lncRNA-Associated ceRNA Networks in Spleen of Nocth1-correlated T-ALL Leukemia Mice

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Research article

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Abstract

Background Acute T-lymphocytic leukemia (T-ALL) is a highly aggressive malignant tumor in leukemia. Nocth1 is considered as an major oncogene in the development of T-ALL. Increasing evidences have revealed that the occurrence and progression of T-ALL referred to abnormal gene expression, pathway activation and the regulation between these genes. However, the potential lncRNA-associated competing endogenous RNA (ceRNA) network involved in spleen of Nocth1-correlated T-ALL leukemia mice remains unclear.

Methods Overexpression of Notch intracellular domain (ICN) of Notch1 by retroviral infection was used to set up mouse T-ALL model. Deep RNA-sequencing analysis was performed the expression of lncRNAs and mRNA in spleen of T-ALL mice and C57BL/6 mice.

Results The deep RNA-sequencing analysis shown that 1833 lncRNAs and 4626 mRNAs were deregulated according to the P-value ($p<0.05$) and fold change (>2-fold) in spleen of T-ALL leukemia mice compared with that of C57BL/6 mice. Gene Ontology(GO) and KEGG pathway analysis were performed to reveal the potential roles of differentiated expressed lncRNAs. Co-expression Network was performed to reveal the regulation relationship between the differentiated expressed lncRNAs and mRNAs. CeRNA prediction constructed the lncRNA-miRNA-mRNA model to find the core ceRNA based on regression model analysis and seed sequence matching methods.

Conclusion This study provided a systematic overview of the altered lncRNAs and mRNA expression, pathway and ceRNA regulation network in the pathogenesis of Nocth1-correlated T-ALL.

Background

T-lymphocytic leukemia (T-ALL) is caused by transformation of immature T-cell progenitors, leading to abnormal proliferation in bone marrow and peripheral blood[1]. T-ALL accounts for 20-25% of the total incidence of acute lymphoblastic leukemia in adults, for 10%-15% of childhood acute lymphoblastic leukemia[2]. The abnormal proliferation T cell can infiltration and damage to various of tissues and affect organ function. Despite the combined chemotherapy and allogeneic hematopoietic stem cell transplantation have applied to clinical treatment of T-ALL, its event-free survival rate is only 30%-50%[3]. Explore the mechanism and pathogenesis of T-ALL is of great significance in improving survival rate.

The formation of T-ALL is a multi-step process which includes the activation of oncogenes and inactivation of tumor suppressor genes[4]. Notch1 is a subtype of Notch receptor in Notch signaling pathway which participated in multiple pathological and physiological processes, including cancer[5]. Approximately 55% of T-ALL patients are related with acquired functional mutations in Notch1[6]. Block Notch signaling pathway causes cell cycle arrest and apoptosis in T-ALL cell lines[7]. Notch1 is a class I transmembrane protein which transduces information from extracellular signals into nucleus directly[8]. The Notch intracellular domain (ICN) of the Notch1 is its active component, which can activate expression of target genes in nucleus. Overexpressed of ICN1 by retroviral infection in hematopoietic progenitor cells or thymocytes promote T ALL tumorigenesis, which was made to set up mouse T-ALL model[9].

lncRNAs are a special class of non-coding RNAs (ncRNAs), participated with varies of physiological cellular processes as well as cancer pathological process[10]. Increasing evidences have revealed that lncRNAs play a critical role in many types of cancers, including hepatocellular carcinoma, renal cell carcinoma and leukemia[11]. Investigating lncRNAs specifcally transcriptional profiles in T-ALL is of great significance to understand the gobal

altered expression of lncRNAs. lncRNAs have diverse mechanism to regulate gene expression. Some studies show that lncRNAs act as competing endogenous RNAs (ceRNA) through a lncRNA-miRNA-mRNA model to regulate gene expression[12]. Based on the lncRNAs and mRNAs specifcally transcriptional profiles, construction the ceRNA network can enrich the raw data in studying the potential mechanism in the formation of T-ALL.

In the present study, we performed the deep RNA-sequence to analyze expression profile in spleen of T-ALL leukemia mice with the aim of explore the lncRNAs catalogue. We showed the differentially expressed lncRNAs and mRNAs. Gene Ontology(GO) and KEGG pathway analysis were performed to reveal the potential roles of these mRNAs. Co-expression Network was performed to reveal the regulation relationship between the differentially expressed lncRNAs and mRNAs. Meanwhile, we performed ceRNA prediction to construct the lncRNA-miRNA-mRNA model. Thus, this study provided a systematic overview of the altered RNAs expression, pathway and ceRNA network in the pathogenesis of Nocth1-correlated T-ALL.

