down       | 4.67        | 7.70E-03 | 1.45 |
| RNF103           | NM_005667      | down       | 4.67        | 9.00E-03 | 1.52 |
| S100A8           | NM_002964      | down       | 4.57        | 1.95E-02 | 1.79 |
| UEVLD            | NM_001040697   | down       | 4.56        | 1.07E-02 | 1.46 |
| PTPRK            | NM_001135648   | down       | 4.53        | 7.70E-03 | 1.41 |
| PKD4             | NM_002612      | down       | 4.40        | 8.40E-03 | 1.31 |
| MAPRE2           | NM_014268      | down       | 4.30        | 8.50E-03 | 1.40 |

NCBI accession: the reference ID of mRNA in NCBI (National Center for Biotechnology Information).
doi:10.1371/journal.pone.0086156.t004
different biological processes [33, 34]. The aberrantly expressed lncRNAs observed may provide clues to the pathophysiological properties of GSV varicosities.

Six validated candidate lncRNAs (AK021444, AF119885, uc.345-, NR_027927, G36810, NR_027830) were identified in GSV varicosities by Q-RT-PCR and their potential functional effects were predicted. AK021444 is a 1611 bp lncRNA with an exon sense-overlapping relationship with POSTN. POSTN encodes the protein periostin, which promotes cardiac repair and cardiomyocyte proliferation [36] and induces vascular cell differentiation and migration during the repair of vascular injury [37]. AF119885 is a 1269 bp lncRNA transcribed from the natural

Table 5. Top 5 Enrichment GO term (BP, CC and MF) from the microarray data.

| GO.ID        | Term                                   | Ontology | Regulation | Enrichment Score | FDR    |
|--------------|----------------------------------------|----------|------------|------------------|--------|
| GO:0044237   | cellular metabolic process              | BP       | down       | 6.96             | 2.47E-04|
| GO:0006996   | organelle organization                 | BP       | down       | 6.75             | 2.47E-04|
| GO:0044238   | primary metabolic process              | BP       | down       | 6.69             | 2.47E-04|
| GO:0008152   | metabolic process                      | BP       | down       | 5.72             | 1.76E-03|
| GO:0044260   | cellular macromolecule metabolic process| BP       | down       | 5.52             | 2.19E-03|
| GO:0044424   | intracellular part                     | CC       | down       | 23.63            | 1.21E-21|
| GO:0005622   | intracellular                          | CC       | down       | 22.97            | 2.77E-21|
| GO:0043229   | intracellular organelle                | CC       | down       | 16.24            | 8.68E-15|
| GO:0043231   | intracellular membrane-bounded organelle | CC       | down       | 16.11            | 8.68E-15|
| GO:0043226   | organelle                              | CC       | down       | 16.06            | 8.68E-15|
| GO:0005488   | binding                                | MF       | down       | 9.43             | 3.30E-07|
| GO:0005515   | protein binding                        | MF       | down       | 6.37             | 1.87E-04|
| GO:0003824   | catalytic activity                     | MF       | down       | 5.59             | 7.50E-04|
| GO:0003723   | RNA binding                            | MF       | down       | 4.94             | 2.51E-03|
| GO:0000166   | nucleotide binding                     | MF       | down       | 4.53             | 5.26E-03|

FDR < 0.05. doi:10.1371/journal.pone.0086156.t005

Figure 1. Validation of microarray data and the Q-RT-PCR data.

Nine lncRNAs were chosen for validation in 32 pairs of VVs samples compared with NVs samples by Q-RT-PCR. seven of the nine lncRNAs showed the same trends with respect to up- or down-regulation as the microarray data and six lncRNAs (AK021444, AF119885, G36810, uc.345, NR_027927 and NR_027830) showed statistically significant differences (P < 0.05). The heights of the columns in the chart represent the mean expression value of log2 fold changes (VVs/NVs) for each of the nine validated lncRNAs in the microarray and Q-RT-PCR data; The bars represent standard errors. The validation results indicated that the microarray data were closely correlate with the Q-RT-PCR results. *: P < 0.05; **: P < 0.01. doi:10.1371/journal.pone.0086156.g001

