Identification Of Exosomal LncRNAs From Peripheral Blood In Spinal Cord Injury Mouse Model

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Abstract

Background

Spinal Cord Injury (SCI) is a disease leading to permanent neurological dysfunction. In recent years, exosomes and non-coding RNAs have been considered as potential therapeutic agents for spinal cord injury. Based on ceRNA regulatory network, the role of non-coding RNAs has been paid attention to, and some genes related to the pathological process after spinal cord injury have been found. However, most gene studies only focus on exosomes and non-coding RNAs in spinal cord injury sites, and few genes related to spinal cord injury repair have been found.

Objective

We aimed to identify exosomes and non-coding RNA in peripheral blood after spinal cord injury, and to predict its role in spinal cord injury according to gene expression profiles.

Materials and methods

After successful modeling of spinal cord injury, rat exosomes were extracted from peripheral blood.Western-Blot was used to identify exosomes. After RNA was extracted from exosomes, total transcriptome sequencing and differential gene GO and KEGG Pathway analysis were performed. We selected potential genes for quantitative Real-Time PCR (qRT-PCR) assays and predicted their potential regulatory networks.

Results

The successful establishment of spinal cord injury model was confirmed by Tarlov's scores, and the extracted exosomes were confirmed by Western-Blot and electron microscopy. Among the significantly differentially expressed lncRNAs, XR_351404, XR_353833, XR_590719, XR_590076, and XR_591455 were associated with miRNA related to repair after spinal cord injury.

Conclusions

The regulatory effect of this network may play a key role in the repair process of SCI. The differential lncRNAs we found may serve as therapeutic targets and diagnostic biomarkers for SCI.

1 Introduction

Spinal cord injury (SCI) is very harmful to humans, usually leading to lesion site and lower limb dysfunction(1). Studies have shown that the incidence of SCI has risen to 12.1–57.8 cases per million worldwide per year(2). Most of the causes are traumatic spinal cord injury, and half of sci patients in North America occur after trauma(3). Most of the causes are fall injury, sports injury, and traffic accidents(4). In clinical treatment, due to the lack of specific and effective therapeutic targets, current treatments include early decompression, glucocorticoid therapy and elevated blood pressure therapy(5). Although some progress has been made in bioengineering, due to the limited understanding of the pathogenesis of SCI, the treatment and prognosis of SCI are still unsatisfactory(6). Therefore, the study of exosomes and non-coding RNA after spinal cord injury is particularly important.Previous studies have shown that the formation of astrocytes scar was considered to be the reason for the failure of axon regeneration in the central nervous system(7). However, the results of Mark A. Anderson 's are different from other cognitions, and it was found that the formation of astrocytes scar helps rather than prevent axon regeneration in the central nervous system(8). The occurrence of inflammation has dual effects of protection and damage to neurons after spinal cord injury(9), so spinal cord injury occurs under the induction of multiple factors.

Exosomes are bilayer membranous nanovesicles involved in various biological activities(10). Current studies have found that exosomes can be used as biomarkers, therapeutics and drug delivery carriers for disease diagnosis(11). A large number of studies suggest that exosomes play an important regulatory role in spinal cord repair(12–14). A study Using exosomes from non-coding RNA (miRNA-29b) injected rats with spinal cord injury, the degree of spinal cord injury relief was significantly improved(14). There are also studies suggesting that the lack of certain non-coding RNA (miR-21) may inhibit the protective effect of exosomes on the spinal cord(15).

Non coding RNA is involved in the process of neuron death, demyelination, neuroglia cell, inflammatory reaction, so it has potential value in the treatment of SCI(16, 17). However, the current research is limited to the role of bone marrow mesenchymal stem cells and the mechanism of exosomes involved in local injur. In terms of treatment, systemic injection of human bone marrow mesenchymal stem cells (hMSCs) exosomes is considered to be the reason for its limited application due to its insufficient therapeutic effect and poor organ targeting ability(18). Therefore, the new SCI diagnosis and treatment method is particularly necessary. In this study, based on the role of exosome signal transduction and non coding RNA regulation, we are committed to discover novel differentially expressed genes and predict their correlation with inflammatory response, scar formation and nerve cell repair.

