

Exploring Long Non-Coding RNAs Associated with IP3/DAG Signaling Pathway as Potential Biomarkers Involved in Mast Cell Degranulation in Chronic Spontaneous Urticaria with 2-Year Follow-Up

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Purpose: Chronic spontaneous urticaria (CSU) pathogenesis involves mast cell degranulation induced by the inositol 1,4,5-trisphosphate/diacylglycerol (IP3/DAG) pathway, but the condition lacks specific biomarkers. This study was performed to investigate long non-coding RNA (lncRNA) expression profiles, identify those associated with IP3/DAG pathway, and assess their diagnostic and prognostic value for CSU.

Methods: Ten samples were selected from CSU and control groups, and microarray was performed to screen differentially expressed (DE) lncRNAs and mRNAs. Bioinformatic and co-expression network analyses were used to identify lncRNAs associated with IP3/DAG pathway. Quantitative real-time polymerase chain reaction was used to validate lncRNA expression levels. Combined with disease characteristics and serum indices detected with enzyme-linked immunosorbent assays, Spearman analysis and logistic regression were applied to analyze lncRNA-associated disease risk. Receiver operating characteristic (ROC) curves and 2-year follow-up data were applied to evaluate lncRNA diagnostic and prognostic value.

Results: A total of 678 up- and 573 downregulated DE lncRNAs and 609 up- and 176 downregulated DE mRNAs were identified. Seven lncRNAs (upregulated T264761, T280622, ENST00000587970, T224062, ENST00000562459, and his-1_RNA_dna; downregulated ENST00000417930) were associated with the IP3/DAG pathway. D-dimer and histamine levels were significantly different between the two groups. Correlation analysis showed that his-1_RNA_dna positively correlated with the frequency of symptom appearance, while his-1_RNA_dna, ENST00000417930, T264761, and T280622 negatively correlated with the maximum wheal diameter. Regression analysis showed T264761 was associated with CSU risk. ROC analysis showed that the specificity of T264761 was 90%, with an area under the curve of 0.666. In follow-up, the rate of well-controlled disease in the low T264761 expression group was 82.61%.

Conclusion: This study established lncRNA and mRNA expression profiles in CSU and identified lncRNAs associated with IP3/DAG pathway, which is mechanistically involved in this disease. T264761 may be a novel biomarker for CSU, but further study is needed to confirm its specific mechanism.

Keywords: lncRNA, biomarker, urticaria, mRNA, microarray

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Introduction

Chronic spontaneous urticaria (CSU) is a common dermatological condition characterized by sudden itchy wheals or angioedema lasting for up to 6 weeks and

occurring twice a week or more.¹ The symptoms are spontaneous, with no known specific triggers. CSU diagnosis is mainly based on clinical symptoms and medical history, the 7-day Urticaria Activity Score (UAS7), quality of life assessments, and other questionnaires that assist in evaluation of the disease and treatment effects. Some reviews suggest that there are statistical differences in the contents of D-dimer, C-reactive protein (CRP), and microRNAs between patients with CSU and healthy controls. These factors may reflect disease course and activity and treatment effects, but they are not specific to CSU and therefore cannot sensitively or accurately distinguish CSU from other diseases.^{2–4} Specific biomarkers of CSU that may facilitate early diagnosis, accurate prognosis, and individualized treatments remain elusive.

Urticaria is a mast cell-driven disease. The high-affinity receptor for immunoglobulin E (IgE) (FcεRI) is considered a key factor in mast cell degranulation, which induces characteristic skin wheals in CSU. Antigen-dependent mast cell activation is regulated by a complex series of intracellular signaling processes that are initiated following FcεRI aggregation.⁵ In downstream signaling processes, phospholipase Cγ1/2 hydrolyzes phosphatidylinositol 4,5-bisphosphate in the cell membrane to produce two second messenger molecules: inositol 1,4,5-trisphosphate (IP3) and diacylglycerol (DAG).⁶ IP3 induces calcium release from the endoplasmic reticulum, triggering extracellular calcium influx; DAG binds to protein kinase C, which is activated by cell membrane and cytoskeleton proteins.⁷ IP3 and DAG combine to trigger mast cell degranulation, increase microvascular permeability, and induce the release of inflammatory mediators such as histamine (HIS), leukotriene B4 (LTB4), prostaglandin D2 (PGD2), and mast cell tryptase (MCT).⁸ Additionally, the mitogen-activated protein kinase and phosphoinositide 3-kinase pathways are mainly involved in generating eicosanoids and cytokines.⁵ The IP3/DAG signaling pathway is thus a key pathway in mast cell degranulation. Currently, there are no known biomarkers for this signaling pathway.

Long non-coding RNAs (lncRNAs) are a group of functional RNAs which non or rarely in coding transcripts that longer than 200 nucleotides.⁹ lncRNAs regulate gene expression at epigenetic and transcriptional levels in the nucleus and at post-transcriptional and translational levels in the cytoplasm. lncRNAs are closely related to the physiology and pathology of allergic diseases including asthma, atopic dermatitis, and rhinitis, suggesting that

they could act as biomarkers and therapeutic targets for allergic disorders, aid in the diagnosis and prognosis of disease, and identify novel therapeutic agents.⁴ Some lncRNAs were shown to be closely related to skin physiology and pathologies. For example, COL1A2-AS1 promotes the apoptosis of normal skin fibroblasts by inhibiting p-Smad3 and promoting β-catenin expression, WAKMAR1 regulates wound healing by promoting keratinocyte migration, and MEG3 affects the proliferation and apoptosis of psoriatic epidermal cells by targeting miR-21/caspase-8.^{10–12} Since CSU pathogenesis is closely related to allergic responses, differential lncRNA expression may therefore serve as a biomarker for this disease.

This study aimed to identify lncRNAs that are closely related to CSU physiology and pathology. lncRNA and mRNA expression profiles in blood samples from CSU patients were compared with those in healthy individuals using microarray technology; bioinformatic analyses, quantitative real-time polymerase chain reaction (qRT-PCR), receiver operating characteristic (ROC) curve analysis, logistic regression analysis, and a 2-year clinical follow-up were used to explore the value of key lncRNAs as biomarkers for CSU.

Materials and Methods

Recruitment and Inclusion/Exclusion Criteria

Patients were recruited from the affiliated Jiangmen Traditional Chinese Medicine Hospital of Jinan University between November 2018 and May 2019. All participants had a documented history of CSU. Patients suffering from the spontaneous appearance of wheals and/or angioedema for ≥6 weeks due to known or unknown causes, and who were aged 18 years or older at the time of recruitment were included in the study. Patients with blood system diseases, tumors or other serious diseases (severe cardiovascular, hepatic, or renal insufficiency), pregnant or lactating women, and those with current drug abuse, alcoholism, or mental illness were excluded. Healthy controls were included in the study if they had no obvious abnormalities on routine physical examination and laboratory imaging, no history of disease or drug treatment before blood collection, and were aged 18 years or older. All subjects signed written informed consent forms prior to participating in this study.

The present study was approved by the Ethics Committee of the affiliated Jiangmen Traditional Chinese Medicine

Hospital of Jinan University (approval KY [2017]-c14) and was conducted in compliance with the Declaration of Helsinki. The study was registered in the Chinese Clinical Trial Registry (No. ChiCTR1800018653).

Blood Sample Collection

Two vacutainer tubes of peripheral blood were collected from all participants. One tube contained ethylene diamine tetraacetic acid (EDTA) and was used for lncRNA microarrays or qRT-PCR, the second standard tube was used for enzyme-linked immunosorbent assay (ELISA). Peripheral blood mononuclear cells (PBMCs) in the EDTA tube were isolated by density gradient centrifugation, and total RNA was isolated from the PBMCs using TRIzol reagent. Blood serum was extracted after centrifugation in the standard tube.

lncRNA Profiling

The Arraystar Human lncRNA microarray v4.0, which contains 40,173 lncRNAs and 20,730 coding transcripts, was used to profile the expression of lncRNAs and mRNAs. Five samples from CSU patients and 5 samples from healthy controls were selected to be detected by Shanghai Kangchen Bio-tech, Inc. (Shanghai, China). After the total RNA was isolated, the concentration and quality were assessed using absorbance spectrometry, measuring absorbance ratios of A260/A280 and A260/230 using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc., Waltham, MA, USA). [Supplementary Table 1](#) shows RNA quantification and quality assurance, and [Supplementary Figure 1](#) shows an assessment of RNA integrity and gDNA contamination test by denaturing agarose gel electrophoresis. According to the manufacturer's instructions and as in previously published research,¹³ RNA samples were labeled by Quick Amp Labeling Kit (Agilent p/n 5190-0442, Santa Clara, CA, USA), and purified by RNeasy Mini Kit (Qiagen p/n 74,104, Hilden, Germany). Microarray hybridization was performed using the Agilent Gene Expression Hybridization Kit (Agilent p/n 5188-5242). After washing and fixing, the hybridized arrays were scanned by Agilent Microarray Scanner (Agilent p/n G2565BA) and data were extracted using Agilent Feature Extract Software (Agilent, v10.5.1.1, Palo Alto, CA, USA). Data were deposited in the Gene Expression Omnibus database (GSE185516). Raw data were normalized using a quantile algorithm in Agilent GeneSpring GX v12.1. Differentially expressed (DE) lncRNAs and mRNAs were identified according to

their fold change (FC) at a threshold ≥ 1.5 -fold with significance $p < 0.05$.

Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) Pathway Analyses

The GO and KEGG databases were used to analyze potential biological functions and signaling pathways associated with DE mRNAs. GO enrichment analysis of target genes was performed by Goseq R package (<http://www.geneontology.org>) and included biological processes (BP), cellular components (CC), and molecular functions (MF). Signaling pathways related to target genes were explored using the KEGG database (<http://www.geneontology.org>) and performed using DAVID software. Target genes with $p < 0.05$ were considered significantly enriched.

Pearson Analysis of DE lncRNAs and mRNAs Associated with IP3/DAG Signaling Pathway

Pearson correlation analysis was carried out on selected DE mRNAs and lncRNAs identified in the KEGG analysis; those with a Pearson correlation coefficient (PCC) ≥ 0.8 with significance $p < 0.5$ and a false discovery rate (FDR) ≤ 1 were selected for further exploration of closely related DE lncRNAs.

Validation of lncRNAs Using qRT-PCR and lncRNA-mRNA Co-Expression Networks

lncRNAs for which the p value of significance is lower, FC is higher, raw intensity is greater than 200, and which were closely related to the IP3/DAG pathway were selected for qRT-PCR validation. Total RNA was extracted using TRIzol reagent, and cDNA was synthesized using EasyScript First-Strand cDNA Synthesis SuperMix (TransGen, Beijing, China). qRT-PCR was performed using SYBR Green (Yeasen, Shanghai, China). The specific primers were designed by LandM Biotech Inc. (Guangzhou, China). Cycle threshold (Ct) values were used to quantify the expression levels of lncRNAs using the $2^{-\Delta\Delta Ct}$ method, and β -actin was used as a control to normalize values.

Selected lncRNAs and total mRNAs with PCC ≥ 0.9 , $p < 0.5$, and FDR ≤ 1 , were used to construct a co-expression network which was visualized using

Cytoscape v2.8.3 (Institute of Systems Biology, Seattle, WA, USA). GO analysis was used to assess the putative biological functions of the co-expressed mRNAs, which likely reflect the biological function of the lncRNAs.

ELISA

The levels of HIS (ab213975, Abcam, Cambridge, UK), MCT (CSB-E09012h, CUSABIO, Wuhan, China), LTB4 (CSB-E08033h), PGD2 (CSB-E13898h), hs-CRP (CSB-E08617h), and D-dimer (CSB-E05175h) were assessed in peripheral blood serum using human ELISA kits according to the manufacturer's instructions. The optical density (OD) value of samples was evaluated at the wavelength of 450 nm.

Statistical Analysis

All data are presented as mean \pm standard deviation (SD), median (range), or *n*, as appropriate. Chi-square and Mann-Whitney *U*-tests were used to analyze qualitative and quantitative data, respectively. Spearman coefficient analysis was performed to assess correlations between lncRNA levels and clinical characteristics of CSU. Logistic regression was used to analyze the CSU risk associated with selected lncRNAs. ROC curves were generated to evaluate the prognostic value of selected lncRNAs. lncRNA expression values were sorted into high- and low-level groups, based on these ROC curves using Youden's index correction.¹⁴ Data were analyzed using SPSS v19.0 (IBM Corp., Armonk, NY, USA) and graphs were drawn using GraphPad Prism v8.3.0 (San Diego, CA, USA). *p* values < 0.05 were regarded as statistically significant.

Results

Figure 1 is a flow diagram summarizing the study methods, participant inclusion and exclusion, and key results.

Patient Demographics

In total, 59 people with CSU and 59 healthy controls were recruited for this study; a summary of their demographic characteristics is shown in Table 1.

lncRNA and mRNA Expression Profiles

A total of 1482 lncRNAs were found to be upregulated and 1775 downregulated with $FC \geq 1.5$ in CSU patients versus healthy control individuals (Figure 2A). Of these up- and downregulated lncRNAs, 678 and 573, respectively, were significantly differentially expressed ($FC \geq 1.5$, $p < 0.05$). ENST00000562459 was upregulated to the greatest extent, with an FC of 6.4280631, while NR_122077 was downregulated to the greatest extent,

with an FC of 5.1422487 (Figure 2B, Table 2). lncRNA expression profiles were distinguishable between CSU patients and healthy control individuals according to the hierarchical clustering (Figure 2C).

A total of 1072 mRNAs were upregulated and 549 downregulated with an $FC \geq 1.5$ in CSU patients versus healthy controls (Figure 3A). Of these up- and downregulated mRNAs, 609 and 176, respectively, were significantly differentially expressed ($FC \geq 1.5$, $p < 0.05$). NM_033130 was upregulated to the greatest extent with an FC of 4.2383847, while NM_002538 was downregulated to the greatest extent with an FC of 11.41218 (Figure 3B, Table 3). mRNA expression profiles were distinguishable between CSU patients and healthy controls according to the hierarchical clustering (Figure 3C).

We constructed a Circos graph to visualize the chromosomal distributions and classifications of the dysregulated lncRNAs and mRNAs (Figure 4). There were 114 lncRNAs in chromosome 2 and 77 mRNAs in chromosome 1.

GO and KEGG Pathway Analyses

Among upregulated target genes, GO analysis showed that vesicle-mediated transport had the highest enrichment score in the BP category (Figure 5A), cytoplasmic vesicle in the CC category (Figure 5B), and carbohydrate binding in the MF category (Figure 5C). Among downregulated target genes, establishment of epithelial cell apical/basal polarity had the highest enrichment score in the BP category (Figure 6A), the apical part of the cell in the CC category (Figure 6B), and intracellular chloride channel activity in the MF category (Figure 6C).

KEGG pathway analysis of all upregulated target genes showed enrichment in 19 pathways including lysosome, osteoclast differentiation and human cytomegalovirus infection, and vascular endothelial growth factor (VEGF) signaling pathways (Supplementary Figure 2, Table 4). Of these, the VEGF pathway was the most closely related to the IP3/DAG pathway (Figure 7), and granules secreted by mast cells contain cytokines including VEGF.¹⁵ VEGF increases microvascular permeability, which is partially consistent with the pathogenesis of urticaria, so we hypothesized that the DE mRNAs in this pathway might be closely related to disease occurrence. Downregulated target genes were enriched in four pathways including salmonella infection, tight junction, and cytokine-cytokine receptor pathways (Supplementary Figure 3, Supplementary Table 2).

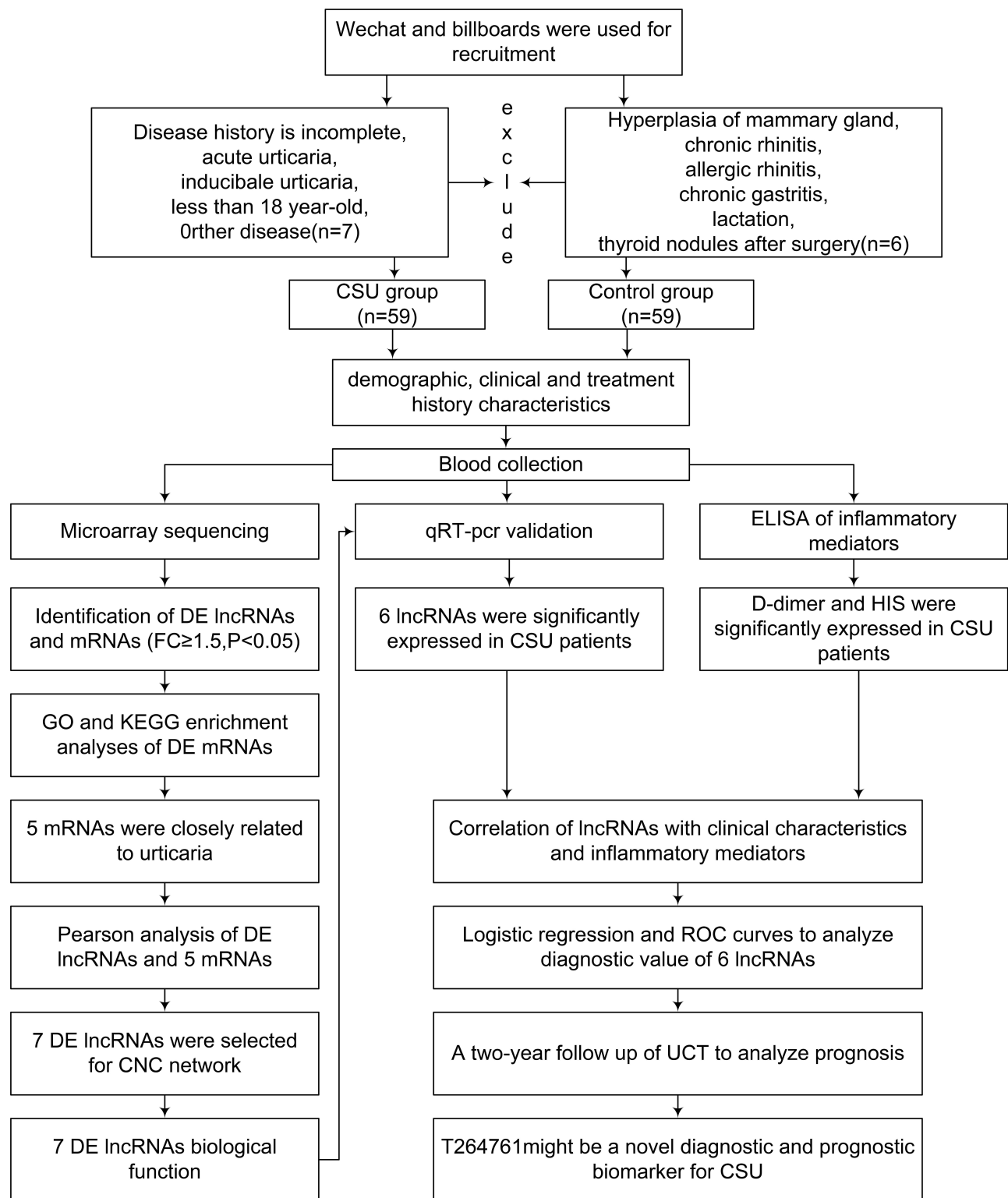


Figure 1 Flow diagram showing recruitment and inclusion/exclusion of participants and a summary of the study methods and key results.