Figure 2. Coding-non-coding gene co-expression network of the four lncRNAs. The network represents co-expression correlations between the four lncRNAs and significantly differentially expressed mRNAs. Only co-expression gene pairs with Pearson coefficient (|r|) > 0.99 are shown. Four separate networks were constructed. They are displayed together in this figure. Gene nodes with a cyan node lines represent lncRNAs and gene nodes without node lines represents a protein-coding RNA (mRNA). Red nodes represent up-regulated RNAs, and blue nodes represent down-regulated RNAs. Solid lines between two nodes indicate positively correlated interactions between RNAs, and dotted lines indicate negatively correlated interactions. Node size represents the node degrees. * indicates protein coding RNA transcribed from natural antisense strands of the gene HOXC4. doi:10.1371/journal.pone.0086156.g002
Table 6. The 11 significantly aberrantly expressed mRNAs correlated with the four validated lncRNAs.

| mRNA gene symbol | NCBI accession | Regulation | Fold change | FDR | SD |
|------------------|----------------|------------|-------------|-----|----|
| CCNO             | NM_021147      | up         | 2.48        | 0.0102 | 1.084 |
| CHAT             | NM_020986      | up         | 2.27        | 0.0090 | 0.792 |
| HOXC4            | NM_014620      | up         | 2.02        | 0.0152 | 0.807 |
| EMX1             | NM_004097      | up         | 2.01        | 0.0223 | 0.876 |
| SHOC2            | NM_007373      | down       | 3.04        | 0.0083 | 1.144 |
| TMEM38B          | NM_018112      | down       | 2.51        | 0.0077 | 0.854 |
| SMC3             | NM_005445      | down       | 2.46        | 0.0086 | 0.850 |
| EPC2             | NM_015630      | down       | 2.37        | 0.0077 | 0.817 |
| FAM13C           | NM_198215      | down       | 2.32        | 0.0077 | 0.749 |
| RTCD1            | NM_003729      | down       | 2.21        | 0.0155 | 0.788 |
| ATXN7            | NM_000333      | down       | 2.02        | 0.0184 | 0.755 |

NCBI accession: the standard reference ID of mRNA in NCBI (National Center for Biotechnology Information).
doi:10.1371/journal.pone.0086156.t006

Table 7. Interactional lncRNAs-mRNAs detected from significantly different expressed lncRNAs and mRNAs.

| LncRNA     | LncRNA fold change | Related mRNA | mRNA fold change | Involved pathway                  | Pathway ID |
|------------|--------------------|--------------|-----------------|----------------------------------|------------|
| uc.345     | 2.06               | NME7         | -2.60           | Pyrimidine metabolism            | hsa00240   |
| G36810     | -2.02              | POLR3F       | -4.18           | Pyrimidine metabolism            | hsa00240   |
|            |                    | IDH1         | -5.08           | Peroxisome                       | hsa04146   |
|            |                    | ALDH2        | -2.31           | Fatty acid metabolism            | hsa00071   |
| AF119885   | 2.05               | ENTPD1       | -2.34           | Pyrimidine metabolism            | hsa00240   |
|            |                    | SLC25A17     | -2.09           | Peroxisome                       | hsa04146   |
|            |                    | PDHA1        | -2.08           | Glycolysis/Gluconeogenesis        | hsa00010   |
| NR_027927  | 2.49               | NEUROG3      | 2.79            | MODY                             | hsa04950   |

LncRNA fold change, Related mRNA, mRNA fold change, Involved pathway, Pathway ID.