Methods

Animals and tissues

We used T-ALL leukemia mice overexpressing the Notch l intracellular domain (NICD) as the research model. T-ALL leukemia cells presented by the Institute of Hematology, Peking Union Medical College, Chinese Academy of Medical Sciences. We cotransfected with retroviral plasmid MSCV-ICN1-IRES-GFP (ICN1-GFP), the reverse transcription packaging protein CMV-VSVG and and Kat into 293T. C57BL/6 mice were divided into two groups randomly, T-ALL mice and C57BL/6 mice, and each group has three mice. T-ALL mice group were treated as follows, collected the viral supernatant to infect C57BL/6 mouse bone marrow cells (Lin-Scal^{+}), and then transplanted into C57BL/6 mice (10^{6} per mouse)after semi-lethal dose irradiation through tail vein injection. C57BL/6 mice group were transplanted culture medium in the same way. Mice were numbered, divided into three cages randomly and reared routinely. C57BL/6 mice, 6~8 weeks female, were purchased from Charles River Experimental Animal Technology Co., Ltd. (Beijing, China). 2 weeks after, the mice were sacrificed by cervical dislocation and removed the spleen by laparotomy(Fig.1A). Mice quarantined in a 12 h light and 12 h dark photoperiod pathogen free environment, received water and food in clean class animal room of Hebei Medical University. All animals were housed and cared in accordance with the Declaration of Helsinki and the guidelines and regulations of the Institutional Animal Care and Use Committee of the Second Hospital of Hebei Medical University.

RNA-Seq

RNeasy mini kit (Qiagen, Germany) was used to isolate the total RNA. TruSeq™ RNA Sample Preparation Kit (Illumina, USA) was used to synthesize paired-end library according to TruSeq™ RNA Sample Preparation Guide. Qubit® 2.0 Fluorometer (Life Technologies, USA) was used to quantified the purified libraries and Agilent 2100 bioanalyzer (Agilent Technologies, USA) was used to confirm the insert size and calculate the mole concentration. Illumina NovaSeq 6000 (Illumina, USA) was used to sequenced the cluster (10 pM) generated by cBot. The library construction, sequencing and bioinformatics analysis were performed at Shanghai Sinomics Corporation.

RNA extraction.

The spleen was homogenized. Total RNA was isolated by E.Z.N.A.® Total RNA Kit I following the manufacturer's Guide. Total RNA were quantifed by Nanodrop 2000 (Nanodrop Technologies, Wilmington, USA) and Agarose gel electrophoresis. RNAs with a 260:280 ratio of ≥1.5 and 28S:18S ratio is approximately 2:1.

qRT-PCR

Differently expressed lncRNAs were selected for validation by qRT-PCR. Total RNA was reverse-transcribed using SureScript™ First-Strand cDNA Synthesis Kit (Genecopoeia, USA) according to the manufacturer's guide. qPCR reactions was performed using SYBR-Green (Invitrogen) according to the manufacturer's guide. BioRad iQ5 Real-Time thermocycler was used to perform qPCR reactions. The Cycling conditions was denaturing at 95°C, followed by 39 cycles of 95℃ (10 s) and 55℃ annealing (30 s). Specific primers of each lncRNA were listed in Table 2.

Statistical analysis

The differentially expression levels of lncRNAs and mRNAs in T-ALL mice or C57BL/6 mice spleen was analyzed by Bioconductor package (limma version 3.26.1) and R (version 3.2.2) software. Spearman correlation test was used to analyze co-expression relationships between the lncRNAs. CeRNA prediction was analyzed by Pearson's correlation coefficients. qRT-PCR data was shown as the means±S.E.M. Differences between two groups were analyzed by Student's *t test*. P<0.05 was considered as significantly.

Results

1. **Deep RNA-sequence lncRNAs and mRNA expression profiles in spleen of T-ALL mice.**

Before injection the mice were healthy and two weeks later, deep RNA-sequence was performed the expression of lncRNAs and mRNA in spleen of T-ALL mice (3 samples) and C57BL/6 mice (3 samples). Compare with control mice, 475 lncRNAs were upregulated and 1398 lncRNAs were downregulated according to the P-value ($p<0.05$) and fold change (>2-fold). Among of them, 89 lncRNAs and 386 lncRNAs were upregulated or downregulated significantly differentially expressed (>10-fold). The differentially expressed lncRNAs were converted into the volcano plot (Fig.1BB) and scatter plot(Fig.1C) to show the differentially expressed lncRNAs visibly. Compared with the spleen of C57BL/6 mice, noncancerous tissues mRNAs expression profiles, 2041 lncRNAs or 3585 lncRNAs were upregulated or downregulated differentially expressed ($p<0.05$, >2-fold). Among them, 252 lncRNAs or 999 lncRNAs were upregulated or downregulated significantly differentially expressed (>10-fold)(Fig.1DE).