Abbreviations: CNC, coding and noncoding co-expression; CSU, chronic spontaneous urticaria; DE, differentially expressed; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; ROC, receiver operating characteristic.

Table 1 Demographic, Clinical, and Treatment History Characteristics

	CSU	Healthy Control
Demographic characteristics		
Age, mean \pm SD, years	37.47 \pm 10.66	31.39 \pm 9.53
Sex, male/female, n	21/38	13/46
Clinical characteristics		
Episodes, n	20	N/A
Duration, median (range), months	48 (2–372)	N/A
Patients reporting provoking factors, n	25	N/A
Frequencies, median (range), times/week	7 (1–21)	N/A
Duration of wheals, median (range), min		
Shortest duration	60 (5–360)	N/A
Longest duration	240 (10–1440)	N/A
Diameter of wheals, median (range), cm		
Minimum	1 (1–8)	N/A
Maximum	5 (1–30)	N/A
Timing of attack period, n		
6:00–12:00	9	N/A
12:00–18:00	5	N/A
18:00–24:00	37	N/A
00:00–6:00	6	N/A
Irregular	13	N/A
Wheal distribution, n		
Head and neck	3	N/A
Trunk	31	N/A
Limbs	28	N/A
Hands and feet	13	N/A
Entire body	16	N/A
Angioedema, n	21	N/A
Complicated disease, n		
Chronic inducible urticaria	23	N/A

(Continued)

Table 1 (Continued).

	CSU	Healthy Control
Autoimmune disease	4	N/A
Allergic history, n		
Personal	15	N/A
Family	18	N/A
Urticaria activity score, median (range)	3 (2–6)	N/A
Urticaria activity score 7, median (range)	28 (2–42)	N/A
Treatment history (n)		
Traditional Chinese medicine	14	N/A
Western medicine	33	N/A
Integrated traditional Chinese and western medicine	5	N/A
Compliance	18	N/A

Abbreviations: N/A, not applicable; SD, standard deviation.

DE lncRNAs Related to the IP3/DAG Pathway

According to KEGG analysis, the VEGF pathway was the most closely related to IP3/DAG pathway. There were five DE mRNAs in the VEGF pathway in our analysis: BAD, PIK3CD, PLA2G4A, PPP3CA, and SRC. Pearson analysis of these five mRNAs and all DE lncRNAs revealed that there were 514 upregulated and 488 downregulated DE lncRNAs related to the 5 VEGF pathway mRNAs ($PCC \geq 0.8$, $p < 0.5$, $FDR \leq 1$). [Supplementary Table 3](#) shows the most closely related lncRNAs based on PCC values.

Validation of lncRNAs Using qRT-PCR and lncRNA–mRNA Co-Expression Networks

In total, seven lncRNAs were selected for validation, of which six were upregulated (T264761, T280622, ENST00000587970, T224062, ENST00000562459 and his-1_RNA_dna) and one was downregulated (ENST00000417930).

These 7 lncRNAs and 959 mRNAs were used to construct a co-expression network ([Figure 8](#)). GO analysis revealed those co-expressed mRNAs were most enriched in the regulation of intracellular transport within the BP category, cell leading edge within the

Table 2 The Top 10 Up- and Downregulated lncRNAs in the CSU Group Compared with the Control Group

Probe Name	p	Fold Change	Up-/Downregulated	Seq Name	Gene Symbol
ASHGV40050043	0.003156575	6.4280631	Up	ENST00000562459	CTD-3064M3.3
ASHGV40049841	0.00804805	5.5837104	Up	T350274	G082673
ASHGV40008202	0.04174666	5.1527168	Up	T056221	G012997
ASHGV40017880	0.018392837	5.0749424	Up	ENST00000567765	CTD-2014E2.6
ASHGV40044980	0.021772955	4.9449214	Up	TCONS_I2_00024198	XLOC_I2_012523
ASHGV40029001	0.00253739	4.6650077	Up	T008095	G001729
ASHGV40002528	0.001698611	4.5717149	Up	ENST00000587970	AC004510.3
ASHGV40041584	0.000217582	4.2496678	Up	T280622	G065252
ASHGV40024779	0.027166343	4.220745	Up	ENST00000595133	CTD-2337J16.1
ASHGV40046473	0.014791186	4.1384602	Up	T323391	G075678
ASHGV40051787	0.039880651	5.1422487	Down	NR_122077	LOC403323
ASHGV40015577	0.020274327	2.7795455	Down	NR_027457	LINC00221
ASHGV40020798	0.039893649	2.4976881	Down	T153603	G035593
ASHGV40041792	0.04932786	2.4358718	Down	T282869	G065898
ASHGV40001993	0.01783377	2.345804	Down	ENST00000545750	RP11-221N13.2
ASHGV40000372	0.005692562	2.2791778	Down	ENST00000415144	RP3-406P24.3
ASHGV40035227	0.034110594	2.2391571	Down	NR_024409	LOC100128164
ASHGV40038911	0.046262907	2.2248124	Down	ENST00000511222	RP11-530I17.1
ASHGV40023570	0.003459644	2.2186053	Down	NR_040034	LOC339298
ASHGV40058780	0.023915348	2.2017553	Down	T274232	G063722

Table 3 The Top 10 Up- and Downregulated mRNAs in the CSU Group Compared with the Control Group

Probe name	p	Fold Change	Up-/Downregulated	Seq Name	Gene Symbol
ASHGV40024700	0.004054487	4.2383847	Up	NM_033130	SIGLEC10
ASHGV40049374	0.016246274	3.9535642	Up	NM_015155	LARP4B
ASHGV40035389	0.000282273	3.6654474	Up	NM_001879	MASPI
ASHGV40010556	0.010148206	3.5672104	Up	NM_018984	SSH1
ASHGV40009825	0.007705098	3.5104154	Up	NM_153634	CPNE8
ASHGV40034070	0.032355514	3.275902	Up	NM_144715	EFHB
ASHGV40019139	0.023197095	3.2613452	Up	NM_017839	LPCAT2
ASHGV40019664	0.010750602	3.0397173	Up	NM_003693	SCARF1
ASHGV40056066	0.006337134	3.0199737	Up	uc001vsc.2	AK055145
ASHGV40024924	0.027606963	2.9908501	Up	NM_004285	H6PD
ASHGV40041861	1.72419E-06	11.41218	Down	NM_002538	OCLN
ASHGV40057642	0.002226629	8.1402545	Down	NM_001080141	CT47A6
ASHGV40054374	0.008951849	6.6993933	Down	NM_001145718	CT47B1
ASHGV40046953	0.007551515	5.7261036	Down	NM_002291	LAMB1
ASHGV40057587	0.04076977	4.8618259	Down	NM_001001888	VCX3B
ASHGV40046418	0.046526571	4.1834572	Down	NM_002192	INHBA
ASHGV40055302	0.015072078	2.7473306	Down	NM_005032	PLS3
ASHGV40027091	0.037930707	2.705742	Down	NM_207328	GPAT2
ASHGV40003239	0.007646155	2.3909454	Down	uc004doa.3	GAGE12F
ASHGV40055533	0.037138567	2.354473	Down	ENST00000370287	CSAG1

CC category, and receptor ligand activity within the MF category (Figure 9). These biological functions are closely related to the process of mast cell degranulation, indicating that the lncRNAs were indeed related to CSU development.