Table S13. The most significantly altered lncRNAs and mRNAs.

| LncRNA     | LncRNA fold change | Related mRNA | mRNA fold change | Involved pathway     | Pathway ID |
|------------|--------------------|--------------|-----------------|----------------------|------------|
| uc.345     | 2.06               | NME7         | -2.60           | Pyrimidine metabolism | hsa00240   |
| G36810     | -2.02              | POLR3F       | -4.18           | Pyrimidine metabolism | hsa00240   |
|            |                    | IDH1         | -5.08           | Peroxisome           | hsa04146   |
|            |                    | ALDH2        | -2.31           | Fatty acid metabolism | hsa00071   |
| AF119885   | 2.05               | ENTPD1       | -2.34           | Pyrimidine metabolism | hsa00240   |
|            |                    | SLC25A17     | -2.09           | Peroxisome           | hsa04146   |
|            |                    | PDHA1        | -2.08           | Glycolysis/Gluconeogenesis | hsa00010   |
| NR_027927  | 2.49               | NEUROG3      | 2.79            | MODY                  | hsa04950   |

LncRNA fold change, Related mRNA, mRNA fold change, Involved pathway, Pathway ID.

doi:10.1371/journal.pone.0086156.t007
lncRNA for Q-RT-PCR validation was very robust. The limitations of this study should also be addressed. First, due to the lack of experimental validation, the bio-computationally derived links between lncRNAs and either individual mRNA or mRNA pathways should be viewed as preliminary. Second, varicose veins typically contain inflamed tissue, and inflammation is an important mechanism for the development of varicose veins. In addition, changes in mRNA and lncRNA levels may reflect pathological changes of inflamed tissue [42,43]. However, since we did not strip the NVs of the additional lymphatic tissue and connective tissue that can build up in these samples, we could not distinguish whether or not the enriched lncRNAs and mRNAs are attributed to the additional cell types that are naturally more prevalent in inflamed tissue, which call for further study.

In summary, we systematically screened, validated, and functional predicted the aberrantly expressed lncRNAs primary varicose GSVs. These findings provide novel insights into physiology of lncRNAs and pathophysiological properties of GSV varicosities. These data may be useful in further investigation. The aberrantly expressed lncRNAs may also serve as new therapeutic targets for varicose veins.

Supporting Information

Table S1 Differentially Expressed LncRNAs. (XLS)
Table S2 Differentially Expressed mRNAs. (XLS)
Table S3 Biological Processes. (XLS)
Table S4 Cellular Components. (XLS)
Table S5 Molecular Functions. (XLS)
Table S6 Pathway Analysis. (XLS)
Table S7 Differentially Expressed Enhancer LncRNA. (XLS)
Table S8 Enhancer LncRNAs nearby coding gene data. (XLS)
Table S9 HOX cluster profiling. (XLS)
Table S10 Differentially Expressed Rinn LincRNAs. (XLS)
Table S11 LincRNAs and their nearby coding gene data table. (XLS)
Table S12 Primers used for Q-RT-PCR. (XLS)
Table S13 Differentially Expressed both LncRNAs and their related mRNAs. (XLS)
Table S14 Function of the genes correlated the candidate LncRNAs. (XLS)

Acknowledgments

We would like to thank all the donors who participated in this program and all those who at KangChen-technology Company of Shanghai for skilled microarray services.
Author Contributions

Conceived and designed the experiments: X-YJ Y-HC. Performed the experiments: XI JW D-YX T-YY. Analyzed the data: JW X-YJ XL.