Based on these, we hypothesized that the cellular behavior of spinal cord injury site is affected by the whole body microenvironment. Exosome mediated non coding RNA differentially expressed genes are associated with spinal cord repair in rat peripheral blood circulation. In order to test the hypothesis, we extracted the blood of abdominal aorta of rats, extracted and determined the non coding RNA of its exosome, then sequenced the gene and screened the genes with significant expression. After quantitative Real-Time PCR assays, lncRNA-miRNA-mRNA network analysis was performed to select the genes

involved in the repair after SCI. Our findings may provide guidance and basis for clinical diagnosis and treatment of SCI.

2. Materials And Methods

2.1 Animals

Six adult female rats, each about 230g, were randomly divided into experimental group and control group with 3 rats in each group. Rats in each group were given the same feeding environment. The rats in the experimental group were operated on for spinal cord injury, while the rats in the control group only injured the skin. Before operation, the rats in the two groups were in the normal environment for one week.

2.2 Spinal Cord Injury Surgery

The model of spinal cord injury was established as previous research(19). Before operation, the skin on the back of T8 and T12 was prepared with scissors. The rats were deeply anesthetized with 10% chloral hydrate solution (3ml/kg). After deep anesthesia, the rats were in the prone position, and the skin incision was made from T8 to T12 of midline of back.. With the dorsal side of the spinal cord as the midline, the scalpel was vertically inserted into the spinal cord on the dorsal midline and moved horizontally in the spinal cord to half cut the right T10. On the contrary, the control group was only made T8 to T12 skin incision. The incisions of both groups were sutured after operation.

All rats were given post operative animal care. 2% Iodophor disinfectant was used to clean the surgical incision once a day, and a heating pad was placed under the rats to maintain the ambient temperature (about 37 ℃). Penicillin solution (5 m/kg) was subcutaneously injected into all rats in the first 3 days after operation to prevent postoperative infection. According to the modified Tarlov's scale, the motor function of the experimental group (S) and the control group (D) were evaluated to determine whether the spinal cord injury model was successfully established.

2.3 Extraction of exosomes

In order to extract exosomes, six blood samples (about 1-2ml) were drawn from the abdominal aorta of all rats. The samples in the experimental group were numbered S1, S2 and S3 respectively; the samples in the control group were numbered D1, D2 and D3 respectively. The blood samples were centrifuged to prepare plasma. Density gradient centrifugation was used to separate exosomes. The mixture of the sample and XBP buffer was added to the exoeasy (Thermo Fisher Scientific, Shanghai, China) column and centrifuged at 500 g for 1 minute, then 10 ml of XWP buffer was added and centrifuged at 5000 g for 5 minutes to remove the residual volume. Four hundred μL-1ml XE buffer was incubated on the membrane for 1 minute, and the eluent was collected after centrifugation. The eluent was added to the exoeasy centrifugation column again and incubated for 1 minute. After centrifugation for 5 minutes, the eluent was collected as exosome. S2, S3, D1, D2 and D3 repeated the procedures of sample S1, and finally extracted 6 exosome samples.

2.4 Identification of exosome protein markers by western blot

Exosomes were identified by Western blot(20). In brief, the vesicles were mixed in RIPA Lysis Buffer (Solarbio, Zhejiang, China). BCA (bicinchoninic acid) method was used to determine the protein concentration in each sample. After mixing, the gel was prepared and detected by electrophoresis. After extracting the protein solution, the sample was denatured in RIPA Lysis Buffer, and then transferred to Millipore PVDF Membrane (Roche, Beijing, China). The PVDF membrane was sealed in 5% BSA blocking buffer (Solarbio, Zhejiang, China) for 2 hours after the end of membrane transfer. TBST buffer (Merck KGaA, Shanghai, China) was used to wash the membrane. After washing the membrane, the specific antibody of marker protein (1:1000) was added. HRP (horseradish perxidase) was used to label secondary antibodies (Jackson 1:2000). After reaction with chemiluminescence detection reagent, the maker protein was observed.

2.5 Electron microscopic examination

TEM h-7000 (Hitachi, Japan) was used to observe exosomes(21). The exosomes were washed with PBS (phosphate buffer saline) and dehydrated with ethanol of different concentration gradients. The exosomes were identified by electron microscope.

2.6 RNA extraction and quality control

Total RNA was extracted from exosome samples using Trizol reagent (life technologies, Shanghai, China). Nanodrop nd-1000 (Thermo Fisher Scientific, Waltham, Ma, USA) was used to measure the RNA concentration of each sample. The extracted RNAs would be subjected to quantitative quality and library quality control.