Samples from 53 CSU patients and 20 healthy controls were selected for qRT-PCR validation. [Supplementary Table 4](#) shows the specific primers sequences for the seven lncRNAs. The results demonstrated that T264761 (1.62-fold), T280622 (3.03-fold), ENST00000587970 (2.67-fold),

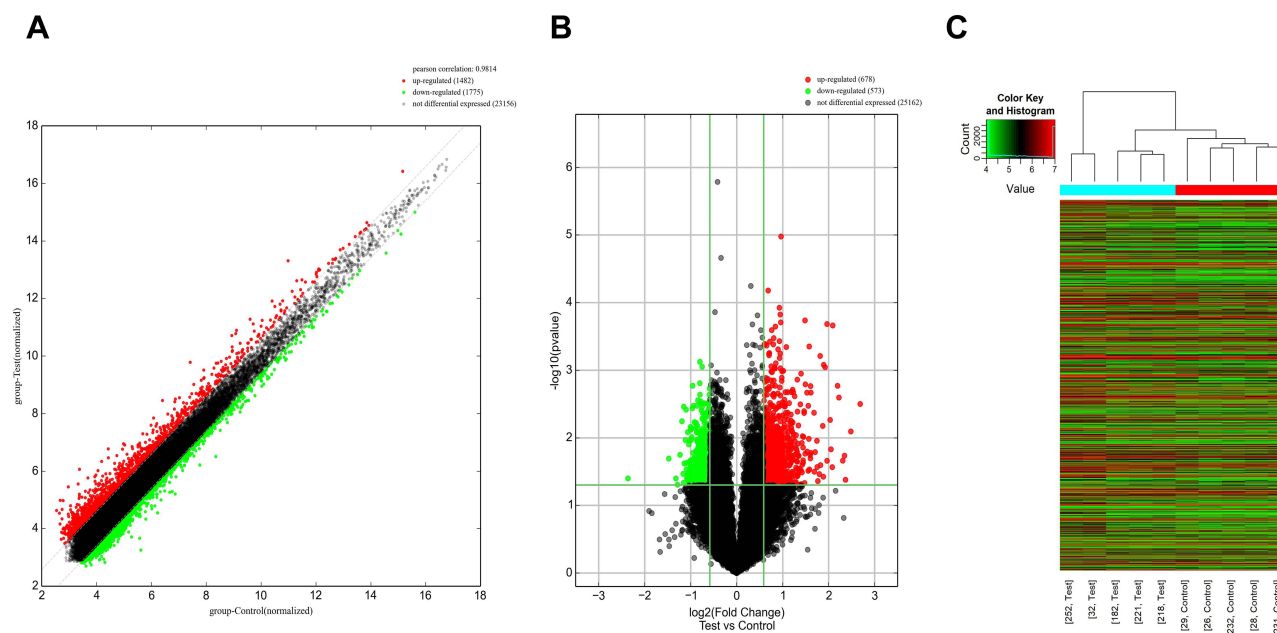


Figure 2 lncRNA expression profile. **(A)** Scatter plot. **(B)** Volcano plot of differentially expressed lncRNA. **(C)** Heatmap analysis. Red and green indicate up- and downregulated lncRNAs, respectively, and black indicates RNAs with no significant differential expression. The five left and right columns represent CSU patients and healthy controls, respectively.

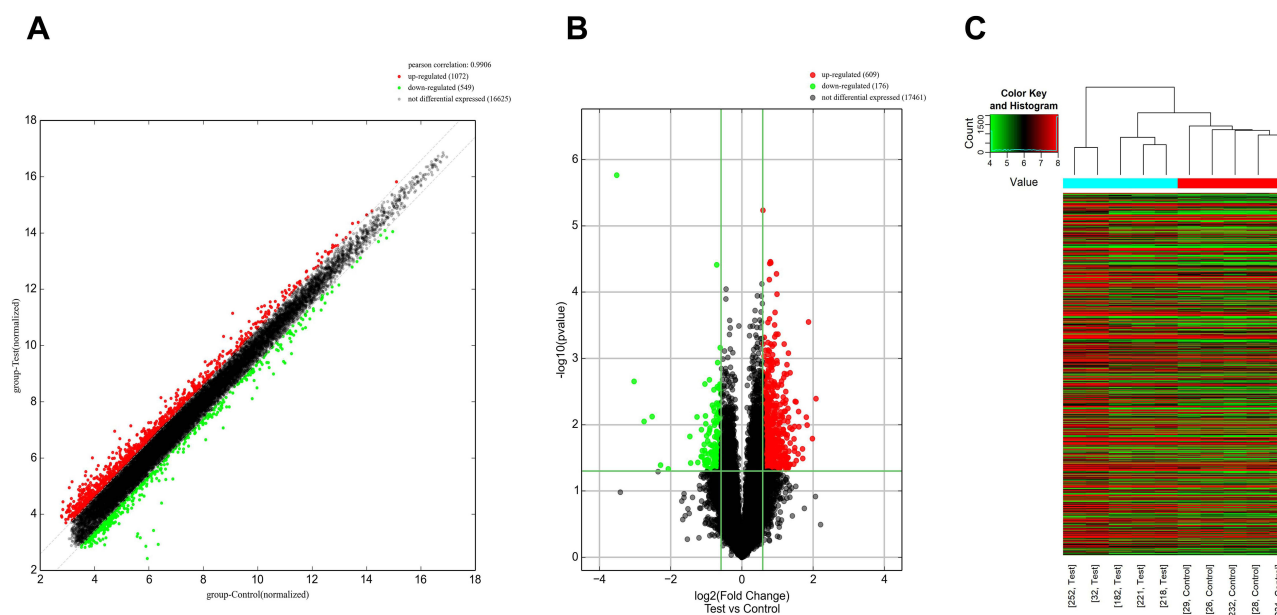


Figure 3 mRNA expression profile of mRNA. **(A)** Scatter plot of mRNA. **(B)** Volcano plot of differentially expressed mRNA. **(C)** Heatmap analysis of mRNA. Red and green indicate upregulated and downregulated mRNAs, respectively, and black indicates mRNAs with no significant differential expression. The five left and right columns represent CSU patients and healthy controls, respectively.

ENST00000562459 (2.70-fold), and his-1_RNA_dna (1.38-fold) were significantly increased in CSU, while ENST00000417930 (2.06-fold) was significantly decreased in CSU (Figure 10, Supplementary Table 5). However, T224062 was not dysregulated in the CSU group.

Correlation of lncRNAs with Clinical Characteristics and Inflammatory Mediators

Samples from 56 CSU patients and 13 healthy controls were selected for ELISA, which showed increased

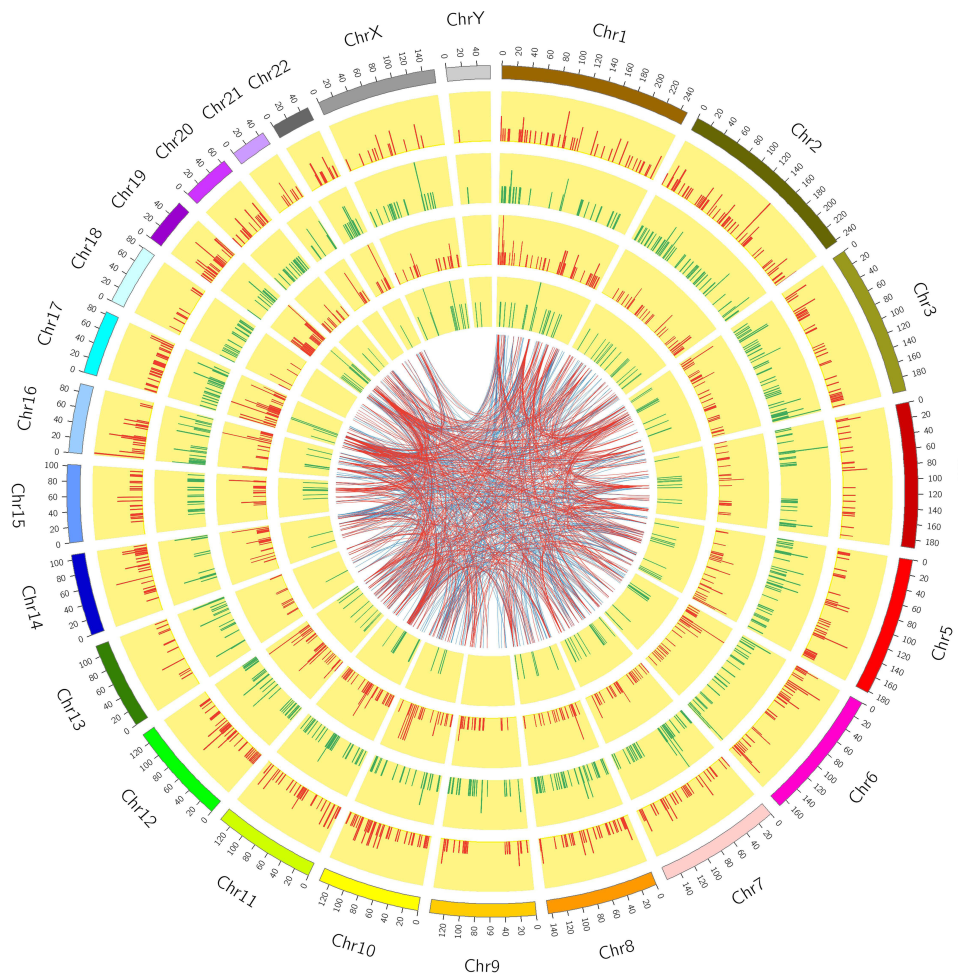


Figure 4 Circos plot showing differentially expressed lncRNAs and mRNAs on human chromosomes. The first circle is the human autosomal distribution map. The second and third circles show the distributions of differentially expressed genes among the chromosomes. Red and green lines show up- and down-regulated RNAs, respectively. A higher column indicates greater differential expression of genes in the region. The fourth and fifth circles show the distributions of differentially expressed lncRNAs on chromosomes. The expression form is related to the expression of RNA. Internal connections indicate relationships between the top 500 co-expressed lncRNAs and mRNAs ($PCC \geq 0.8$, $p < 0.5$). Red and blue show positive and negative correlations, respectively.