References

1. Fowkes F, Lee A, Evans C, Allan P, Bradford A, et al. (2001) Lifestyle risk factors for lower limb venous reflux in the general population: Edinburgh Vein Study. International journal of epidemiology 30: 846–852.
2. van Groenendael L, van der Vliet JA, Flinkenflo¨gel L, Roovers EA, van Sterkenburg SM, et al. (2009) Treatment of recurrent varicose veins of the great saphenous vein by conventional surgery and endovenous laser ablation. Journal of vascular surgery 50: 1106–1113.
3. Lim C, Davies A (2009) Pathogenesis of primary varicose veins. British Journal of Surgery 96: 1231–1232.
4. Lahropoulos N, Tsiougos J, Pryor L, Tassopoulos AK, Kang SS, et al. (2003) Definition of venous reflux in lower-extremity veins. Journal of vascular surgery 38: 793–798.
5. Faffet D, Khalil R (2008) Mechanisms of varicose vein formation: valve dysfunction and wall dilatation. Phlebology 23: 85–98.
6. Meisner MH, Gloviczki P, Bergan J, Kistner RL, Morrison N, et al. (2007) Primary chronic venous disorders. Journal of vascular surgery 46: 534–536.
7. Lee J, Lai C, Yang W, Lee T (2012) Increased expression of hypoxia-inducible factor-1a and metallothionein in varicose and veins. Phlebology 27: 409–415.
8. Spraque AH, Khalil RA (2009) Inflammatory cytokines in vascular dysfunction and varicose vein disease. Biochemical pharmacology 78: 539.
9. Asher E, Jacob T, Hingorani A, Tsvetkhnin B, Gundray U (2001) Expression of molecular mediators of apoptosis and their role in the pathogenesis of lower-extremity varicose veins. Journal of vascular surgery 33: 1080–1086.
10. Takase S, Bergan JJ, Schmid-Schönbein G (2000) Expression of adhesion molecules and cytokines on saphenous veins in chronic venous insufficiency. Annals of Vascular Surgery 14: 427–432.
11. Furuno M, Pang KC, Ninnomiya N, Fukuda S, Frith MC, et al. (2006) Clusters of internally primed transcripts reveal novel long noncoding RNAs. PLoS genetics 2: e37.
12. Wiltse JE, Sunwoo H, Specter DL (2009) Long noncoding RNAs: functional surprises from the RNA world. Genes Dev 23: 1494–1504.
13. Pan Y-f, Feng L, Zhang X-q, Song L-j, Liang H-x, et al. (2011) Role of long non-coding RNAs in gene regulation and oncogenesis. Chinese Medical Journal 124: 3571–3583.
14. Yang F, Zhang L, Huo XS, Yuan JH, Xu D, et al. (2011) Long noncoding RNA high expression in hepaticcellular carcinoma facilitates tumor growth through enhancement of zeste homolog 2 in humans. Hepatology 54: 1679–1689.
15. Gupta RA, Shah N, Wang KC, Kim J, Hohlmg HM, et al. (2010) Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. Nature 464: 1071–1076.
16. Premser JR, Chimniyan AM (2011) The emergence of IncRNAs in cancer biology. Cancer discovery 1: 391–407.
17. Faghihi MA, Modarresi F, Khalil AM, Wood AM, DE, Sahagan BG, et al. (2008) Rapid feed-forward regulation of a secreted factor by long noncoding RNAs transcriptionally regulated by Oct4 and Nanog modulate pluripotency in mouse embryonic stem cells. Rna 16: 324–337.
18. Folkeren T, Kyriakou T, Geel A, Peden J, Malarstig A, et al. (2009) Relationship between CAD risk genotype in the chromosome 9p21 locus and gene expression. Identification of eight new ANRIL splice variants. PLoS One 4: e7677.
19. Iishi N, Ozaki K, Sato H, Mizuno H, Saito S, et al. (2006) Identification of a novel non-coding RNA, MIAT, that confers risk of myocardial infarction. Journal of human genetics 51: 1087–1099.
20. Folkersen L, Kyriakou T, Goel A, Peden J, Malarstig A, et al. (2009) HBEFGF, SRA1, and IK: Three coregulating genes as determinants of cardiomyopathy. Genome Research 19: 395–403.
21. Schlothoff C, Voehrler-Mahlein F, Dahnke IN, Mahlknecht U (2012) On the epigenetics of vascular regulation and disease. Clinical Epigenetics 4: 7.
22. Lorenzen JM, Martino F, Thun T (2012) Epigenetic modifications in cardiovascular disease. Basic research in cardiology 107: 1–10.
