Research Article

Analysis of Differentially Expressed Long Noncoding RNA in Renal Ischemia-Reperfusion Injury

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Keywords

Long noncoding RNA · Renal ischemia-reperfusion injury · RNA sequencing

Abstract

Background: Renal ischemia-reperfusion (IR) injury is one of the major causes of acute renal failure which seriously endangers the health and life of patients. Currently, there is still lack of comprehensive knowledge of the molecular mechanism of renal IR injury, and the regulatory role of long noncoding RNA (lncRNA) in renal IR damage remains poorly understood. **Aim:** The aim of this study was to analyze the expression spectrum of lncRNA in renal IR damage in mice and to explore specific lncRNA that may be involved in regulating the development of human renal IR injury. **Methods:** RNA-Seq was used to investigate the lncRNA profile of renal IR injury in a mouse model, and conservation analysis was performed on mouse lncRNAs with differential expression (fragments per kilobase of transcript per million mapped reads ≥ 2) by BLASTN. The potential functions and associated pathways of the differentially expressed lncRNA were explored by bioinformatics analysis. The cell hypoxia model was used to detect the expression of the candidate lncRNA. **Results:** Of the 45,923 lncRNA transcripts detected in the samples, and 5,868 lncRNAs were found to be significantly differentially expressed ($p < 0.05$ and fold change ≥ 2) in 24-h IR kidney tissue compared to the expression in the control group. It was found that 56 differently expressed mouse lncRNA transcripts have human homology by analyzing the conserved sequences. We also found that lncRNA-NONHSAT183385.1 expression significantly increased in HK2 cells after 24 h of hypoxia and

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increased further 6 h after reoxygenation, and after 24 h of reoxygenation it was dramatically downregulated, indicating that NONHSAT183385.1 may be involved in the pathophysiological process of renal tubular epithelial cells in response to ischemia in human renal IR.

Conclusion: Our study revealed differentially expressed lncRNAs in renal IR damage in mice and identified a set of conserved lncRNAs, which would help to explore lncRNAs that may play important regulatory roles in human renal IR injury.

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Introduction

Renal ischemia-reperfusion (IR) injury is a clinically common and complicated pathophysiological event with a high incidence and mortality rate. A violent surge in reactive oxygen, excessive release of inflammatory mediators, massive infiltration of neutrophils, cellular calcium overload, and mitochondrial dysfunction are pivotal elements leading to renal functional cell damage [1–3]. So far, the detailed molecular mechanism of the progression of renal IR injury has not been fully understood, and effective clinical therapies are still lacking. Although much progress has been made in the research of the function of protein-coding genes in renal IR injury, the specific regulatory mechanisms leading to the development and progression of renal IR damage remain unclear. Long noncoding RNAs (lncRNAs) are a class of transcripts that are longer than 200 nucleotides, with little coding capacity for proteins [4–7]. lncRNAs are classified as intergenic, antisense, sense overlapping, bidirectional, and intronic lncRNA according to the genomic location with respect to adjacent protein-coding genes [8] and interfere with gene expression in multiple ways [9–11]. Recent studies have shown that lncRNA plays a key regulatory role in diverse biological processes such as cell cycle, proliferation, differentiation, apoptosis, and metabolism [12, 13]. Regulation of lncRNA is necessary to maintain tissue homeostasis, and its dysregulation contributes to human disease [14–17]. It has been reported that lncRNAs are involved in various kidney diseases [18–20]. However, their specific role in the physiopathology of renal IR injury is still largely unknown. Here, we investigated the expression patterns of lncRNA after 24 h of renal IR by RNA sequencing using a mouse model, aiming to better understand the gene regulatory rules of renal IR injury in humans. In addition, we performed a conservative analysis of differently expressed lncRNAs in renal IR lesions of the mouse with human lncRNAs, which will help to further reveal lncRNAs that may play key roles in the regulation of human renal IR damage.

Materials and Methods

Renal IR Injury Model

To investigate the involvement of lncRNA in kidney tissue injured by IR, we induced renal IR injury in mice. Experiments were performed on adult male C57BL/6 mice (6–8 weeks old and weighing 20–25 g) purchased from the Second Military Medical University of China. Animals were maintained under conditions free of specific pathogens. Induction of renal IR injury was performed as described previously [21]. Briefly, mice were intraperitoneally anesthetized with 4% chloral hydrate (10 μ L/g). The kidneys were exposed by abdominal incision and the right kidney was removed following ligation of the right ureter. The left renal pedicle was clamped completely for 45 min using a microvascular clamp. Mice were kept well hydrated with warm saline and a homeothermic station to maintain body temperature at 37°C. Sham control animals were subjected to the same procedure without left renal pedicle

clamping. Mice were sacrificed 24 h after reperfusion. Blood samples were collected at the same time for biochemical analyses, and the left kidneys harvested were cut in half longitudinally. Half of each kidney was either snap-frozen in liquid nitrogen and stored at -80°C for subsequent transcriptome sequencing and quantitative real-time PCR (qRT-PCR) analysis or perfusion-fixed with 4% paraformaldehyde for histological analysis.

Serum Biochemical Parameters and Histological Examination

Serum creatinine and blood urea nitrogen concentrations were measured using a 7180 Automatic Analyzer (Hitachi, Japan) to assess renal function. Kidney tissue samples fixed in 4% paraformaldehyde were embedded in paraffin and stained with hematoxylin and eosin for histological analysis of tubular injury.