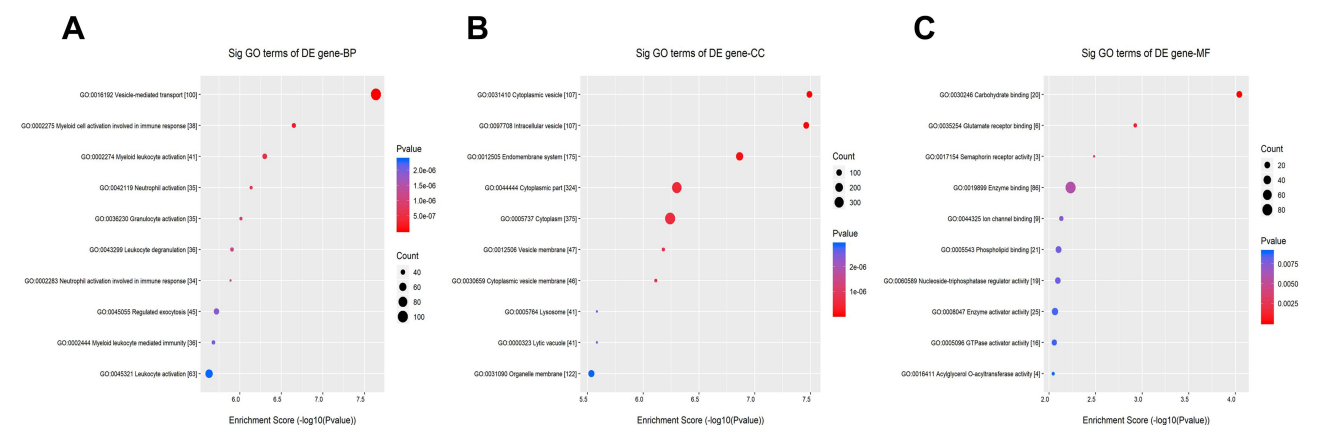


Figure 5 GO enrichment analyses showing upregulated differentially expressed mRNAs. (A) Biological process (BP) analysis. (B) Cellular component (CC) analysis. (C) Molecular function (MF) analysis.

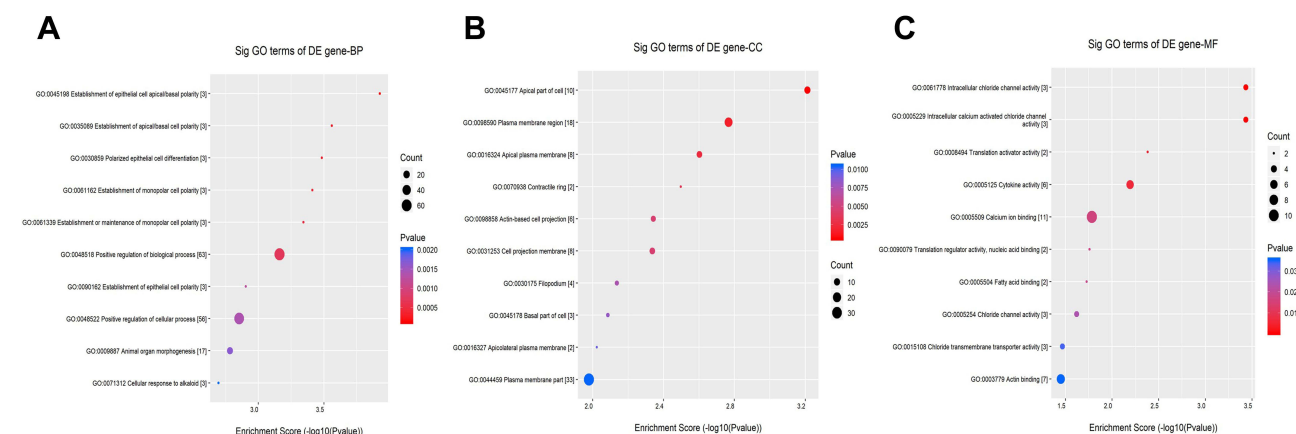


Figure 6 GO enrichment analyses showing downregulated differentially expressed mRNAs. **(A)** Biological process (BP) analysis. **(B)** Cellular component (CC) analysis. **(C)** Molecular function (MF) analysis.

expression levels of D-dimer and HIS in the serum of CSU patients (Figure 11, Supplementary Table 6).

Spearman coefficient analysis was used to assess the correlations of six lncRNAs (T264761, T280622, ENST00000587970, ENST00000562459, his-1_RNA_dna, and ENST00000417930) with clinical characteristics (age, disease duration, frequency of symptom occurrence, duration of wheals, size of wheals, and UAS7 score) and inflammatory mediators (D-dimer and HIS). A positive correlation was observed between his-1_RNA_dna and the frequency of symptom occurrence, and negative correlations were noted between four lncRNAs (T264761, T280622, his-1_RNA_dna, ENST00000417930) and the maximum size of wheals (Figure 12, Supplementary Table 7).

ROC Curves of Selected lncRNAs for CSU Risk Prediction

Logistic regression was used to analyze the CSU risk associated with selected lncRNAs. Six lncRNAs with significant differences in univariate analysis were included as independent variables, with CSU occurrence as the dependent variable (1 = occurrence, 0 = no occurrence). ENST00000417930 (odds ratio [OR] = 0.385; 95% confidence interval [CI] = 0.156–0.948) and T264761 (OR = 1.266, 95% CI = 1.023–1.567) were identified as risk factors for CSU (Table 5). Furthermore, at the cut-off value of 4.138, the area under the ROC curve for T264761 was 0.666, and the sensitivity and specificity values were 49.06% and 90%, respectively (Figure 13, Supplementary Table 8). This finding suggested that the T264761 expression level

may be useful for differentiating CSU patients from healthy controls.

Prognostic Value of T264761

The last follow-up data was collected in 4th September 2021. A total of 47 patients were included in this analysis. According to a previous study in China, a Urticaria Control Test (UCT) score ≥ 12 indicates well-controlled CSU.¹⁶ Based on a T264761 expression level cut-off value of 4.138, CSU patients were sorted into high and low expression groups.¹⁴ The rate of well-controlled disease in those with low T264761 expression was 82.61% compared with 54.17% in those with high T264761 expression. Table 6 shows that lower T264761 expression could predict higher disease control rates.

Discussion

In this study, we identified several DE lncRNAs and mRNAs in CSU patients compared with healthy controls using microarray sequencing. Some were found to be closely related to IP3/DAG signaling pathway, a key pathway in the induction of mast cell degranulation, which is involved in CSU. Importantly, we identified a potential biomarker for this disease, where no known biomarkers existed previously: lncRNA T264761 was found to have diagnostic and prognostic value for CSU in our study.

Upregulated lncRNAs and mRNAs outnumbered those that were downregulated in CSU samples compared with healthy controls, indicating the activation of multiple biological processes or signaling pathways under pathological conditions. In our study, we identified DE lncRNAs using a FC threshold of 1.5, compared to 2 in other

Table 4 Signaling Pathways Associated with Differentially Expressed Upregulated mRNAs

Pathway	Fisher p	Count	Genes
Lysosome - <i>Homo sapiens</i> (human)	2.44228E-05	14	API53//AP3S2//ATP6V0A1//ATP6V0C//CD68//CTSD//CTSS//DNASE2//GAA//GM2A//IDUA//MAN2B1//SORT1//SUMF1
Tuberculosis - <i>Homo sapiens</i> (human)	0.000420099	15	ATP6V0A1//ATP6V0C//BAD//BAX//C3//CALML4//CTSD//CTSS//CYP27B1//FCGR1A//IL18//ITGAX//PPP3CA//RAB7A//SRC
Amphetamine addiction - <i>Homo sapiens</i> (human)	0.001121431	8	ATF2//ATF6B//CALML4//FOSB//GRIN3A//PPP1CB//PPP3CA//STX1A
Osteoclast differentiation - <i>Homo sapiens</i> (human)	0.006252124	10	BTK//CYBA//FCGR1A//FOSB//LILRA2//LILRA5//LILRB1//LILRB4//PIK3CD//PPP3CA
Phagosome - <i>Homo sapiens</i> (human)	0.007451336	11	ATP6V0A1//ATP6V0C//ATP6V1B2//C3//CANX//COLEC12//CTSS//CYBA//FCGR1A//RAB7A//TUBB6
Human cytomegalovirus infection - <i>Homo sapiens</i> (human)	0.01001837	14	ADCY9//ATF2//ATF6B//BAX//CALML4//CTNNB1//CXCR2//GNAI1//GNB4//GNG7//PIK3CD//PPP3CA//RB1//SRC
Kaposi sarcoma-associated herpesvirus infection - <i>Homo sapiens</i> (human)	0.01271734	12	BAX//C3//CALML4//CTNNB1//GNB4//GNG7//MAP2K4//PIK3CD//PPP3CA//RB1//SRC//TRAF3
Pentose phosphate pathway - <i>Homo sapiens</i> (human)	0.01308713	4	FBP1//H6PD//PGM2//PRPS1L1
Epithelial cell signaling in <i>Helicobacter pylori</i> infection - <i>Homo sapiens</i> (human)	0.01836361	6	ATP6V0A1//ATP6V0C//ATP6V1B2//CXCR2//MAP2K4//SRC
<i>Vibrio cholerae</i> infection - <i>Homo sapiens</i> (human)	0.01868265	5	ADCY9//ATP6V0A1//ATP6V0C//ATP6V1B2//KDELR2
Relaxin signaling pathway - <i>Homo sapiens</i> (human)	0.01937107	9	ACTA2//ADCY9//ATF2//ATF6B//GNB4//GNG7//MAP2K4//PIK3CD//SRC
Glutamatergic synapse - <i>Homo sapiens</i> (human)	0.02490071	8	ADCY9//GLUL//GNB4//GNG7//GRIN3A//HOMER3//PLA2G4A//PPP3CA
cGMP-PKG signaling pathway - <i>Homo sapiens</i> (human)	0.02987496	10	ADCY9//ATF2//ATF6B//BAD//CALML4//GNAI1//MRV11//PPP1CB//PPP3CA//SLC8A1
AMPK signaling pathway - <i>Homo sapiens</i> (human)	0.03252643	8	ACACA//CAB39//CAMKK2//FBP1//PIK3CD//PPP2R3B//RAB2A//STRADB
Histidine metabolism - <i>Homo sapiens</i> (human)	0.03295433	3	ALDH3B1//CARNMT1//HAL
VEGF signaling pathway - <i>Homo sapiens</i> (human)	0.03535321	5	BAD//PIK3CD//PLA2G4A//PPP3CA//SRC
Axon guidance - <i>Homo sapiens</i> (human)	0.04515047	10	FES//PARD3//PIK3CD//PLXNB2//PLXNC1//PPP3CA//SEMA3C//SRC//SRGAP3//SSH1
Viral carcinogenesis - <i>Homo sapiens</i> (human)	0.04766081	11	ATF2//ATF6B//BAD//BAX//C3//HNRNPK//PIK3CD//RB1//SRC//TRAF3//YWHAE
Collecting duct acid secretion - <i>Homo sapiens</i> (human)	0.04975539	3	ATP6V0A1//ATP6V0C//ATP6V1B2