2. **mRNA GO and KEGG pathway analysis**

In order to clarify the biological processes, cellular components and molecular functions of differentially expressed mRNAs, we performed GO terms enrichment and KEGG pathway analysis. The GO terms enrichment for differentially expressed mRNAs were related to melanocyte differentiation, myosin complex and translation repressor activity in biological processes(Fig.2A), cellular component(Fig.2B) or molecular function(Fig.2C), respectively. KEGG pathway analysis showed that showed that 303 pathways were significantly enriched among the transcripts(Fig.2D). Acute myeloid leukemia, protein export and glycosaminoglycan biosynthesis - keratan sulfate were the 3 significantly enriched pathways.

3. **Coding/non-coding co-expression analysis**

To predict the functions of lncRNAs, we constructed lncRNA-mRNA co-expression network(Fig.3).

4. **Construction of a ceRNA network.**

lncRNAs could function as ceRNA to competing the binding between miRNA and mRNA. Analysis of ceRNA network helped to understand the characterization of lncRNAs. As shown in Fig.4, 71 differentially expressed lncRNAs and

123 expressed mRNAs were selected, which predicted 11 miRNAs sharing binding sites with the differentially expressed lncRNAs and mRNAs.

5. **Validation of differentially lncRNAs**

Nice differentially expressed lncRNAs from ceRNA network prediction were selected to confirm RNA sequence results by qRT-PCR(Table 1). Ten pairs of mice spleen which contain both T-ALL leukemia mice or C57BL/6 mice were selected. 10 downregulated lncRNAs($p<0.05$; FC>5-fold) were selected. All these lnRNAs expression were downregulated and consistent with RNA sequence results($P<0.05$). Moreover, NONMMUT026003.2 was maximal changed lncRNAs in these lncRNAs.

Discussion

Notch1 is one of the major driving oncogene in T-ALL. About 55% of T-ALL patients occurred Notch1 mutation in the trans-membrane region and the intracellular PEST domain, which resulted abnormal activation of the Notch signaling pathway. Overexpression of ICN1 by retroviral infection in hematopoietic progenitor cells or thymocytes promote T ALL tumorigenesis, which was made to set up mouse T-ALL model. Over the past decades, the molecular mechanism of Notch1-correlated T-ALL has been extensively investigated. However, the precise pathogenesis of Notch1-correlated T-ALL is still unknown. Recent years, ncRNAs, including lncRNAs, have been found to be related with humorous number of biological regulatory functions. lncRNA NALT activating Notch signaling pathway promoted cell proliferation in T-ALL[13]. lncRNA-IUR acted as a tumor suppressors by suppressing the STAT5-CD71 pathway in Bcr-Abl-mediated tumorigenesis of T-ALL[14]. To further confirm the significant differences in the expression of lncRNAs and mRNAs, we removed the spleen from T-ALL leukemia mice and C57BL/6 mice and performed deep RNA sequence to study the different expression of lncRNAs and mRNAs. We found that lncRNAs' expression altered significantly in the spleen of Notch1-correlated T-ALL leukemia mice compared with that of C57BL/6 mice which was the first time to report the altered expression of lncRNAs. 1873 lncRNAs and 5626 mRNAs were differentially expressed in the spleen from T-ALL leukemia mice compared with that of C57BL/6 mice.

we performed GO terms enrichment to study the biological functions of differently expressed mRNAs. The most enriched GO terms for differentially expressed mRNAs were related to melanocyte differentiation, myosin complex and translation repressor activity in biological processes, cellular component or molecular function. To understand the function of differentially expressed mRNAs further, we performed KEGG pathway analysis and found that 303 pathways were significantly enriched among the altered transcripts. Acute myeloid leukemia, Protein export ad Glycosaminoglycan biosynthesis-keratan sulfate were the 3 significantly enriched pathways. Apoptosis, Notch signaling pathway and PI3K-Akt signaling pathway which was closely related with the pathology process of T-ALL was involved in the enriched pathways[15]. Furthermore, we constructed co-expression network to investigate the relation between lncRNAs and the coding genes.

Competing endogenous RNAs (ceRNAs) was raised that ceRNA molecules could sponge miRNA through miRNA response elements (MREs) and regulate gene expression. Numbers of molecules could act as ceRNAs, including lncRNAs, circRNAs or pseudogene. Wang et al found that lncRNA CHRF regulates cardiac hypertrophy by targeting miR-489[16]. circRNA MTO1 sponges miR-9 to suppress hepatocellular carcinoma progression[17]. Chan et al found that A FTH1 gene:pseudogene, can sponge miRNAs to regulates tumorigenesis in prostate cancer[18]. In this study, a lncRNA-associated ceRNA network analysis was perform and showed that 71 differentially expressed lncRNAs and 123 expressed mRNAs were selected, which predicted 11 miRNAs sharing binding sites with the differentially expressed lncRNAs and mRNAs.