RNA Extraction, Library Preparation, and Next-Generation Sequencing

Total RNA was isolated from renal tissue using RNAiso Plus Total RNA extraction reagent (Takara) following the manufacturer's directions. RNA was qualitatively and quantitatively analyzed using a NanoDrop ND-2000 spectrophotometer and checked for a RIN number to inspect RNA integrity by an Agilent Bioanalyzer 2100 (Agilent). Qualified total RNA was further purified by RNAClean XP Kit (Beckman) and RNase-Free DNase Set (QIAGEN). The Shanghai Biotechnology Corporation constructed the RNA-Seq libraries and performed the high-throughput sequencing on the Illumina HiSeq 2500 system (Illumina) using paired-end technology.

Prediction of Conserved lncRNAs

Most lncRNAs display a low conservative property of sequence between humans and mice. In order to investigate the possible regulatory role of lncRNA in human renal tissue after IR injury, we used BLASTN to make a conservative evaluation of candidate mouse lncRNAs with differential expression. To facilitate further study, these differentially expressed lncRNA transcripts were selected by filtering on the basis of the following parameters: (1) fragments per kilobase of transcript per million mapped reads (FPKM) ≥ 2 in either control or IR treatment groups, and (2) differential expression of the target gene. Those lncRNAs with the best high degree of similarity (E value $\leq 10^{-5}$) in bidirectional comparison were considered as potential orthologs.

Cell Culture and Hypoxia

HK2 cells were maintained in DMEM/F12 supplemented with 10% fetal bovine serum (Bioind) at 37°C with 5% CO_2 . For hypoxia-reoxygenation, cells cultured in serum-free medium for 24 h were placed in a hypoxia incubator (Sanyo, Japan) under anoxic conditions at 5% CO_2 , 94% N_2 , and 1% O_2 for 24 h followed by normoxic culture for 3, 6, and 24 h. Cells cultured under normoxic conditions were used as control and hypoxia-treated cells confirmed by immunoblot analysis for hypoxia-inducible factor 1α .

Quantitative Real-Time PCR

lncRNAs were randomly selected to perform qRT-PCR validation. Total RNA of HK2 cell was isolated as described above. cDNA was synthesized from 1 μg of total RNA per sample using a Thermo Fisher Scientific™ RevertAid First-Strand cDNA Synthesis kit (Thermo Fisher Scientific, Inc.). qRT-PCR was performed on an Applied Biosystems ViiA™ 7 Dx (Thermo Fisher Scientific, Inc.) in a total volume of 10 μL using the SYBR-Green PCR mix (Thermo Fisher Scientific, Inc.). Primers designed using the Primer Express 3.0 software are presented in Table 1. The PCR reaction conditions were as follows: denaturation at 95°C for 5 s, followed by 40 cycles at 95°C for 10 s and 60°C for 30 s. All amplification reactions were carried out

Table 1. Quantitative real-time PCR primers

Gene	Primer sequence	
NONMMUT103346.1	forward	5'-CATGGGAGGAAGGATCAGAA-3'
	reverse	5'-AAGGCAAAATGTGGAAATGC-3'
NONMMUT035300.2	forward	5'-ATGGCACCTTTAAGCATTGG-3'
	reverse	5'-CTCAGAAGGTGGCAGGTAGC-3'
NONMMUT059145.2	forward	5'-GGACATCTGGGTCTCTTCCA-3'
	reverse	5'-GTGGGATTGCAAGCTGGTAT-3'
NONMMUT022589.2	forward	5'-CTCTGCCACCATGGCTATTT-3'
	reverse	5'-GATCTCTCCAAATGGGCAAA-3'
NONMMUT096389.1	forward	5'-GTGGACAAGTGCTGTGGATG-3'
	reverse	5'-TTTTGAGCGGACACACAATC-3'
NONMMUT014879.2	forward	5'-TGTTTCCCTTGCCCCACAT-3'
	reverse	5'-ATGGCTAAGATCAAAAACCCAAGT-3'
NONHSAT183385.1	forward	5'-ACGACCCCTAACAGACGTAAGG-3'
	reverse	5'-CTAAACGCTTGTCTGCCAAT-3'
ENSMUST00000120275	forward	5'-AGAGATTCAACTGGTCTGTGTGAGA-3'
	reverse	5'-TCTTCTACAACCTTTGGTGCCTCTAT-3'
Hspe1	forward	5'-GCTGCCGAACTGTAACCAA-3'
	reverse	5'-CCAACCTTTCACACTGACAGGC-3'
Beta-actin	forward	5'-CTGGAACGGTGAAGGTGACA-3'
	reverse	5'-CGGCCACATTGTGAACCTTTG-3'

in triplicate, and the specificity of the amplified fragments was checked according to the generated melting curve. Target gene expression was normalized to the expression of β -actin and all real-time data were analyzed using the comparative CT method ($2^{-\Delta\Delta CT}$).

Statistical Analysis

Data analysis was performed using the SPSS software and results were shown as mean $\pm$ standard deviation. Statistical differences between groups were determined using the Student *t* test. Analysis of variance was used to determine significant differences among groups. *p* values <0.05 were considered statistically significant.

Results

Establishment of the Ischemic Injury Model

To confirm the successful establishment of a renal IR injury model in mice, we determined serum creatinine and blood urea nitrogen concentrations. As expected, levels of serum creatinine ($158.9 \pm 17.7 \mu\text{mol/L}$) were significantly increased in mice subjected to ischemia for 45 min followed by reperfusion for 24 h compared to sham-operated mice ($43.73 \pm 5.38 \mu\text{mol/L}$). Blood urea nitrogen levels were also observed to be significantly higher in IR injury mice than in sham-operated animals ($57.1 \pm 2.91 \text{ mmol/L}$ vs. $9.7 \pm 0.986 \text{ mmol/L}$) (Fig. 1A, B). Consistent with renal insufficiency, hematoxylin and eosin staining of kidney tissue revealed IR-induced tubular dilatation, epithelial cell disruption and desquamation, brush border loss, and tubular lumen obstruction and congestion (Fig. 1C).