studies.^{13,17,18} A lower threshold in this study may be related to the characteristic CSU symptoms of sudden itchy wheals or angioedema. Most of the CSU patients were in remission, and were therefore symptomatically similar to healthy control individuals except for those who experienced sudden attacks, so the overall differential expression may have been relatively low.

DE mRNAs were analyzed using GO term and KEGG pathway enrichment analyses. GO analysis showed that most upregulated mRNAs were associated with vesicle-mediated transport within the BP category, cytoplasmic

vesicle within the CC category and carbohydrate binding within the MF category. This pattern is consistent with the process of mast cell degranulation, which involves the secretion of mediators via a vesicle secretion system.^{19,20} Mast cells also require carbohydrates for granule generation and maintenance.²¹ Our KEGG analysis showed that the VEGF signaling pathway was the most closely related to the occurrence of urticaria. VEGF is a mediator of vascular permeability that could be involved in inducing and maintaining urticaria symptoms; the IP3/DAG pathway is known to be involved in urticaria pathology and is

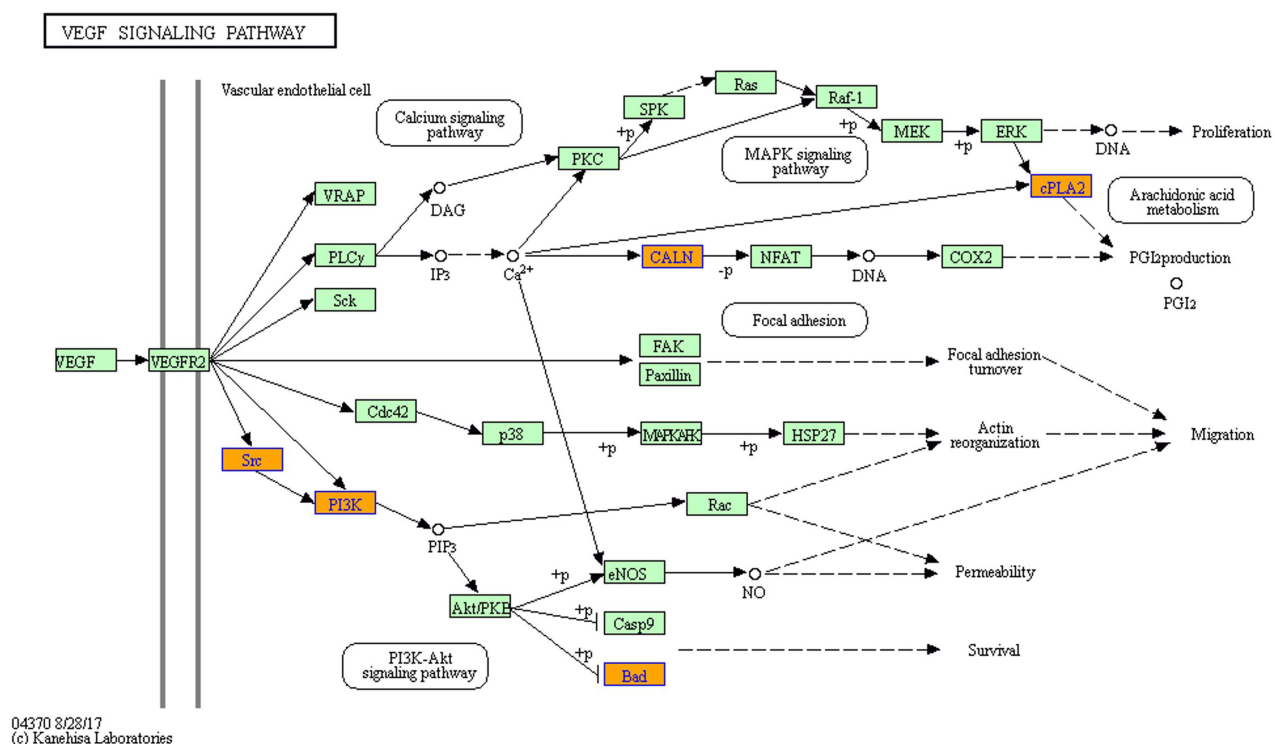


Figure 7 KEGG pathway annotations within the vascular endothelial growth factor (VEGF) signaling pathway.

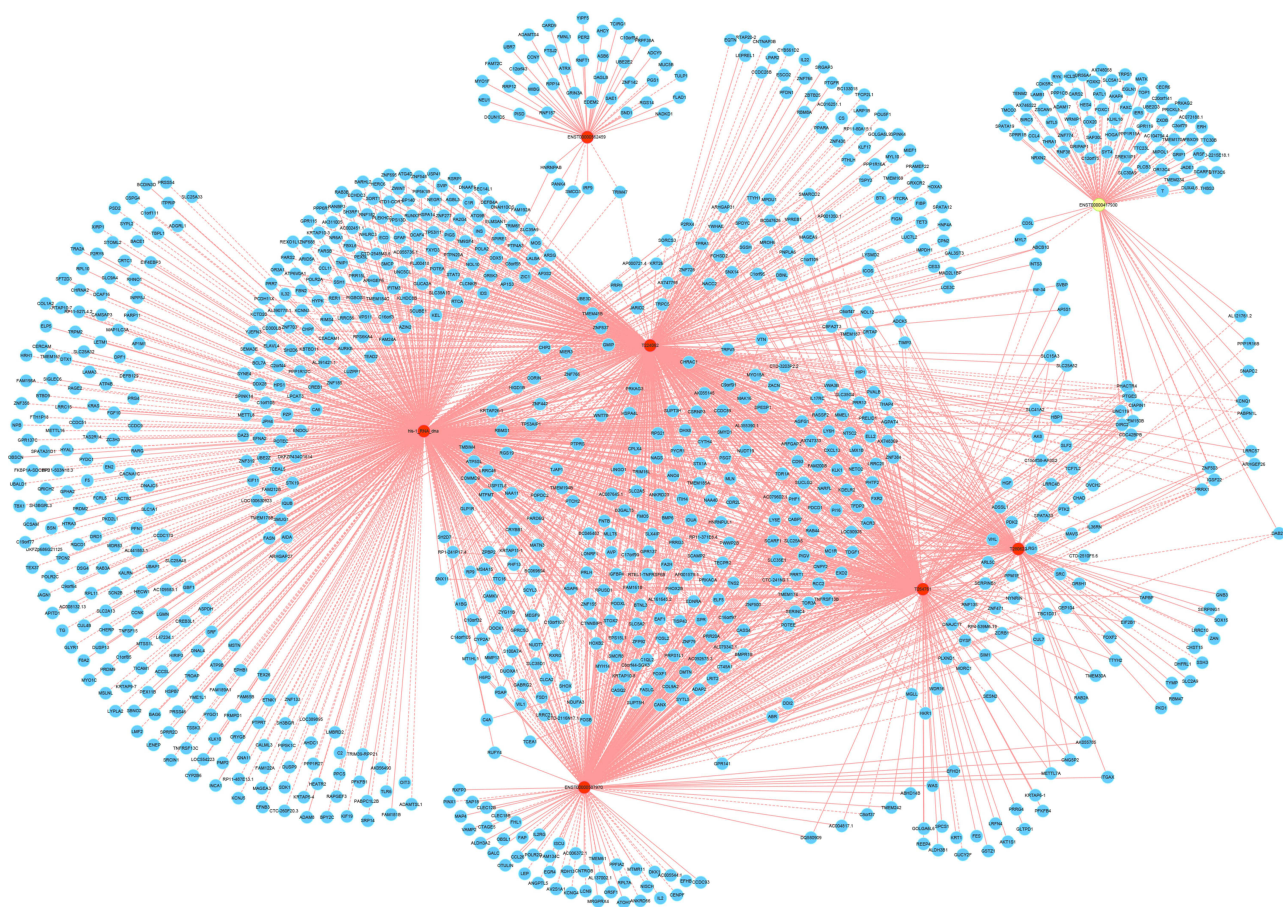


Figure 8 Co-expression network of lncRNAs and mRNAs. Red and yellow nodes represent up- and downregulated lncRNAs, respectively, and blue nodes represent mRNAs. Dotted and solid lines indicate negative and positive correlations, respectively.