Nice differentially expressed lncRNAs from ceRNA network prediction were selected to confirm deep RNA sequence results by qRT-PCR. We selected ten pairs of mice spleen which contain both T-ALL leukemia mice or C57BL/6 mice to further validation. All of the nice downregulated lncRNAs expression were consistent with RNA sequence results. NONMMUT117521.1 was located in intron of ECE-1 which has endopeptidase activity and membrane-bound metalloprotease. Bao et al found that ECE-1 promoted Ischemia/Reperfusion-Induced Injury[19]. ENSMUST00000195494 was located in Pfkfb3 which participated in the glucose metabolism and promoted cell proliferation, apoptosis and autophage in many types of cancer[20]. NONMMUT008951.2 was located in Bcl11a which inhibited proliferation and promoted apoptosis in B lymphoma cell lines[21]. NONMMUT026003.2, in the ceRNA network of differentially expressed lncRNAs, was located Bcl6 which participated humorous of processes, including inflammatory response, growth and differentiation[22].

This study provides the first Notch1-correlated ceRNA network prediction of lncRNAs and mRNAs which needed some studies to explore the roles of these differentially expressed lncRNAs. Because of the limitation of mice model, patients' samples addition is valuable.

Abbreviations

T-ALL Acute T-lymphocytic leukemia

ceRNA competing endogenous RNA

ICN Notch intracellular domain

GO Gene Ontology

KEGG Kyoto Encyclopedia of Genes and Genomes

MiRNA microRNA

qRT-PCR Quantitative real time polymerase chain reaction

NALT NOTCH1 associated lncRNA in T cell acute lymphoblastic leukemia 1

IUR Imatinib-upregulated lncRNA

STAT5 Signal transducer and activator of transcription 5

CD71 Cluster of Differentiation 71

PI3K Phosphatidylinositol 3 kinase

MREs miRNA response elements

MTO1 mitochondrial tRNA translation optimization 1

CHRF Cardiac hypertrophy related factor

FTH1 Ferritin heavy chain 1

ECE-1 Endothelin converting enzyme 1

Pfkfb3 6-phosphofructo-2-kinase/fructose-2,6-biphosphatase 3

Declarations

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Availability of data and materials

RNA-seq data are included in additional files.

Authors' contributions

M.S prepared Figures.

S.W wrote manuscript text.

X.L prepared the tissues of animals.

Y.L. designed, directed the experiment and analyzed data and wrote the manuscript text.

All authors reviewed the manuscript.

Ethics approval

All animals were housed and cared for according to the guidelines and regulations of the Institutional Animal Care and Use Committee of the Second Hospital of Hebei Medical University.

Consent for publication

This work described was original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part.

Competing interests

The authors have declared that no competing interest exists.

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Tables

Table 1 Basic information of selected nice lncRNAs for qRT-PCR confirmation.

Seqname	Source	RNA length	Locus	P-value	Fold change	Regulation
NONMMUT117521.1	PMID24463510	863	4:137882371-137883938	0.007679055	7.33	DOWN
NONMMUT021309.2	PMID23951020	2508	14:65114738-65117245	0.005231906	5.65	DOWN
ENSMUST00000195494	noncodeV3	2099	2:11532596-11534694	0.000178499	10.60	DOWN
NONMMUT008951.2	noncodeV3	3828	11:24103379-24107440	4.34E-05	12.53	DOWN
NONMMUT008779.2	noncodeV3	3379	11:20461259-20464664	0.00264931	9.67	DOWN
NONMMUT026003.2	noncodeV3	2078	16:23981313-23983390	0.004331438	5.59	DOWN
NONMMUT041921.2	NONCODEv4	3925	2:181405971-181409895	0.000122807	7.66	DOWN
NONMMUT147972.1	-	1666	4:32239436-32241101	0.001742802	8.78	DOWN
NONMMUT029828.2	NONCODEv3	2875	17:46424215-46427089	3.45E-05	32.22	DOWN

Table 2 Specific primers of each lncRNA.

Seqname	Forward	Reverse
NONMMUT117521.1	GATGCTGAGCAAGTGGGTGA	TGGATAGGTGGATGTGAGCG
NONMMUT021309.2	TCCAACATTGCTCACCCGAG	AGCATGGCGCTGTCAATGTA
ENSMUST00000195494	GGATCTGCAGAGGGGCTTAC	GCACCAGGGAACCCATTGTA
NONMMUT008951.2	CAGGACGAGTCAGGCACATT	CAAGCCTTCGGTCTGTGTCT
NONMMUT008779.2	AGTCCTTGTGTGAGCATCCG	CCCATGTGTTCCGTGGAGAA
NONMMUT026003.2	AAGAGGCTGGCTAGACCTGA	AAGGAGTTTGAAGGCGGGAG
NONMMUT041921.2	AAGATGTAAGGGCCACCAGC	TGTGAGGCTGTTCCAAGTCC
NONMMUT147972.1	ACCCAACAAGATGCTCCTCG	AAGTCGCAGTTGGGTCTCTG
NONMMUT029828.2	TTCAGGTTCAGCTGTTCGGG	GAGTGGGGAGCCGTAAGAAC