Fig. 1. Functional and histological changes in the kidney after IR injury. **A, B** Blood urea nitrogen (BUN, **A**) and serum creatinine (SCR, **B**) levels of IR 24 h and sham mice ($n = 3$). **C** Renal tissue sections were stained with hematoxylin and eosin. Arrows indicate injured tubules. * $p < 0.05$ versus sham control. Scale bar, 100 μm . IR, ischemia-reperfusion.

Differential Gene Expression Characters

A total of 45,923 lncRNAs were detected in the samples by transcriptome sequencing, and 5,868 distinctly differentially expressed lncRNAs ($p < 0.05$ and fold change ≥ 2) were found in IR 24 h kidney tissues compared to the expression in control groups, among which 2,908 lncRNAs were observed to be upregulated and 2,960 downregulated (Fig. 2A). To assess the possible function of lncRNA, we performed protein-coding gene sequencing in the same samples and predicted the potential targets of lncRNAs in cis or trans-regulatory relationships. The differentially expressed lncRNAs were predicted to target 5,101 mRNAs, of which 3,052 differentially expressed lncRNAs were predicted to target 1,471 (621 up- and 850 downregulated genes) differentially expressed mRNAs. According to their genomic location, these lncRNAs targeted differential expression of protein-coding genes portioned into 155 bidirectional lncRNAs, 276 exonic antisense lncRNAs, 435 exonic sense lncRNAs, 70 intronic antisense lncRNAs, 835 intronic sense lncRNAs and 1,281 intergenic lncRNAs (Fig. 2C). Renal IR conditions led to the differential expression of 5,160 protein-coding genes (2,384 up- and 2,776 downregulated mRNAs) (Fig. 2B). The above data indicate that approx-

Fig. 2. Features of lncRNA expression. Heatmaps displayed different expression levels of lncRNAs (**A**) and mRNAs (**B**) between the control and the IR group. Each column represents a sample and each row represents a lncRNA or mRNA transcript. Red stripes imply high levels of gene expression, while green stripes imply the opposite. **C** Distribution of lncRNA subtypes. **D** Distribution of differentially expressed lncRNA-target genes/ differentially expressed protein-coding genes. Orange indicates upregulation of mRNAs after IR 24 h and gray indicates the opposite. IR, ischemia-reperfusion; lncRNA, long noncoding RNA.

imately 26% of upregulated mRNA and 30.6% of downregulated mRNA in mouse renal tissue can be regulated by lncRNA after 24 h of IR (Fig. 2D).

qRT-PCR Validation

To confirm the reliability of the RNA sequencing data, we randomly selected seven differentially expressed lncRNAs for validation by qRT-PCR (upexpressed: NONMMUT103346.1, NONMMUT035300.2, NONMMUT059145.2; downexpressed: NONMMUT022589.2, NONMMUT014879.2, NONMMUT096389.1, ENSMUST00000120275). The expression patterns of these lncRNAs validated with qRT-PCR were mostly consistent with lncRNA-Seq results (Fig. 3).

Fig. 3. Validation of randomly selected differentially expressed lncRNAs for RNA-Seq by quantitative real-time PCR. lncRNAs, long noncoding RNAs.

Fig. 4. Functional annotation of lncRNAs. **A** lncRNA GO BP enrichment (top 20). **B** Sequence conserved lncRNA GO BP enrichment (top 20). **C** lncRNA KEGG pathway enrichment (top 20). **D** Sequence conserved lncRNA KEGG pathway enrichment (top 20). The horizontal axis represents the p value ($-\log_{10}$). BP, biological process; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes; lncRNA, long noncoding RNA.