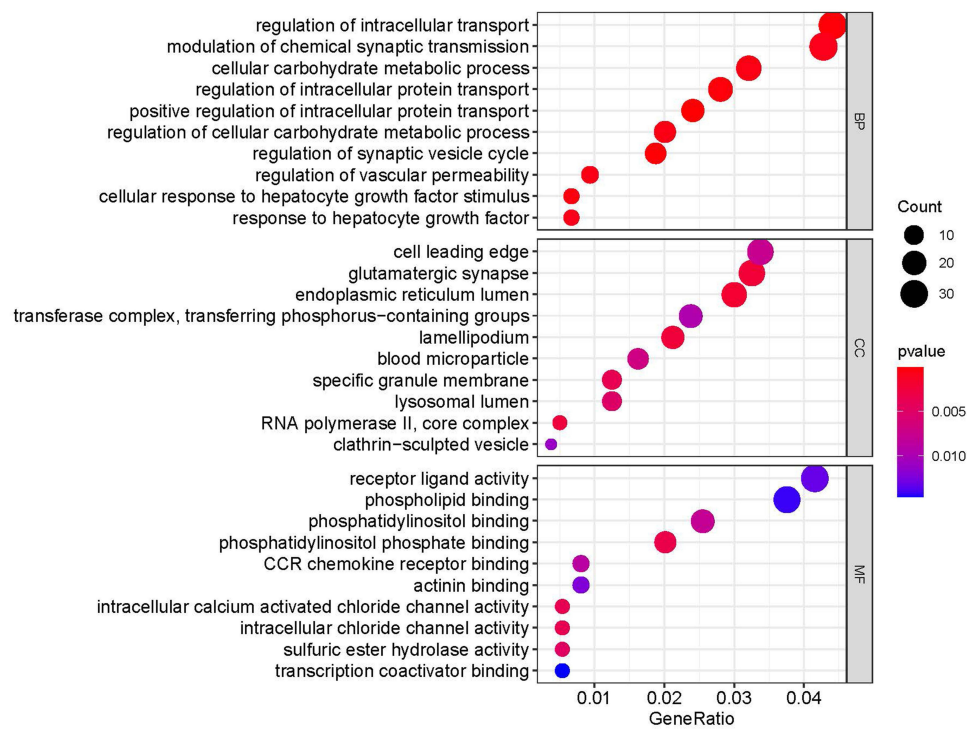


Figure 9 GO enrichment analyses of co-expressed mRNAs associated with seven biomarker candidate lncRNAs.

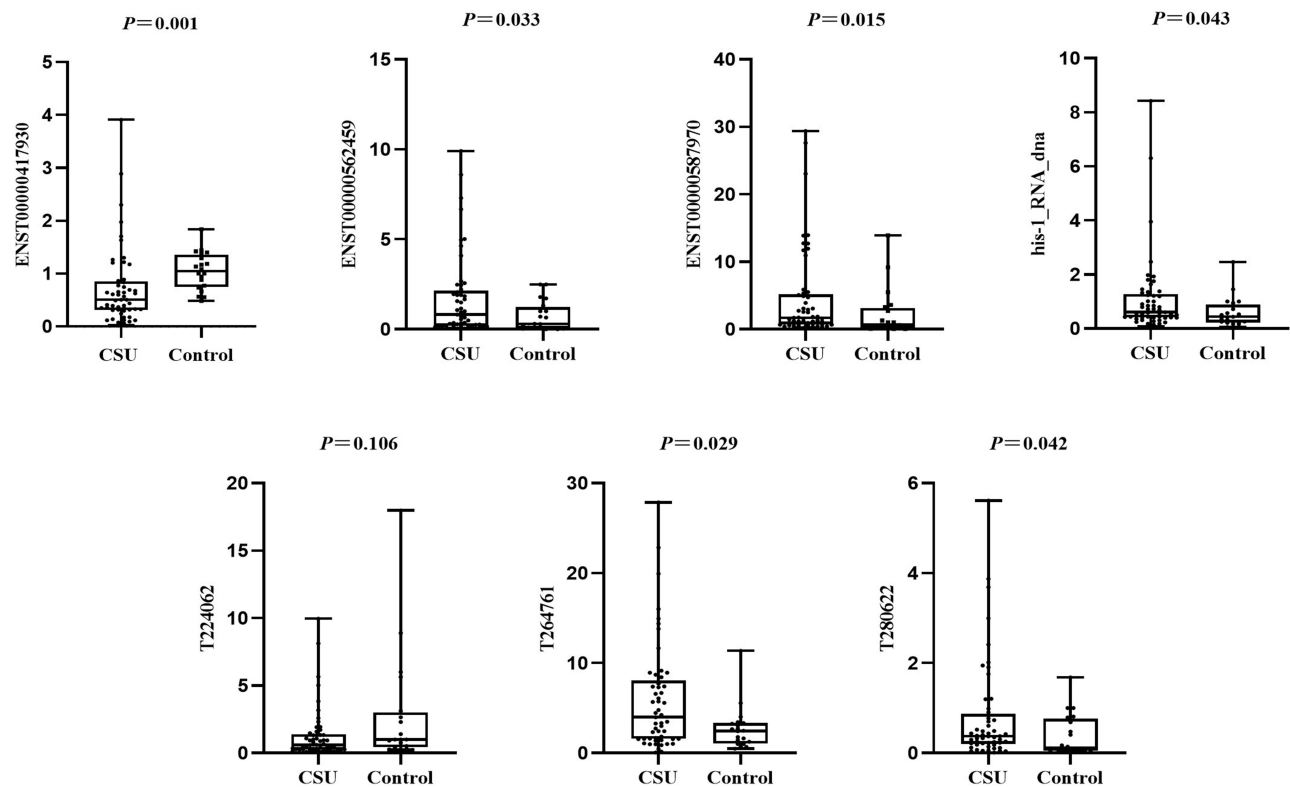


Figure 10 Expression levels of seven lncRNAs in the CSU ($n = 53$) and healthy control ($n = 20$) groups measured by qRT-PCR. Six of seven lncRNAs were confirmed to be significantly differentially expressed; T224062 was the exception.

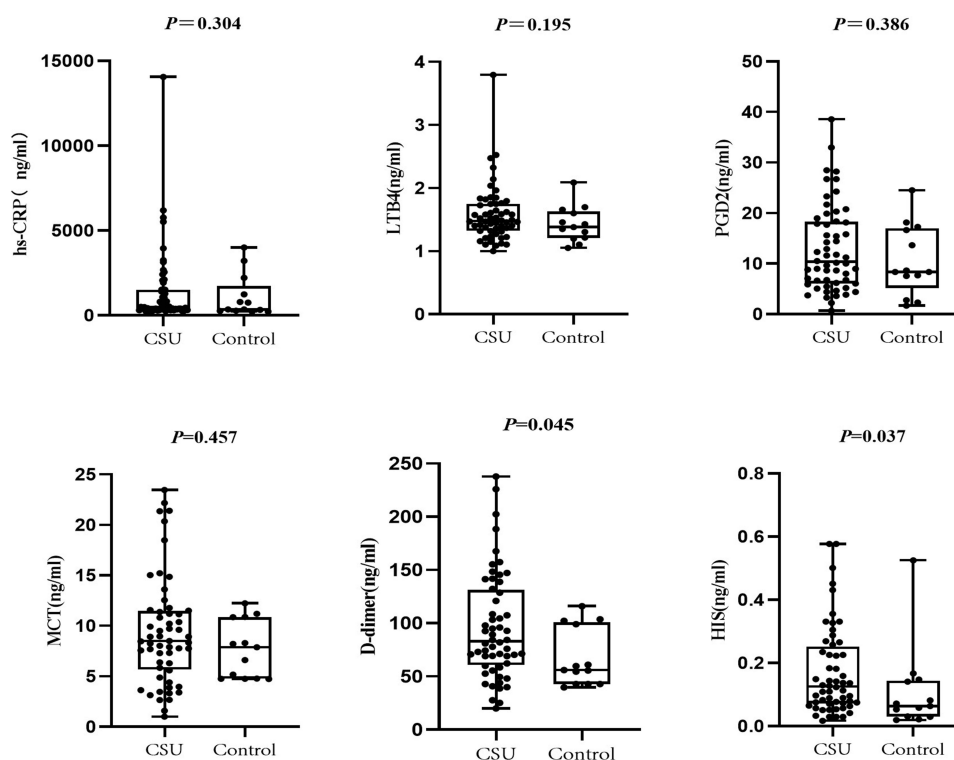


Figure 11 Serum concentrations of hs-CRP, LTB4, PGD2, MCT, D-dimer and HIS in the CSU (n = 56) and healthy control (n = 13) groups. Differences between groups were assessed using Mann-Whitney U-tests; experiments were repeated three times.

Abbreviations: hs-CRP, high sensitivity C-reactive protein; LTB4, leukotriene B4; PGD2, prostaglandin D2; MCT, mast cell tryptase; HIS, histamine.