2.7 Gene sequencing and enrichment analysis

Ribosomal RNA (rRNA) was removed from the samples using the nebnext rRNA Removal Kit (New England Biolabs, Inc., Massachusetts, USA). NEBNext® Ultra ™ II directional RNA library prep Kit (New England Biolabs, Inc., Massachusetts, USA) was used to construct sequencing library. After quality control and quantification of the library by Bioanalyzer 2100 system (Agilent Technologies, USA), the RNA was sequenced 150 BP. After that, high quality reads were compared to rat reference genome (UCSC RN5) using hisat2 (v2.0.4) software. cuffdiff software (part of cufflinks) was used to get the FPKM as the expression profiles of lncRNAs and mRNAs(22), and fold change and p-value were calculated based on FPKM, differentially expressed LncRNAs and mRNAs were identified. Based on differentially expressed mRNAs and lncRNAs, GO and KEGG pathway enrichment analysis were performed.

2.7 qRT-PCR assays

In order to confirm the reliability of the results, we selected 10 potential lncRNAs for qRT-PCR. qPCR SYBR Green master mix(CloudSeq, Shanghai China)would be used for qRT-PCR assays. The target RNA and internal reference of each sample were detected by real time PCR. Glyceraldehyde-3-phosphate

dehydrogenase (GAPDH) mRNA was used as the internal control of differentially expressed genes. The primers are shown in Table 1. The data were analyzed by $2^{-\triangle\triangle CT}$ method.

Table 1
The sequence of primers

	Gene	Sequence*	
1	ENSRNOT00000067908	1-Forward	CGGTGCCCAACTTTGAACTG
		1-Reverse	GGAGGTTGTTGTGCCTACGA
2	XR_590093	2-Forward	TGAGGCTGCAAGAGAAGGTG
		2-Reverse	GAGAGAGCCCTCCTCAGACA
3	XR_351404	3-Forward	CGAGCGGGACCACATATGAA
		3-Reverse	GCCGTGCAGACCTATACTCC
4	XR_591426	4-Forward	CTGTGACCCCAAGGATGGAC
		4-Reverse	CCTTTGCCCCCATGGATCTT
5	XR_591455	5-Forward	TGTGGAAGAGCACCGCTATG
		5-Reverse	TGAAGCTGAGGTTCCTGCTG
6	XR_353833	6-Forward	CATGCAGGACACAAGCACAC
		6-Reverse	GAGTCTTGGGCAGCAACTCT
7	XR_360081	7-Forward	GTTCCTACGCAGATAGCCCC
		7-Reverse	CCGCTTGGAATATTGGCTGC
8	XR_346933	8-Forward	CTGAGCCCCTTCCTCTTTGG
		8-Reverse	GGGAGTCTGCTTGGCTTTCT
9	XR_590076	9-Forward	GCAGGAGGAGCTGAGAGTTC
		9-Reverse	TGGCAGGTCGTAGTTGACAC
10	XR_590719	10-Forward	TGTCTTAGGAACGGGTTGGC
		10-Reverse	GTTGTGTTCACGGCCACTTC
11	GAPDH	11-Forward	GACATGCCGCCTGGAGAAAC
		11-Reverse	AGCCCAGGATGCCCTTTAGT
* All primers were provided by Shanghai CloudSeq Biotechnology Co., Ltd.			

2.8 LncRNA-miRNA-mRNA Network

Based on the ceRNA (competing endogenous RNAs) regulatory mechanism, the lncRNA-miRNA-mRNA network was construted using the 10 selected lncRNAs, which might predict the correlation between them.

3 Results

3.1 Spinal cord hemisection model

According to modified Tarlov's scale, the hind limb function of S1, S2 and S3 rats in the experimental group was scored(23). The results showed that the Tarlov scores of S1, S2 and S3 were 1, 1 and 2 respectively. As shown in Fig. 1(a), there was significant difference betwwen the two groups. The motor function of hind limbs was significantly inhibited after spinal cord hemisection, which indicated that the SCI model was successfully established.

3.2 Identification of exosomes

In order to identify the exosomes of six samples, Western blot was used to detect the exosomes. As shown in Fig. 1(b), CD9, the typical exsome maker protein, was positive in all samples. The results showed that exosomes were extracted successfully. As shown in Fig. 1 (c), the diameter of exosomes was 200 nm, and the membrane was clear and intact. The results showed that the extracted exosomes met the standard and could be used in subsequent experiments.