Table 2. lncRNA sequence alignment results

Mouse	m-start	m-end	Human	h-start	h-end	Pident	E value
MSTRG.28171.1	286	575	NONHSAT093240.2	1,893	1,594	88.41	2E-94
MSTRG.28171.1	290	575	NONHSAT196556.1	1,890	1,595	88.59	8E-94
NONMMUT041012.2	1,621	1,860	NONHSAT079881.2	2,472	2,724	81.1	2E-46
NONMMUT032806.2	888	1,526	NONHSAT107017.2	106	744	87.66	0
NONMMUT032806.2	944	1,017	NONHSAT107018.2	77	4	91.89	9E-21
NONMMUT037264.2	595	777	NONHSAT185675.1	475	293	88.52	1E-56
NONMMUT022589.2	588	1,545	NONHSAT098468.2	6,206	7,168	72.63	2E-74
ENSMUST00000131345	1,868	2,027	NONHSAT149522.1	1	160	87.5	9E-45
NONMMUT014994.2	1	435	NONHSAT037726.2	145	579	83.6	2E-111
NONMMUT014879.2	1,734	2,141	NONHSAT183385.1	718	309	82.81	2E-98
NONMMUT014879.2	1,844	2,145	ENST00000554777	296	1	83.77	5E-74
NONMMUT014879.2	1,844	2,145	ENST00000559254	299	1	83.11	3E-71
NONMMUT014879.2	1,834	2,136	ENST00000550617	308	10	82.57	2E-68
NONMMUT014879.2	1,842	2,102	ENST00000604681	316	59	84.29	4E-65
NONMMUT014879.2	1,846	2,091	ENST00000406997	276	34	83.74	2E-58
NONMMUT014879.2	1,844	2,140	NONHSAT008913.2	287	6	80.47	2E-53
NONMMUT014879.2	1,844	2,139	ENST00000479457	281	1	80.41	7E-53
NONMMUT014879.2	1,843	2,102	ENST00000538133	284	27	78.63	4E-40
NONMMUT014879.2	1,844	1,953	ENST00000426640	339	233	86.36	2E-24
NONMMUT028972.2	2,472	2,596	NONHSAT163238.1	128	5	82.54	1E-21
NONMMUT063900.2	131	750	NONHSAT016736.2	654	36	77.22	4E-85
NONMMUT063900.2	874	1,070	NONHSAT156247.1	65	260	83.08	8E-42
ENSMUST00000180638	1,047	1,133	NONHSAT007235.2	4,462	4,372	91.21	5E-26
NONMMUT015136.2	1,959	2,284	NONHSAT217397.1	628	299	85.15	6E-90
NONMMUT015136.2	1,975	2,306	ENST00000482744	335	1	82.79	1E-77
NONMMUT015136.2	1,979	2,306	ENST00000429309	323	1	82.83	6E-75
NONMMUT015136.2	1,979	2,244	ENST00000457129	304	35	84.13	1E-66
NONMMUT015136.2	2,011	2,140	ENST00000501016	920	1,052	87.97	2E-35
ENSMUST00000120275	17	281	NONHSAT183385.1	340	611	86.45	9E-78
ENSMUST00000120275	24	280	ENST00000604681	59	321	84.09	5E-65
ENSMUST00000120275	24	273	ENST00000559254	44	299	84.44	2E-64
ENSMUST00000120275	17	275	ENST00000339579	34	298	82.4	9E-58
ENSMUST00000120275	25	273	ENST00000479457	39	281	81.64	7E-49
ENSMUST00000120275	25	273	NONHSAT008913.2	45	287	81.64	7E-49
ENSMUST00000120275	24	280	ENST00000421494	54	315	79.92	4E-46
ENSMUST00000120275	17	281	ENST00000605812	36	301	79.42	9E-43
ENSMUST00000180427	1,881	2,248	NONHSAT165747.1	568	940	86.9	2E-113
NONMMUT059145.2	496	798	NONHSAT184603.1	305	1	84.74	3E-80
NONMMUT059145.2	496	664	NONHSAT070548.2	359	191	83.72	6E-37
NONMMUT059145.2	225	432	NONHSAT204518.1	1,649	1,442	76.92	3E-25
ENSMUST00000189634	31	289	NONHSAT152317.1	2	247	79.23	4E-40
ENSMUST00000189634	31	289	NONHSAT152316.1	9	253	78.85	2E-38
ENSMUST00000189634	46	289	NONHSAT006509.2	7	240	79.27	6E-38
ENSMUST00000189634	46	289	NONHSAT152318.1	97	327	78.78	4E-35
NONMMUT033609.2	2,945	3,877	NONHSAT022124.2	5,990	6,966	81.97	0
NONMMUT033609.2	6,024	6,142	NONHSAT022124.2	9,647	9,774	87.5	8E-31
NONMMUT033609.2	4,480	5,861	NONHSAT022135.2	4,433	5,824	80.19	0
NONMMUT033609.2	6,024	6,142	NONHSAT022135.2	6,016	6,143	87.5	8E-31
NONMMUT033609.2	2,945	3,877	NONHSAT022145.2	3,198	4,174	81.97	0
NONMMUT033609.2	6,024	6,142	NONHSAT022145.2	6,736	6,863	87.5	8E-31
NONMMUT033609.2	4,480	5,861	NONHSAT022136.2	3,972	5,363	80.19	0
NONMMUT033609.2	6,024	6,142	NONHSAT022136.2	5,555	5,682	87.5	8E-31
NONMMUT033609.2	2,945	3,877	ENST00000534336	4,495	5,471	81.97	0
NONMMUT033609.2	6,024	6,142	ENST00000534336	8,152	8,279	87.5	8E-31
NONMMUT033609.2	3,310	3,877	NONHSAT022132.2	3,450	4,042	82.39	9E-135
NONMMUT033609.2	2,038	2,774	NONHSAT022125.2	2,483	3,272	80.22	4E-148
NONMMUT033609.2	2,945	3,877	NONHSAT022137.2	3,298	4,274	81.97	0
NONMMUT033609.2	6,024	6,142	NONHSAT022137.2	6,622	6,749	87.5	8E-31

Table 2 (continued)