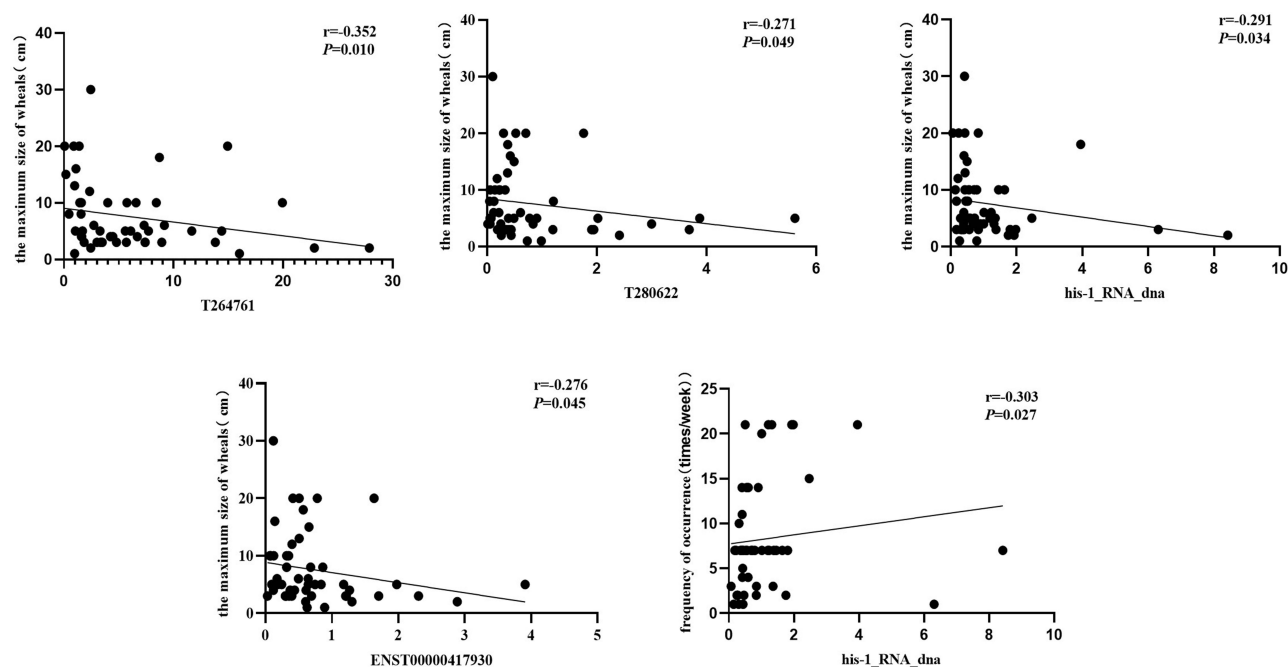


Figure 12 Correlations between lncRNA levels and clinical characteristics in CSU patients. The expression levels of T264761, T280622, his-1_RNA_dna, and ENST00000417930 were associated with the maximum size of wheals. The expression level of his-1_RNA_dna was associated with the frequency of symptom occurrence.

Table 5 Logistic Regression Analyses to Assess the Potential Value of lncRNAs in the Prediction of CSU Risk

Factor	B	SE	Wald	p	OR	95% CI
ENST00000417930	-0.955	0.460	4.310	0.038	0.385	0.156–0.948
T264761	0.236	0.109	4.687	0.030	1.266	1.023–1.567

Abbreviations: B, beta; CI, confidence interval; OR, odds ratio; SE, standard error.

included in the pathway diagram in Figure 7.²² We therefore identified five DE mRNAs in the VEGF pathway that were used to identify seven lncRNAs closely related to the IP3/DAG pathway (T264761, T280622, ENST00000587970, T224062, ENST00000562459, his-1_RNA_dna, and ENST00000417930). Co-expression network and GO analyses confirmed that these lncRNAs were closely related to the development of CSU.

Co-expressed mRNAs were found to be enriched in the regulation of intracellular transport within the BP category, cell leading edge within the CC category, and receptor ligand activity within the MF category. Many high-affinity receptors for IgE are expressed on the mast cell surface, and the intracellular transportation and release of pro-inflammatory mediators following antigen-induced aggregation causes allergic reactions.^{5,23} Our qRT-PCR results suggested that T224062 was not significantly dysregulated in CSU patients, which contradicted our microarray sequence data; however, this may be due to the relatively small sample size.

Given that our bioinformatics analysis revealed putative associations between several lncRNAs and CSU, we then assessed their correlations with clinical characteristics of the disease to determine their usefulness as disease indicators. Our ELISA results revealed that D-dimer and HIS expression were increased in CSU. Previous studies showed a positive

correlation between D-dimer levels and the severity of CSU activity.^{24,25} However, we found no correlation between the seven lncRNAs we identified in our bioinformatics analysis and D-dimer or HIS levels. Some of the lncRNAs were correlated with the frequency of symptom occurrence and the maximum size of wheals. It suggested that lncRNAs did not seem to be promising biomarkers for disease activity. Logistic regression showed that high levels of T264761 could indicate a greater risk of CSU. The ROC curve analysis suggested that T264761 may differentiate CSU patients from healthy control individuals with high specificity; however, the sensitivity was not high. A combination of medical history and symptom evaluation could improve its diagnostic value. We performed a 2-year follow-up to assess the prognostic value of T264761 in CSU patients based on the UCT score. This assessment was the first valid and reliable tool to assess disease control in patients with CSU.²⁶ This test was rarely used in CSU clinical research in China before 2020; however, Yu et al translated the UCT into Chinese and assessed the reliability, validity, sensitivity, and screening accuracy of the new scale, enabling its application in China.¹⁶ In our follow-up, lower T264761 expression (≤ 4.138) could predict a higher disease control rate. Notably, in five women, CSU resolved after pregnancy during the follow-up period; further studies of lncRNA levels in pregnant and postnatal women with CSU could help identify the underlying mechanism.

Many recently developed techniques have enabled the identification of potentially useful CSU biomarkers, including proteomic analysis, transcriptome analysis, microRNA or gene sequencing, and gut microbiome analysis.^{27–31} However, this study is the first to assess the diagnostic and prognostic value of lncRNAs as CSU biomarkers using microarrays, bioinformatics analyses, and co-expression network construction. T264761 was found to be related to the IP3/DAG signal pathway, which is known to induce mast cell degranulation that is involved in CSU. This lncRNA was also associated with the maximum size of the wheals that are symptomatic of this disease. High levels of T264761 may indicate an increased CSU risk with high specificity. This lncRNA

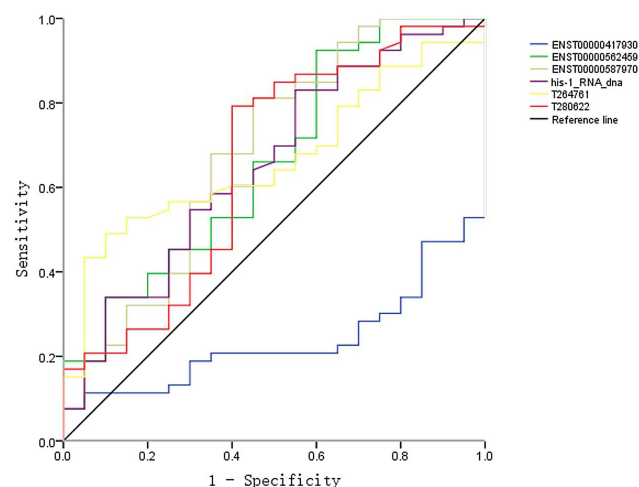
**Figure 13** The ROC curve for lncRNAs.

Table 6 Correlations Between T264761 Levels and UCT Scores in CSU Group After 2-Year Follow-Up

T264761 Level	UCT Score (<12, n)	UCT Score (≥12, n)	Rate of Well-Controlled Disease (%)	Pearson Value	p
Low level (<4.138)	4	19	82.61	4.372	0.037*
High level (≥4.138)	11	13	54.17		

Notes: A Chi-square test was used to compare groups. *Represents $p < 0.05$.

Abbreviation: UCT, Urticaria Control Test.

may also predict longer-term disease control status. This study provides novel insights into CSU biomarkers and guide further basic and clinical research.

Our results should be considered in the context of some limitations. First, the samples were only from CSU patient and healthy controls; in future studies we will include more samples including those from subjects acute urticaria and other similar allergic diseases as controls to further investigate the diagnostic value of lncRNAs. Secondly, the frequency and duration of follow-up were relatively short, because urticaria has a self-healing tendency without treatment, so the findings should be interpreted with caution. Thirdly, the results of this study are mostly based on bioinformatics predictions, and further cell culture and animal experiments are needed to clarify the specific roles of lncRNAs in CSU. Additionally, it is worth mentioning that although some CSU patients included were complicated with chronic idiopathic urticaria, the results are still plausible. This is consistent with clinical practice and other relevant CSU clinical literatures.^{28,32}

Conclusions

Our study established lncRNA and mRNA expression profiles in CSU using microarrays. Some lncRNAs and mRNAs were differentially expressed in CSU patients compared with healthy controls; these were shown to be involved in diverse biological pathways related to mast cell degranulation, which is associated with CSU, and the IP3/DAG signal pathway that is involved in producing the characteristic wheals and itchiness associated with CSU. The lncRNA T264761 may be a clinically useful novel diagnostic biomarker for CSU, with lower levels predicting a better UCT score and thus indicating less severe disease. Further investigations are required to clarify the specific function of T264761 in CSU pathogenesis.

Data Sharing Statement

Supporting data used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Disclosure

The authors report no conflicts of interest in this work.

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