Figures

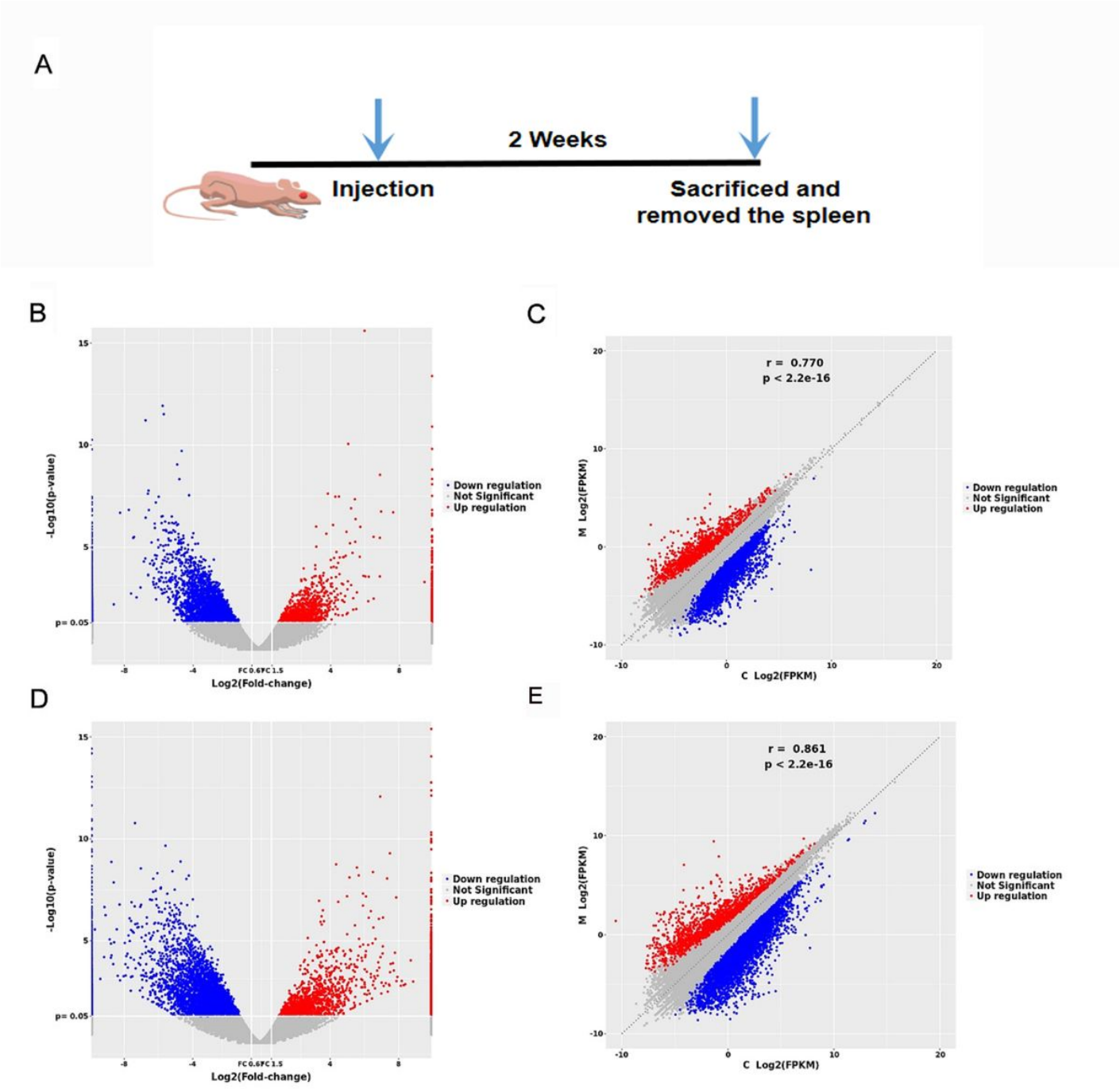

Figure 1

Differentially expressed lncRNAs and mRNAs in spleen of mice. The time-line of study design (A). Differentially expressed lncRNAs are shown in volcano Plot(B) 16 and scatter-Plot(C). Differentially expressed mRNAs are shown in volcano plot(D) and scatter plot(E). For volcano plot, the abscissa is fold change(fold change≥2) and the ordinate is p value(p-value≤0.05). The red color is the up-regulated genes and the blue is the down-regulated genes. For scatter plot, The abscissa and ordinate represent two sets of samples. The red color is the up-regulated genes and the blue is the down-regulated genes.