Mouse	m-start	m-end	Human	h-start	h-end	Pident	E value
NONMMUT033609.2	4,480	5,861	NONHSAT022138.2	4,565	5,956	80.19	0
NONMMUT033609.2	6,024	6,142	NONHSAT022138.2	6,148	6,275	87.5	8E-31
NONMMUT033609.2	2,038	2,557	NONHSAT022143.2	2,173	2,722	80.82	2E-106
NONMMUT033609.2	3,310	3,877	NONHSAT022144.2	3,339	3,931	82.39	9E-135
NONMMUT033609.2	3,310	3,765	NONHSAT022134.2	3,440	3,920	80.82	2E-92
NONMMUT033609.2	4,480	5,813	NONHSAT022142.2	3,902	5,238	80.42	0
NONMMUT033609.2	2,945	3,877	NONHSAT022147.2	1,367	3,91	81.97	0
NONMMUT033609.2	4,480	5,403	NONHSAT022129.2	4,684	5,599	82.27	0
NONMMUT033609.2	4,480	5,403	NONHSAT022131.2	3,267	4,182	82.27	0
NONMMUT033609.2	4,480	5,403	NONHSAT022126.2	4,767	5,682	82.27	0
NONMMUT033609.2	6,024	6,142	NONHSAT022126.2	6,125	6,252	87.5	8E-31
NONMMUT033609.2	4,480	5,196	NONHSAT022139.2	4,792	5,483	83.13	1E-173
NONMMUT033609.2	2,038	2,774	NONHSAT022139.2	2,216	3,005	80.22	4E-148
NONMMUT033609.2	6,024	6,142	NONHSAT022133.2	6,380	6,507	87.5	8E-31
NONMMUT033609.2	2,945	3,314	NONHSAT022146.2	390	2	81.31	2E-76
NONMMUT033609.2	2,038	2,774	NONHSAT022130.2	1,410	621	80.22	4E-148
NONMMUT033609.2	2,945	3,300	NONHSAT022130.2	384	11	81.63	3E-75
NONMMUT033609.2	3,310	3,877	ENST00000508832	550	1,142	82.39	9E-135
NONMMUT033609.2	4,480	4,981	ENST00000618132	207	701	84.14	3E-129
NONMMUT033609.2	4,670	5,196	ENST00000610851	1	503	82.31	6E-117
NONMMUT033609.2	3,305	3,765	NONHSAT159249.1	192	677	80.81	1E-93
NONMMUT033609.2	5,012	5,403	ENST00000618227	11	408	81.84	1E-83
NONMMUT033609.2	2,945	3,307	NONHSAT022110.2	1,050	669	81.49	8E-76
NONMMUT033609.2	3,402	3,765	ENST00000618925	1	392	79.09	8E-61
NONMMUT033609.2	3,013	3,300	NONHSAT022148.2	302	5	81.58	1E-58
NONMMUT033609.2	6,024	6,109	NONHSAT159253.1	744	829	95.35	3E-30
NONMMUT015894.2	1	88	ENST00000554735	76	162	92.05	2E-26
NONMMUT015894.2	1	88	ENST00000557109	68	154	92.05	2E-26
NONMMUT015894.2	1	89	ENST00000557661	96	184	91.11	6E-26
NONMMUT015894.2	1	89	ENST00000554441	40	128	91.11	6E-26
NONMMUT015894.2	1	89	ENST00000553729	57	145	91.11	6E-26
NONMMUT015894.2	1	87	ENST00000554694	68	153	91.95	6E-26
NONMMUT015894.2	1	87	NONHSAT040031.2	68	153	91.95	6E-26
NONMMUT015894.2	1	89	ENST00000617327	13	101	91.11	6E-26
NONMMUT015894.2	1	87	ENST00000555174	87	172	91.95	6E-26
NONMMUT015894.2	1	87	ENST00000557532	68	153	91.95	6E-26
NONMMUT012993.2	662	913	NONHSAT056324.2	300	547	81.96	1E-51
NONMMUT013845.2	618	765	NONHSAT212507.1	159	12	93.92	4E-57
NONMMUT013845.2	620	765	NONHSAT212506.1	887	742	93.84	5E-56
NONMMUT005589.2	1,799	2,136	NONHSAT032437.2	2,424	2,769	81.3	2E-69
NONMMUT001329.2	37	857	NONHSAT186261.1	510	390	96.69	3E-50
NONMMUT096389.1	52	745	NONHSAT163707.1	708	8	85.51	0
ENSMUST00000185789	44,632	45,362	ENST00000597346	47,341	48,075	91.72	0
ENSMUST00000185789	32,357	32,747	ENST00000597346	34,980	35,369	86.29	1E-114
NONMMUT022058.2	1,327	1,500	NONHSAT166937.1	787	960	90.23	9E-58
NONMMUT024839.2	311	938	NONHSAT163497.1	244	875	87.89	0
ENSMUST00000056590	31	288	NONHSAT152317.1	2	247	79.54	8E-42
ENSMUST00000056590	46	288	NONHSAT006509.2	7	240	79.59	5E-39
ENSMUST00000056590	46	288	NONHSAT152318.1	97	327	79.1	8E-37
NONMMUT063462.2	714	1,584	NONHSAT141042.2	1,393	2,297	85.07	0
NONMMUT063462.2	1,010	1,218	ENST00000568354	383	176	87.2	3E-60
MSTRG.8913.4	17,954	18,141	NONHSAT103797.2	187	1	85.42	7E-46
MSTRG.8913.4	17,998	18,193	ENST00000512933	201	1	82.18	1E-38
NONMMUT065808.2	1,759	2,876	NONHSAT199361.1	55	1,175	87.13	0
NONMMUT035300.2	2,886	3,164	NONHSAT016573.2	1,894	2,178	87.76	1E-87
NONMMUT011815.2	153	255	NONHSAT053356.2	2,324	2,214	90.99	3E-32
NONMMUT016470.2	1,920	2,535	NONHSAT120258.2	1,022	1,676	84.93	2E-177
NONMMUT019025.2	119	340	ENST00000603072	1	218	81.17	1E-42
NONMMUT016807.2	1,508	2,484	NONHSAT108043.2	12	989	91.57	0

Table 2 (continued)