3.3 RNA quantitative quality control

After RNA extraction, RNA quantification and quality assurance by NanoDrop nd-1000 (Nanodrop, USA). The OD260 / OD280 values were used as RNA purity index for RNA quantitative quality control. Table 2 presents the results of sequencing library RNA quantitative quality control showed that the OD260/OD280 values of six groups of RNA samples were in the range of 1.8 ~ 2.1, and the RNA quantity was more than 1μL. The results of quantitative quality control were qqualified. The RNA quality control showed that the extracted RNAs was qualified and could be used in subsequent experiments. Sequencing library quantitative was determined by Agilent 2100 Bioanalyzer using the Agilent DNA 1000 chip kit (Agilent, part # 5067 − 1504, USA). The results showed that the size of RNA was about 300 bp, and the mass concentration was more than 1cg / μ L. The above results showed that the RNA samples were qualified, as shown in Table 3.

Table 2
RNA quantitative quality control

Sample ID	Sample Name	OD260/280 Ratio	Conc. (cg/μl)	Volume (μl)	Quantity (μg)	QC Results
1	S1	1.85	20.34	11	0.22	Pass
2	S2	1.86	19.67	11	0.22	Pass
3	S3	1.86	21.76	11	0.24	Pass
4	D1	1.95	21.76	11	0.24	Pass
5	D2	1.85	17.38	11	0.19	Pass
6	D3	1.84	20.04	11	0.22	Pass

OD260/280 Ratio between 1.8 and 2.1 indicates that RNA purity is qualified (record as "pass"). There is no unqualified in the table.

Table 3
RNA library quality control

Sample Name	Size (bp)	Conc. (cg/μl)	Conc. (noml/L)	Volume (μg)	Total Amount (ng)
S1	277	2.72	14.9	10	27.2
S2	293	2.02	10.5	10	20.2
S3	304	2.73	13.6	10	27.3
D1	300	1.77	8.9	10	17.7
D2	302	1.75	8.8	10	17.5
D3	303	2.30	11.5	10	23.0

3.4 Gene expression profile and enrichment analysis

Seventy six differentially expressed lncRNAs were screened, of which 30 were up-regulated and 46 were down-regulated. There were 372 up-regulated and 635 down regulated differentially expressed mRNAs were detected. Based on FPKM, the heat maps and hierarchical clustering of differentially expressed lncRNAs and mRNAs were constructed (Fig. 2). As it was shown in volcano plot (Fig. 3), there were significant difference of gene expression after SCI. Go and KEGG pathway enrichment analysis was used to further evaluate the function of genes with obvious expression trend. Because the expression of lncRNAs is closely related to the adjacent coding genes, we selected the target genes of lncRNAs for enrichment analysis to speculate the function of lncRNAs(24). GO analysis and KEGG analysis were performed to infer the function and signal pathway of differentially expressed mRNAs and lncRNAs. GO terms with p-value < 0.05 were considered statistically significant. KEGG pathway takes p-value < = 0.05

as the threshold of significant enrichment. Figure 4 shows the top ten enrichment scores (- log10 (p-value)) of enrichment pathways.

3.5 qRT-PCR assays

In order to further verify the reliability of the data, 10 potential lncRNAs (ENSRNOT00000067908, XR_590093 et al, see Table 1 Primer) were identified by qPCR. QuantStudio 5 Real-Time PCR System (Thermo Fisher, Shanghai, China) was used for qPCR assays. The target RNA and internal reference of each sample were detected by Real-Time PCR.The data were analyzed by $2^{-\triangle\triangle CT}$ method, and there were significant differences in all selected RNAs ($P < 0.05$). We constructed the amplification curve (Fig. 5) and dissolution curve (Fig. 6) of 10 genes. The results showed that the expression profiles of mRNAs and lncRNAs were reliable.

3.6 LncRNA-miRNA-mRNA network analysis

The lncRNA-miRNA-mRNA network (Fig. 7) was construted to predict their correlation. The network consisted of 10 lncRNAs, 41 miRNAs and 198 mRNAs nodes. Edge was taken as the attribute of each connector in the graph, which indicated the combination of two genes. Previous studies had shown that rno-miR-34b-5p, rno-miR-210-3p, rno-miR-219a-5p, rno-miR-347 could regulate neuronal apoptosis, inhibited inflammatory response and astrocyte formation. The network diagram showed that these miRNAs were closely related to XR_351404, XR_353833, XR_590719, XR_590076, XR_591455. We found that miR-210-3p was combined with XR_591455 and XR_590719 respectively; XR_351404 was combined with rno-miR-34b-5p and rno-miR-219a-5p respectively;XR_353833 was combined with rno-rno-miR-219a-5p□rno-miR-347 was combined with XR_590076. In the lncRNA expression profile, the expression of XR_351404, XR_353833, XR_590719 and XR_590076 was up-regulated, but XR_591455 was down-regulated.