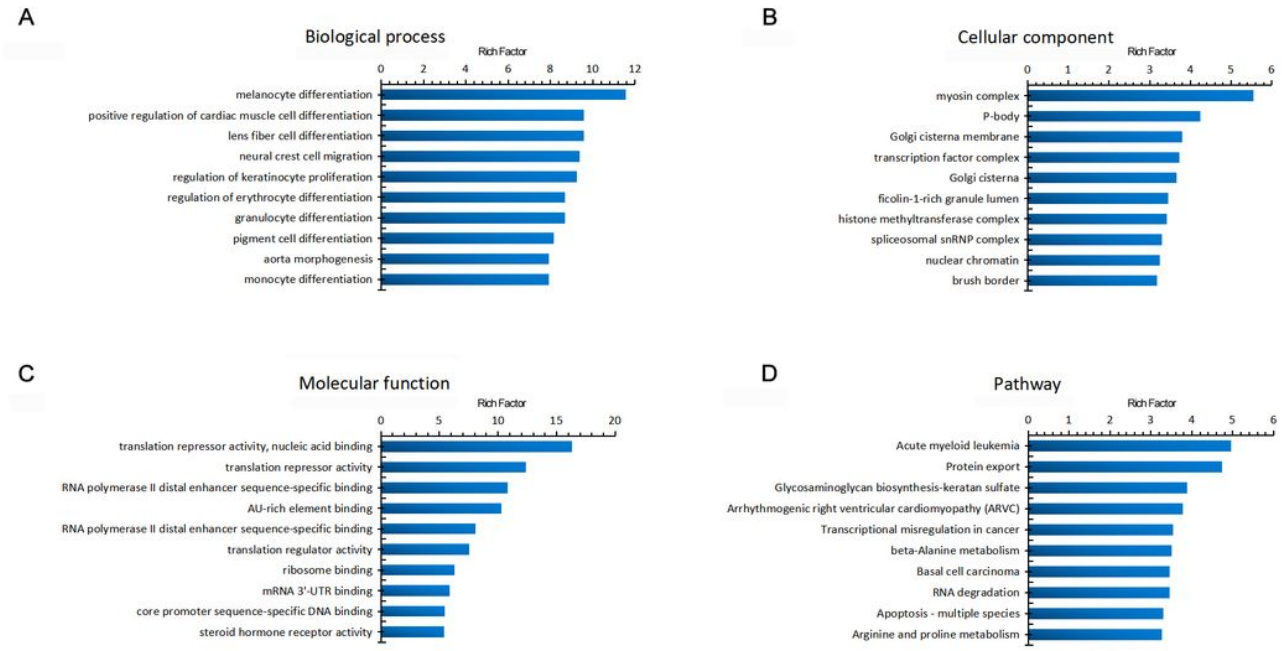

Figure 2

mRNA GO and KEGG pathway analysis in spleen of mice. TOP10 enriched GO terms for Biological process(A), cellular component(B) and molecular function(C). TOP10 enriched KEGG pathway was shown in corresponded to differentially expressed transcripts(D).