23. Schonrock N, Harvey RP, Mattick JS (2012) Long non-coding RNAs in cardiac development and pathophysiology. Circulation Research 111: 1349–1362.
24. Ekdör B, Rutherford R, Bergan J, Carpenter P, Gloviczki P, et al. (2004) American Venous Forum International Ad Hoc Committee for Revision of the CEAP Classification. Revision of the CEAP classification for chronic venous disorders: consensus statement. J Vasc Surg 40: 1240–1252.
25. Bergan JJ, Schmid-Schönbein GW, Smith PDC, Nicoladines A, Boisseau M, et al. (2006) Chronic venous disease. New England Journal of Medicine 355: 481–490.
26. Kanelisa M, Goto S, Furumichi M, Tanabe M, Hirakawa M (2010) KEGG for representation and analysis of molecular networks involving diseases and drugs. Nucleic acids research 38: D355–D360.
27. Liao Q, Liu C, Yuan X, Kang S, Miao R, et al. (2011) Large-scale prediction of long non-coding RNA functions in a coding-non-coding gene co-expression network. Nucleic acids research 39: 3864–3878.
28. Stuart JM, Segal E, Koller D, Kim SK (2003) A gene coexpression network for global discovery of conserved genetic modules. Science 302: 249–253.
29. Oron UA, Derrien T, Beringer M, Guaridakis V, Gardini A, et al. (2010) Long noncoding RNAs with enhancer-like function in human cells. Cell 143: 46–58.
30. Harrow J, Drenauel F, Frankish A, Reymond A, Chen-K, et al. (2006) ENCODE: producing a reference annotation for ENCODE. Genome Biol 7: S4.
31. Mitchell Guttman IA, Garber M, French C, Lin MF, Feldser D, et al. (2009) Chromatin signature reveals over a thousand highly conserved large non-coding RNAs in mammals. Nature 458: 223–227.
32. Khalil AM, Guttman M, Huarte M, Garber M, Raj A, et al. (2009) Many human large intergenic non-coding RNAs associated with chromatin-modifying complexes and affect gene expression. Proceedings of the National Academy of Sciences 106: 11667–11672.
33. Rinn JL, Kertesz M, Wang JK, Squazzo SL, Xu X, et al. (2007) Functional Demarcation of Active and Silent Chromatin Domains in Human HOX Loci by Noncoding RNAs. Cell 129: 1311–1323.
34. Kuhn B, del Monte F, Hajjar RJ, Chang Y-S, Lebeche D, et al. (2007) Periostin induces proliferation of differentiated cardiomyocytes and promotes cardiac repair. Nature medicine 13: 962–969.
35. Landhe V, Wang SQ, Conley BA, Friesel RE, Vary GP (2005) Vascular Injury Induces Expression of Periostin Implications for Vascular Cell Differentiation and Migration. Arteriosclerosis, thrombosis, and vascular biology 25: 77–83.
36. Wanders R, Ferdinandusse S, Brites P, Kemp S (2010) Peroxisomes, lipid metabolism, ageing and apoptosis. Histochemistry and Cell Biology 129: 341–350.
37. Lu J, Sun A, Sun X, et al. (2013) Large-scale detection of non-coding RNAs in human and mouse genomes. Cell 152: 185–197.
38. Ray SK, Banik NL (2003) Calpain and its involvement in the pathophysiology of CNS injuries and diseases: therapeutic potential of calpain inhibitors for prevention of neurodegeneration. Current Drug Targets-CNS & Neurological Disorders 2: 173–189.
39. Kurz T, Terman A, Gustafsson B, Brunk UT (2008) Lysosomes in iron metabolism, ageing and apoptosis. Histochemistry and cell biology 129: 309–406.
40. Exton J (1987) Mechanisms of hormonal regulation of hepatic glucose metabolism. Diabetess/metabolism reviews 3: 163–183.
41. Wadkars R, Ferdinandusse S, Brites P, Kemp S (2010) Peroxisomes, lipid metabolism and lipotoxicity. Biochimica et Biophysica Acta (BBA)/Molecular and Cell Biology of Lipids 1801: 272–280.
42. Carpenter S, Auriolle D, Atiamand MK, Ricci EP, Gandhi P, et al. (2013) A long noncoding RNA mediates both activation and repression of immune response genes. Science 341: 789–792.
43. Somers P, Kaanemen P (2006) The histopathology of varicose vein disease. Angiology 57: 546–555.

Contributed reagents/materials/analysis tools: JG G-JC LX D-SZ HZ. Wrote the paper: X-YJ XL Y-HC.