4 Discussion

SCI is a serious traumatic disease. When spinal cord injury occurs, it will lead to a large number of neuron apoptosis, leading to paralysis of trunk and limbs. Studies have shown that inflammation has a dual role of protecting and injuring nerves(25). In the same way, astrocytes can protect peripheral neurons by scarring local lesions after spinal cord injury(26), but it hinders the growth and repair of neurons in the same time(7, 27). Therefore, we should pay attention to the effects of inflammation and astrocyte formation in the process of spinal cord repair. Recent studies have shown that exosomes can be used as a carrier of non-coding RNA to the central nervous system, and play a regulatory role in the repair of SCI(28). Because of the involvement of apoptosisMesenchymal stem cell (MSC) exosomes in inhibiting inflammatory response, scar formation and reducing neuronal, MSC transplantation is considered to be an effective therapeutic strategy for traumatic spinal cord injury(29, 30). More and more attention has been paid to the role of exosomes in the treatment of spinal cord injury.

LncRNAs in exosomes plays an important role in the regulation of spinal cord injury. In this study, we mainly predicted the possible role of lncRNAs in SCI. A large number of studies have shown that lncRNAs

can competitively inhibit the expression of miRNAs to play the role of gene regulation based on the mechanism of ceRNA(25). Therefore, lncRNAs are related to the process of various diseases. In recent years, the researches on SCI have confirmed that lncRNAs has obvious expression difference after SCI, and is closely related to neuroprotection, scar formation and inflammatory response, such as lncrna-scir1, lncrna Xist and other genes(31, 32). However, many lncRNAs related to SCI repair have not been found. Although a study has showed that exosomes in peripheral blood have diagnostic and therapeutic value for SCI(33), the research on exosomes in peripheral blood is insufficient, and few genes with therapeutic and diagnostic value are found.Based on these reasons, the purpose of our study is to explore the differences of gene expression of non-coding RNAs in peripheral blood after SCI, and to analyze the potential role of these genes in diagnosis or treatment.

In this study, the exosomes in the peripheral blood of rats were extracted and verified. After that, the extracted RNA was sequenced. Heat maps and volcano maps proved that lncRNAs and mRNAs had significant differential expression. These up-regulated genes and down regulated genes may be involved in the development and repair of SCI. According to the scores of GO and KEGG pathway enrichment analysis, we selected 10 potential lncRNAs from the differentially expressed lncRNAs. Finally, we constructed the lncRNA- miRNA-mRNA network, in which we found five lncRNAs related to the recovery of spinal cord injury (XR_591455, XR_351404, XR_353833, XR_590719, XR_590076).

We forecasted that XR_351404 was combined with miR-34b-5p after SCI. A study has shown that inhibition of up regulation of miR-34b-5p can reduce the release of inflammatory cytokines in the lung(34), suggesting miR-34b-5p may be the target of inflammatory response treatment. Another study showed that miR-34b-5p can be used as a regulator of astrocyte apoptosis(35). After SCI, XR_351404 was up-regulated and combined with miR-34b-5p, which regulated the pathological process of SCI based on ceRNA mechanism. In addition, miR-219a-5p is the common target gene of XR_351404 and XR_353833 in lncRNA-miRNA-mRNA network. According to previous studies, miR-219a-5p is related to the regulation of neuronal apoptosis(36), so we predict that the expression of XR_351404 is related to inhibiting inflammatory reaction and promoting apoptosis of astrocytes, XR_351404 and XR_353833 may be a therapeutic target for inhibiting neuronal apoptosis.