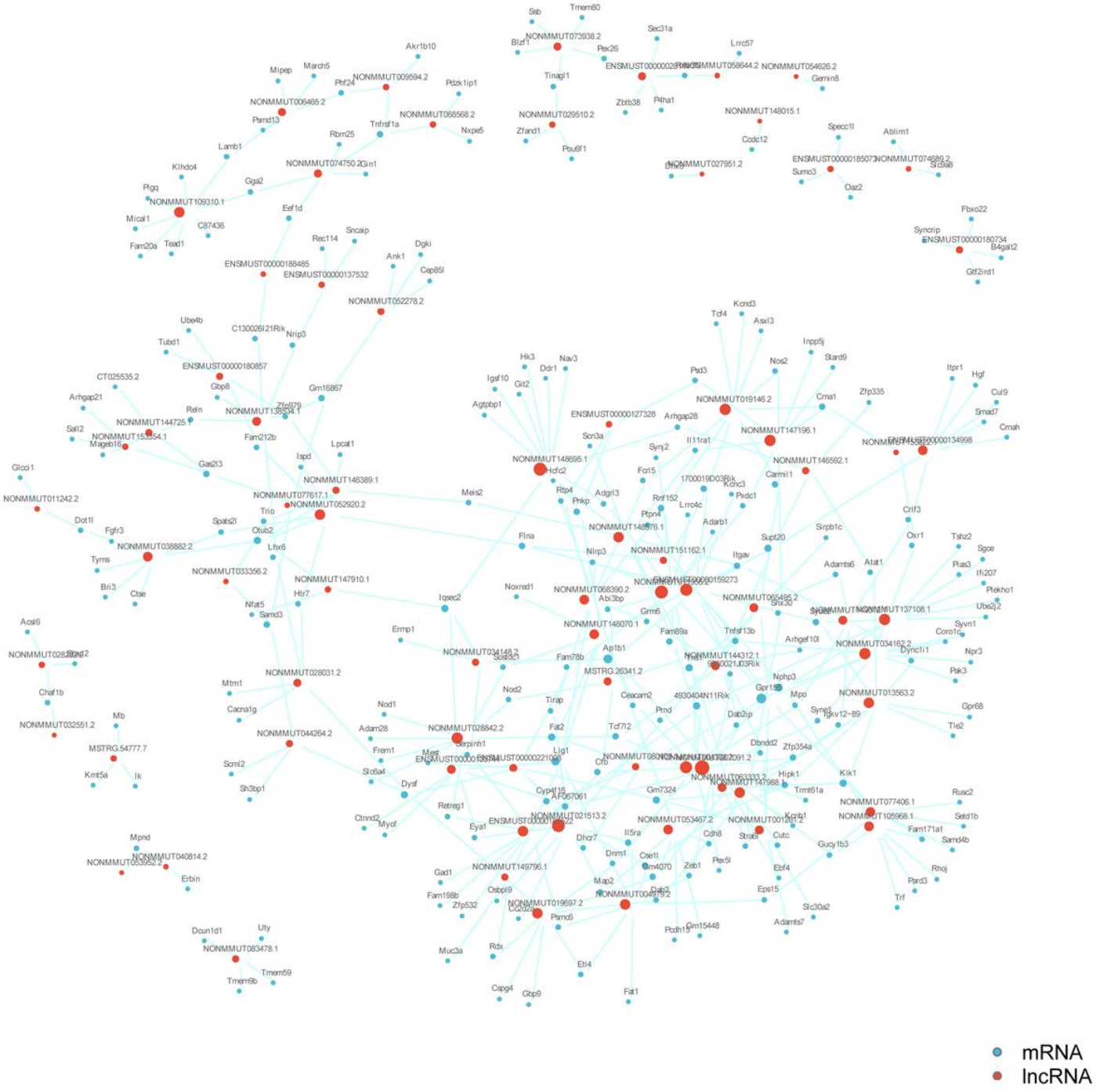

Figure 3

lncRNAs and mRNAs co-expression analysis. This network shown the co-expression correlations between lncRNAs and mRNAs.