Mouse	m-start	m-end	Human	h-start	h-end	Pident	E value
NONMMUT016807.2	2,028	2,461	NONHSAT208863.1	466	56	91.03	5E-159
NONMMUT016807.2	1,715	1,946	NONHSAT208863.1	699	468	96.98	8E-107
NONMMUT103346.1	641	1,118	NONHSAT203300.1	2,550	3,028	82.45	5E-112
NONMMUT110083.1	293	584	NONHSAT189708.1	2	284	85.08	5E-75
NONMMUT004318.2	2,478	2,795	NONHSAT150492.1	1	327	93.31	9E-132
NONMMUT014997.2	314	757	NONHSAT037726.2	136	579	83.71	9E-115
NONMMUT018006.2	1,821	2,040	ENST00000619044	1,010	1,229	77.93	2E-29
NONMMUT018006.2	1,821	2,040	ENST00000427548	1,373	1,592	77.93	2E-29
ENSMUST00000180643	3,197	3,566	NONHSAT169452.1	289	660	83.2	2E-90
ENSMUST00000180643	1,738	1,855	NONHSAT169452.1	170	287	89.83	8E-35
ENSMUST00000145410	1,202	1,406	NONHSAT196345.1	372	576	96.1	7E-90
NONMMUT125899.1	1	571	ENST00000537019	179	788	83.71	2E-151
NONMMUT040114.2	94	582	NONHSAT189325.1	14	509	81.2	1E-106
ENSMUST00000185516	3,416	3,531	NONHSAT186099.1	1	119	89.08	1E-32
NONMMUT009081.2	1,059	1,307	NONHSAT184647.1	294	543	82.21	5E-53
ENSMUST00000176760	506	858	NONHSAT163337.1	1,792	2,144	86.69	2E-107
ENSMUST00000176760	487	786	NONHSAT108187.2	495	197	80.07	1E-55
ENSMUST00000176760	487	786	NONHSAT108186.2	495	197	80.07	1E-55
NONMMUT046485.2	2,521	3,080	NONHSAT114055.2	6,867	7,442	78.25	2E-93
NONMMUT023603.2	369	450	NONHSAT217573.1	602	682	98.78	8E-33
NONMMUT023603.2	374	447	NONHSAT128599.2	71	144	100	1E-30
NONMMUT015630.2	4,282	4,529	NONHSAT168911.1	316	70	96.37	2E-111
NONMMUT069701.2	1,824	2,079	ENST00000558888	1,724	1,470	85.16	9E-68
NONMMUT069701.2	1,824	2,079	NONHSAT046577.2	1,724	1,470	85.16	9E-68
NONMMUT021187.2	1	253	ENST00000425586	132	405	82.85	2E-57
NONMMUT021187.2	49	253	ENST00000621282	1	226	79.2	9E-31
NONMMUT021187.2	49	253	NONHSAT033777.2	1	226	79.2	9E-31
ENSMUST00000128176	422	622	NONHSAT176094.1	60	270	89.57	3E-67
ENSMUST00000128176	1,798	1,914	ENST00000625127	1,425	1,541	95.73	4E-46
ENSMUST00000128176	1,798	1,914	NONHSAT052946.2	933	1,049	95.73	4E-46
ENSMUST00000191701	313	551	ENST00000515522	189	421	80.91	5E-44
ENSMUST00000172812	2,956	3,888	NONHSAT022124.2	5,990	6,966	81.97	0
ENSMUST00000172812	2,956	3,888	ENST00000534336	4,495	5,471	81.97	0
ENSMUST00000172812	3,321	3,888	NONHSAT022132.2	3,450	4,042	82.39	1E-134
ENSMUST00000172812	6,499	6,759	NONHSAT022132.2	6,723	6,995	90.11	2E-92
ENSMUST00000172812	2,956	3,888	NONHSAT022125.2	3,509	4,485	81.97	0
ENSMUST00000172812	6,499	6,759	NONHSAT022125.2	7,166	7,438	90.11	2E-92
ENSMUST00000172812	2,049	2,785	NONHSAT022129.2	2,307	3,096	80.22	5E-148
ENSMUST00000172812	4,139	5,878	NONHSAT022131.2	2,425	4,182	79.64	0
ENSMUST00000172812	2,956	3,888	NONHSAT022137.2	3,298	4,274	81.97	0
ENSMUST00000172812	6,499	6,759	NONHSAT022137.2	6,622	6,894	90.11	2E-92
ENSMUST00000172812	4,955	6,336	NONHSAT022135.2	4,433	5,824	80.19	0
ENSMUST00000172812	6,499	6,759	NONHSAT022135.2	6,016	6,288	90.11	2E-92
ENSMUST00000172812	2,956	3,888	NONHSAT022145.2	3,198	4,174	81.97	0
ENSMUST00000172812	6,499	6,759	NONHSAT022145.2	6,736	7,008	90.11	2E-92
ENSMUST00000172812	4,955	6,336	NONHSAT022136.2	3,972	5,363	80.19	0
ENSMUST00000172812	6,499	6,759	NONHSAT022136.2	5,555	5,827	90.11	2E-92
ENSMUST00000172812	4,955	6,336	NONHSAT022138.2	4,565	5,956	80.19	0
ENSMUST00000172812	6,499	6,626	NONHSAT022138.2	6,148	6,284	88.32	9E-36
ENSMUST00000172812	4,955	6,288	NONHSAT022143.2	3,886	5,222	80.42	0
ENSMUST00000172812	4,955	6,288	NONHSAT022144.2	4,910	6,246	80.42	0
ENSMUST00000172812	4,955	6,288	NONHSAT022134.2	4,403	5,739	80.42	0
ENSMUST00000172812	4,377	4,696	NONHSAT022134.2	3,918	4,235	82.53	1E-69
ENSMUST00000172812	4,955	6,288	NONHSAT022142.2	3,902	5,238	80.42	0
ENSMUST00000172812	2,049	2,769	NONHSAT022142.2	2,173	2,946	79.82	4E-139
ENSMUST00000172812	2,956	3,888	NONHSAT022147.2	1,367	391	81.97	0
ENSMUST00000172812	4,955	5,878	NONHSAT022126.2	4,767	5,682	82.27	0
ENSMUST00000172812	6,499	6,759	NONHSAT022126.2	6,125	6,397	90.11	2E-92

Table 2 (continued)