Neural stem cell transplantation plays a key role in the repair of SCI, mainly due to its neural differentiation potential and neurotrophic effect(37). Transplantation of homologous neural stem cells can regenerate the injured spinal cord after SCI, which indicates that neural stem cells (NSCs) have great potential to reconstruct the injured spinal cord nerve(37, 38). Recent studies have found that MSC-EVs can prevent NSCs from hypoxic injury by promoting the expression of miR-210-3p, which may reduce NSC apoptosis(39). In our experiments, miR-210-3p was combined with XR_591455 and XR_590719 respectively. Based on the mechanism of ceRNA, both XR_591455 and XR_590719 competitively binds to miR-210-3p. After SCI, the expression of XR_591455 was down-regulated and XR_590719 was up-regulated. So, we speculated that XR_591455 and XR_590719 together interfere with the binding of miR-210-3p to target genes, thereby regulating the hypoxic injury of NSCs. In addition, we found that XR_590076 was combined with miR-347. According to a study, miR-347 has been proved to promote

neuronal apoptosis after focal ischemia(40). Therefore, XR_590076 may inhibit miR-347 and reduce the loss of neuronal cells.

In this study, we extracted exosomes from peripheral blood and predicted the role of new genes in exosomes. It was concluded that the differentially expressed lncRNAs minght be involved in the regulation of inflammatory response, inhibition of scar formation, and regulation of neuronal apoptosis after SCI. The whole body microenvironment might influence the cell behavior of spinal cord injury site through exosomal lncRNAs. They might be the therapeutic targets and biomarkers for diagnosis of SCI.

Declarations

Ethics approval

This study was approved by the medical ethics committee of Tianjin Hospital.

Consent for publication

All patients gave their approval for participation.

Conflicts of interest

All authors declare that there is no conflict of interest.

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Authors' contributions

Jianan Li: Design of study; Writing of manuscript. **Mingpeng Shi:** Writing of manuscript; Interpretation of data. **Zhenhua Li:** Implementation of methodology and soft-ware; Analysis of data. **Wanfu Wei** Implementation of the experiment; Analysis of data. **Lin Cong, Mingyu Gu, Yiheng Chen and Siyi Wang:** Implementation of the experiment.