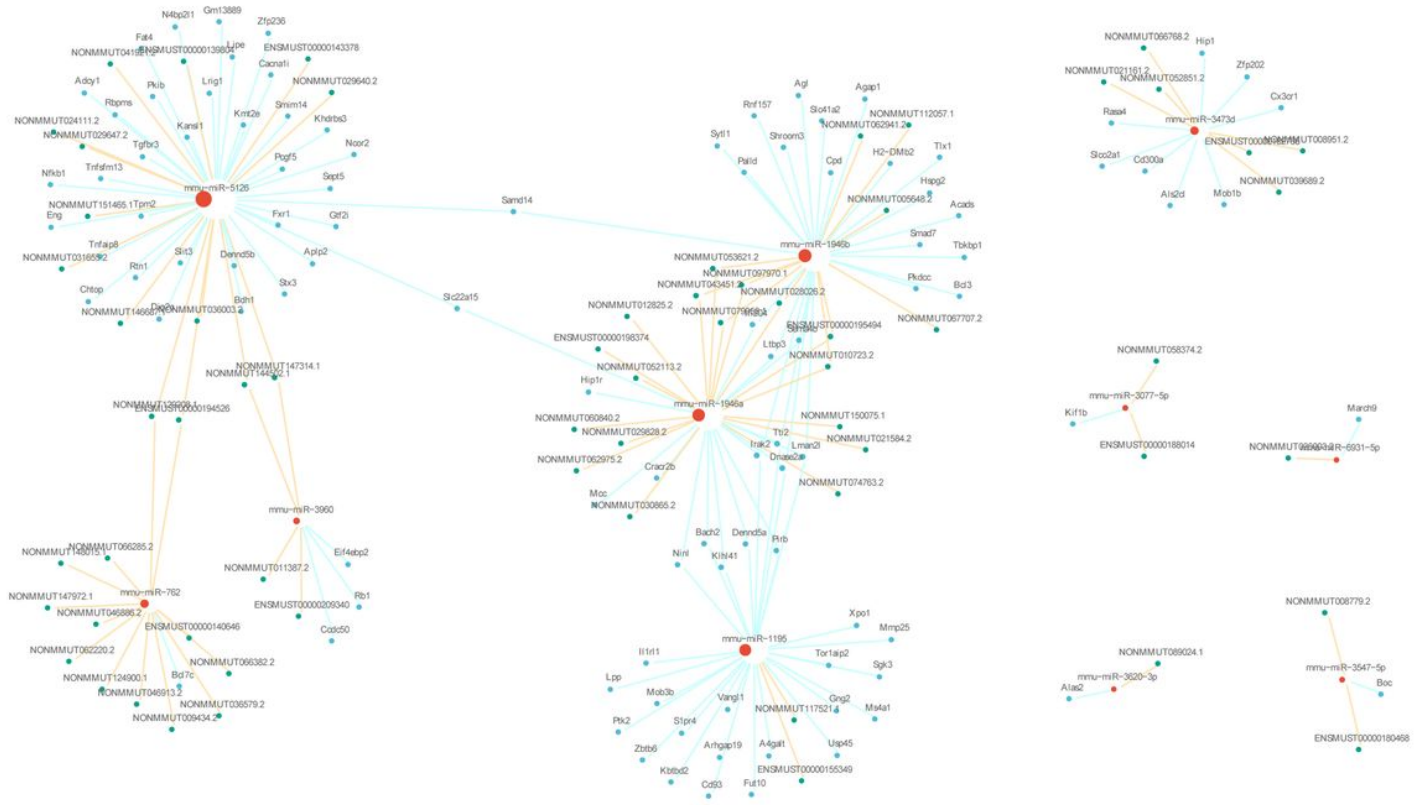

Figure 4

Construction of a ceRNA network. Global view of the ceRNA network. Six networks were presented.

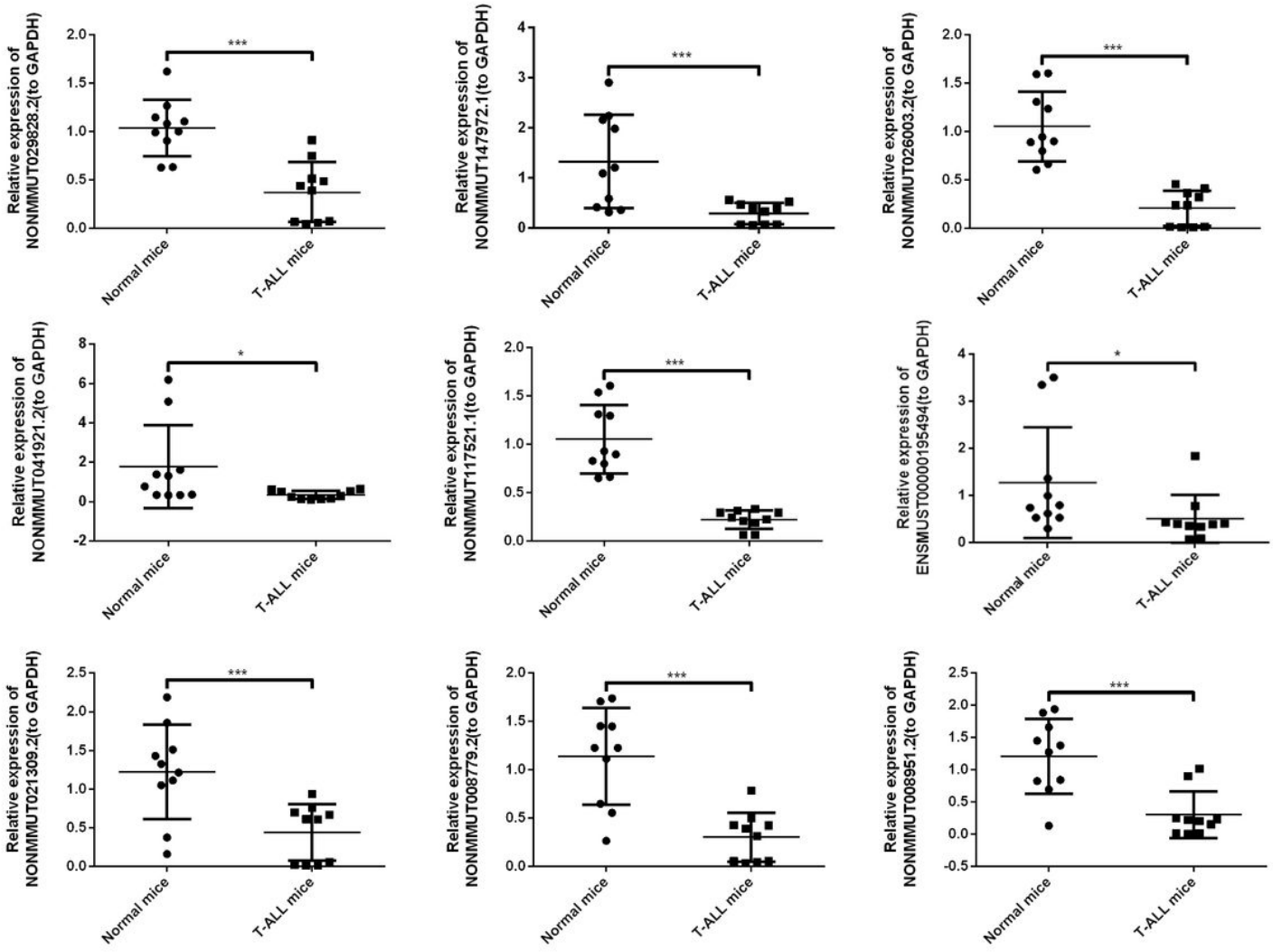

Figure 5

Validation of deep RNA sequence results by qRT-PCR from spleen of T-ALL mice or C57BL/6 mice.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- additionaldata.xls
- ARRIVEGuidelinesChecklist.pdf