Mouse	m-start	m-end	Human	h-start	h-end	Pident	E value
ENSMUST00000172812	4,955	5,671	NONHSAT022139.2	4,792	5,483	83.13	1E-173
ENSMUST00000172812	3,321	3,888	NONHSAT022133.2	3,450	4,042	82.39	1E-134
ENSMUST00000172812	6,499	6,759	NONHSAT022133.2	6,380	6,652	90.11	2E-92
ENSMUST00000172812	2,956	3,325	NONHSAT022146.2	390	2	81.31	2E-76
ENSMUST00000172812	2,049	2,785	NONHSAT022130.2	1,410	621	80.22	5E-148
ENSMUST00000172812	2,956	3,311	NONHSAT022130.2	384	11	81.63	3E-75
ENSMUST00000172812	3,321	3,888	ENST00000508832	550	1,142	82.39	1E-134
ENSMUST00000172812	4,955	5,456	ENST00000618132	207	701	84.14	4E-129
ENSMUST00000172812	5,145	5,671	ENST00000610851	1	503	82.31	6E-117
ENSMUST00000172812	4,139	4,574	NONHSAT159250.1	129	572	81.58	1E-93
ENSMUST00000172812	3,316	3,776	NONHSAT159249.1	192	677	80.81	1E-93
ENSMUST00000172812	4,377	4,574	NONHSAT159249.1	675	880	85.51	2E-51
ENSMUST00000172812	5,487	5,878	ENST00000618227	11	408	81.84	1E-83
ENSMUST00000172812	2,956	3,318	NONHSAT022110.2	1,050	669	81.49	9E-76
ENSMUST00000172812	3,413	3,776	ENST00000618925	1	392	79.09	9E-61
ENSMUST00000172812	3,024	3,311	NONHSAT022148.2	302	5	81.58	1E-58
ENSMUST00000172812	6,499	6,584	NONHSAT159253.1	744	829	95.35	3E-30
NONMMUT003914.2	1	117	NONHSAT163674.1	11	127	91.45	4E-38

lncRNA, long noncoding RNA.

Functional Enrichment Analyses of Differentially Expressed lncRNAs

To understand the potential role of lncRNAs in the development of renal IR injury, we performed Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analyses for differentially expressed genes. A total of 1,471 differentially expressed genes with functional annotation information for differentially expressed lncRNAs were assigned to 3,023 GO terms; 2,347 of the GO terms significantly enriched for biological process, which are primarily associated with positive regulation of tumor necrosis factor-mediated signaling pathway, cell migration by VEGF signaling pathway, regulation of NLRP3 inflammasome complex assembly, TRAIL-activated apoptotic signaling pathway, and metabolic including ubiquitin, insulin, phospholipid, DNA, quinolate, etc. (Fig. 4A). Furthermore, KEGG pathway analysis revealed that the differentially expressed genes were found to be mainly enriched in metabolism, SNARE interactions in vesicular transport, peroxisome, microRNAs in cancer, Notch signaling pathway, and sphingolipid signaling pathway (Fig. 4C).

Analysis of Conserved lncRNA

For searching out the putative human orthologs of differentially expressed mouse lncRNAs in renal IR, 1,135 candidate lncRNAs in mice were compared to lncRNAs in humans through sequence identity. Fifty-six mouse lncRNA transcripts were found to have potential human orthologs (Table 2). While compared to all differentially expressed lncRNAs, the GO biological process terms for conserved lncRNAs tend to be more enriched in the regulation of growth, cell cycle, cytoskeleton organization, platelet aggregation, mitochondrial membrane potential, TNF α -mediated signaling pathway, intrinsic apoptotic signaling pathway, protein stabilization and transport, and Wnt signaling pathway (Fig. 4B). Accordingly, KEGG pathway analyses showed the most significant enrichment of conserved lncRNAs in several novel roles including the VEGF signaling pathway, Gap junction, ABC transporters, the B cell receptor signaling pathway, and the chemokine signaling pathway (Fig. 4D). These results suggest that conserved lncRNAs are closely

Fig. 5. Conserved gene expression levels after IR 24 h and hypoxia. **A** The expression of NONMMUT014879.2 and its target Hspe1, as examined by quantitative real-time PCR, was markedly decreased after IR 24 h, which was consistent with the results of RNA-Seq. **B** Protein levels of hypoxia-inducible factor 1α (Hif1a) were significantly upregulated in hypoxic HK2 cells. **C** NONHSAT183385.1 was highly expressed in HK2 cells under hypoxia for 24 h and further increased after reoxygenation for 3 and 6 h. After 24 h of reoxygenation, the expression of NONHSAT183385.1 was dramatically downregulated. * $p < 0.05$ versus control, ** $p < 0.001$ versus control. IR, ischemia-reperfusion.

correlated with the regulation of inflammation, vascular biology, apoptosis, and cell cycle during renal IR injury.

To survey whether these corresponding human lncRNAs with sequence homology have functional homology, we preliminarily observed the expression changes of human lncRNA NONHSAT183385.1 whose mouse counterpart is NONMMUT014879.2 under hypoxia and reoxygenation conditions in vitro. As shown in Figure 5C, the expression of NONHSAT183385.1 was markedly induced in HK2 cells after 24 h of hypoxia and peaked at 6 h following restoring normoxic culture. At 24 h after recovery from normoxia, the expression of NONHSAT183385.1 exhibited a sharp decline. The results suggest that NONHSAT183385.1 may exert an essential action in human renal IR injury. The validation of the expression change of NONMMUT014879.2 and its potential target Hspe1 in renal IR tissues are shown in Figure 5A; the fold changes of NONMMUT014879.2 and Hspe1 were both significantly decreased, which was roughly consistent with the lncRNA-Seq results.