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Figures

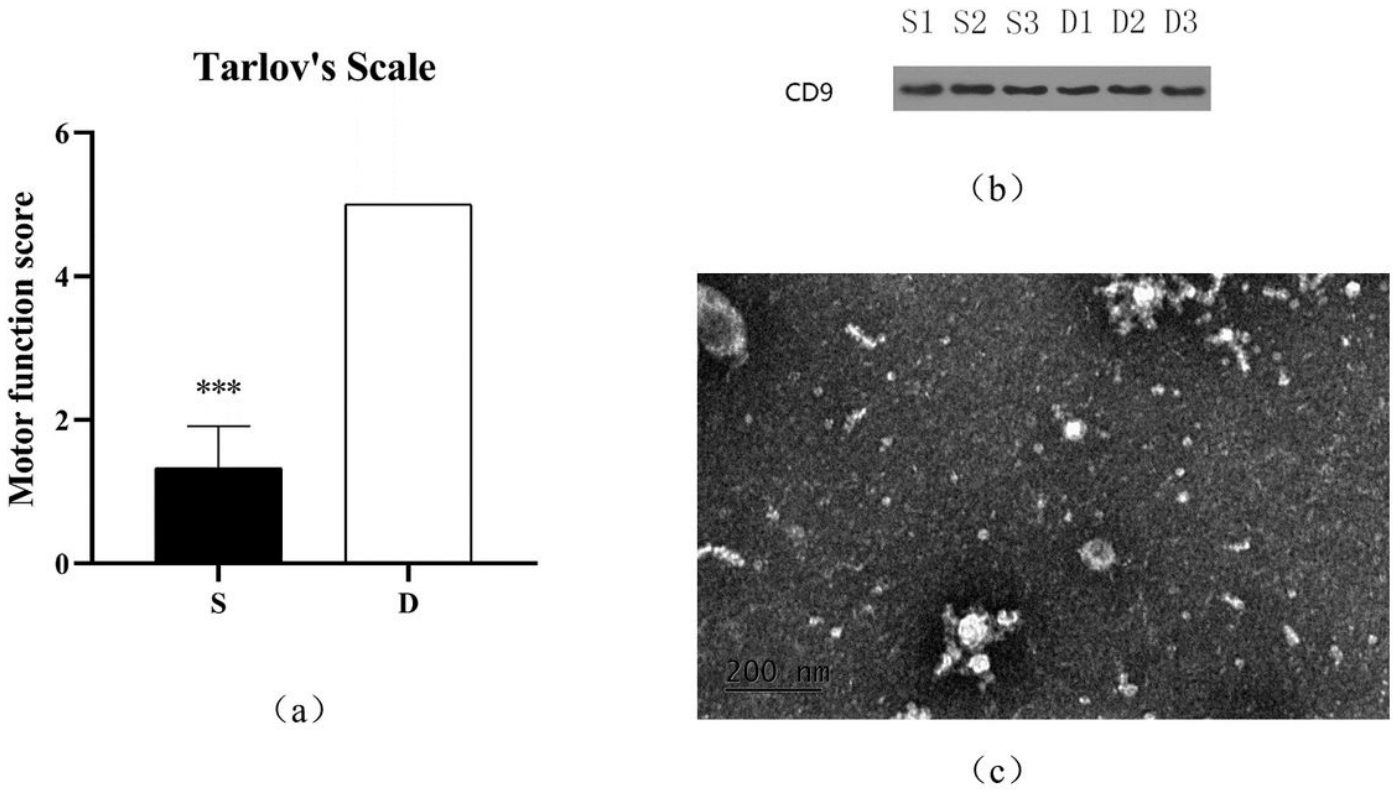

Figure 1

The modified Tarlov's scale(a). Western blot images of the exosomes(b): The stained exosomes enhanced the clarity of membrane structure (bar = 200 nm)