Discussion

Renal IR injury is a common clinical disorder characterized by significant additional damage to ischemic tissue after the restoration of blood supply. During reperfusion, cells are exposed to aggravated reactive oxygen, intracellular calcium overload, and inflammatory factor stimulation. Regulation of gene expression is a critical event for cells in response to harmful stress under these pathological conditions. Accumulating evidence has suggested that lncRNAs regulate these pathological processes in renal injury. The lncRNAs MALAT1, TapSAKI, and PVT1 were the most reported lncRNAs involved in the pathogenesis of acute renal injury in humans by modulating inflammation, cell death, and survival [22–24]. However, due to the lack of extensive research, the role of lncRNAs in renal IR injury is still largely unknown.

In the present study, we established a lncRNA expression profile of renal IR injury in mice. It was found that 5,101 mRNAs, including 26% of the upregulated mRNAs and 30.6% of the downregulated mRNAs, were the target gene of the differentially expressed lncRNAs by prediction of cis and trans functions. Among a multitude of different signaling pathways induced by renal ischemia injury in mice, these target gene products of differentially expressed lncRNAs tend to primarily function in the regulation of the Notch and sphingolipid signaling pathways. The Notch signaling pathway is a highly conserved intercellular signaling cascade system and crucial for embryogenesis, cellular differentiation, proliferation, and tissue homeostasis in mammals [25]. Previous studies have shown that the Notch signaling pathway is significantly involved in renal IR injury. Gupta et al. [26] found that pretreatment with the Notch ligand DLL4 enhanced recovery from acute renal failure induced by IR in rats. Chen et al.'s study [27] showed that Notch-2 signaling upregulated the expression of survivin in renal proximal tubule epithelial cells, aiding in the functional and structural recovery of the kidney from acute renal injury. Kobayashi et al.'s study [28] suggested that the Delta-1/Notch-2/Hes-1 signaling pathway may regulate the regeneration and proliferation of renal tubules during acute renal injury. The sphingolipid signaling pathway plays a critical role in IR-induced cellular damage [29, 30]. The above indicate that lncRNAs may play key regulatory roles in cell survival, apoptosis, and tissue repair in the development of renal IR injury.

Although mice share a high genetic similarity with humans and lots of known human protein-coding genes have functional homologs in mice, most lncRNA transcripts tend to exhibit species specificity and low DNA sequence conservation [31, 32]. Only about 20% of lncRNA homologous genes were discovered in humans and mice [33], which limits the full understanding of human lncRNAs involved in IR injury by the use of animal models. For all that, it is still of great significance for the functional study of lncRNAs to search for relatively conservative lncRNAs between humans and mice. Conservative lncRNAs often suggest that they may have important biological functions in IR injury. In this work, 56 lncRNAs in the mouse had putative orthologs corresponding to 129 lncRNA transcripts of humans through sequence conservation analysis. It was observed that the conserved lncRNAs in mice were mainly involved in the VEGF signaling pathway, the B cell receptor signaling pathway, and the chemokine signaling pathway, which showed that conserved lncRNAs may play major roles in adjusting kidney microvasculature responses to stress stimuli from ischemia or hypoxia and in regulating the inflammatory cascade after renal IR.

Further, to investigate lncRNA involved in human renal IR injury, we tentatively selected the human lncRNA NONHSAT183385.1, whose mouse counterpart NONMMUT014879.2 had abundant expression based on FPKM expression level in our sequencing results, to perform hypoxic stimulation in vitro for mimicking ischemia in vivo. Since the main pathological feature of renal IR injury is associated with the damage of tubular epithelial cells, which is one of the major functional cells in the kidney, we surveyed the expression changes of the candidate

human lncRNA in HK2 cells undergoing hypoxia for up to 24 h followed by restoration of normoxic culture for 3, 6, and 24 h. We found that NONHSAT183385.1 was significantly induced by hypoxia in tubular epithelial cells and dramatically reduced after 24 h of reoxygenation, which suggested that lncRNA NONHSAT183385.1 may be involved in renal IR injury. According to the trans analysis of our sequencing results, the predicted gene of mouse homologous lncRNA-NONMMUT014879.2 corresponded to NONHSAT183385.1 is Hspe1. Based on our sequence data, Hspe1 expression was markedly decreased after 24 h of IR as with NONMMUT014879.2. Hspe1 (also known as Hsp10), an important member of the heat shock protein family, is a 10-kDa, highly conserved molecular chaperone across species and classically works together with 60-kDa heat shock protein (Hsp60) in folding or driving the degradation of mitochondrial proteins and maintenance of homeostasis in response to adverse stressors [34]. Renal IR injury induces mitochondrial protein misfolding, and disruption of mitochondrial function is the underlying cause of apoptosis in renal tubular epithelial cells. The expression level of Hspe1 is associated with increasing severity of renal IR damage. Whether NONHSAT183385.1 is involved in the progress of human renal IR injury through interaction with the heat shock protein pathway is unclear, and the specific function related to renal IR needs further analysis.

Conclusion

In summary, the study established a lncRNA expression profile of renal IR injury using a mouse model and conducted a conservative analysis of differentially expressed lncRNAs to hunt their putative human orthologs. Future studies are needed to dissect the role of these homologous human lncRNAs in renal cells. Our work will contribute to reveal important lncRNAs involved in human renal IR injury.

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Statement of Ethics

All animal procedures were conducted after approval by the Animal Committee of Nanchang University (License No. SYXK [Jiangxi] 2015-0001). All experiments were implemented in compliance with the principles of the Institutional Animal Care and Use Committee of Nanchang University.

Conflict of Interest Statement

The authors have no conflicts of interest to declare.

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Author Contributions

F. Liu, Y. Yang, T. Liu, J. Deng, H. Zhang, and D. Luo performed the experiments. Y.-L. Lou, F. Liu, and Y. Yang analyzed the data. Y.-L. Lou, F. Liu, and Y. Yang wrote the paper. Y.-L. Lou, F. Liu, and Y. Yang reviewed and edited the manuscript.

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