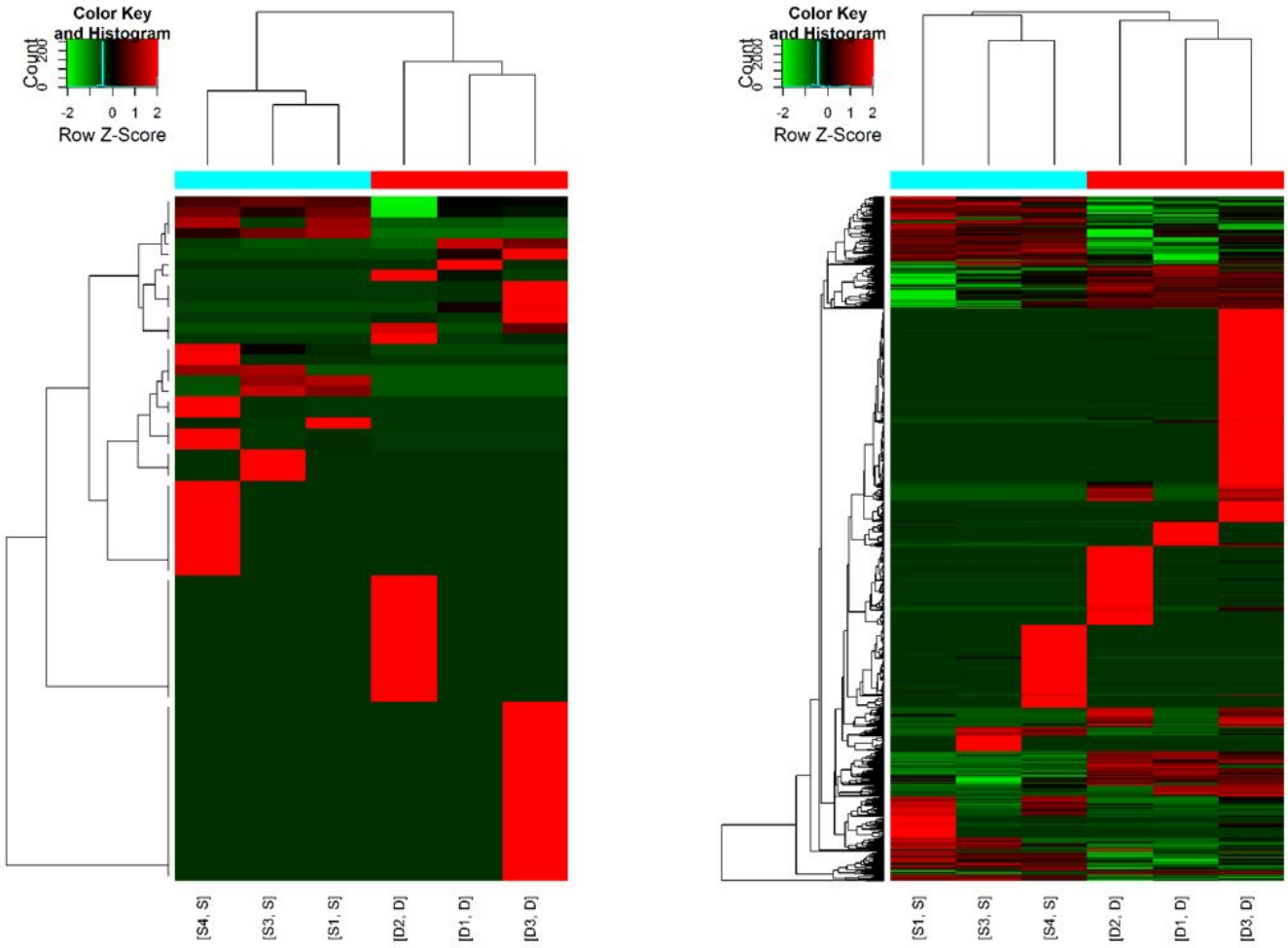

Figure 2

The expression of lncRNAs (a) and mRNAs (b) was significantly different between the two groups. Red represents a high level of expression. Green represents low expression level. Black represents the average expression level. Each column represents a group, and each row represents an RNA.

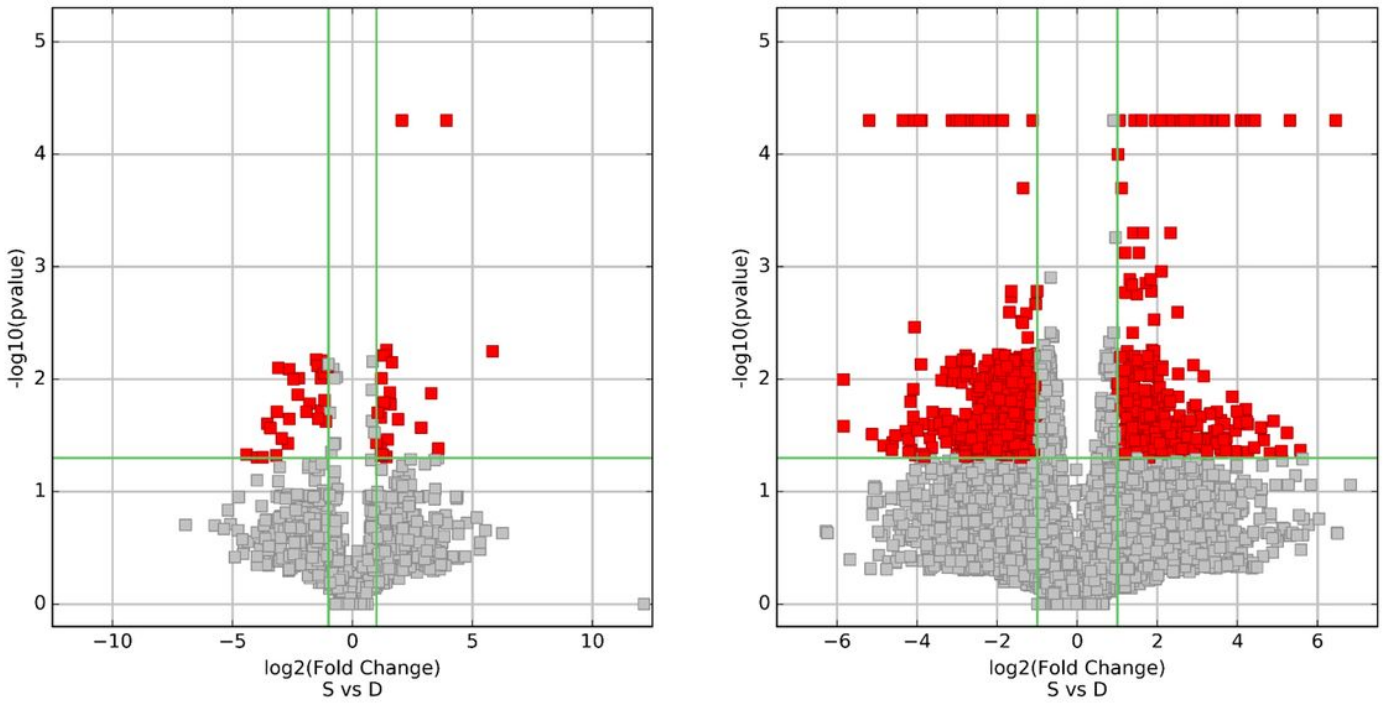

Figure 3

According to the fold change and p-value, the volcano map was drawn. (a) LncRNA, (b) mRNA. The red rectangle represents the differentially expressed mRNA or lncRNA, P < = 0.05, fold change > = 2.0.

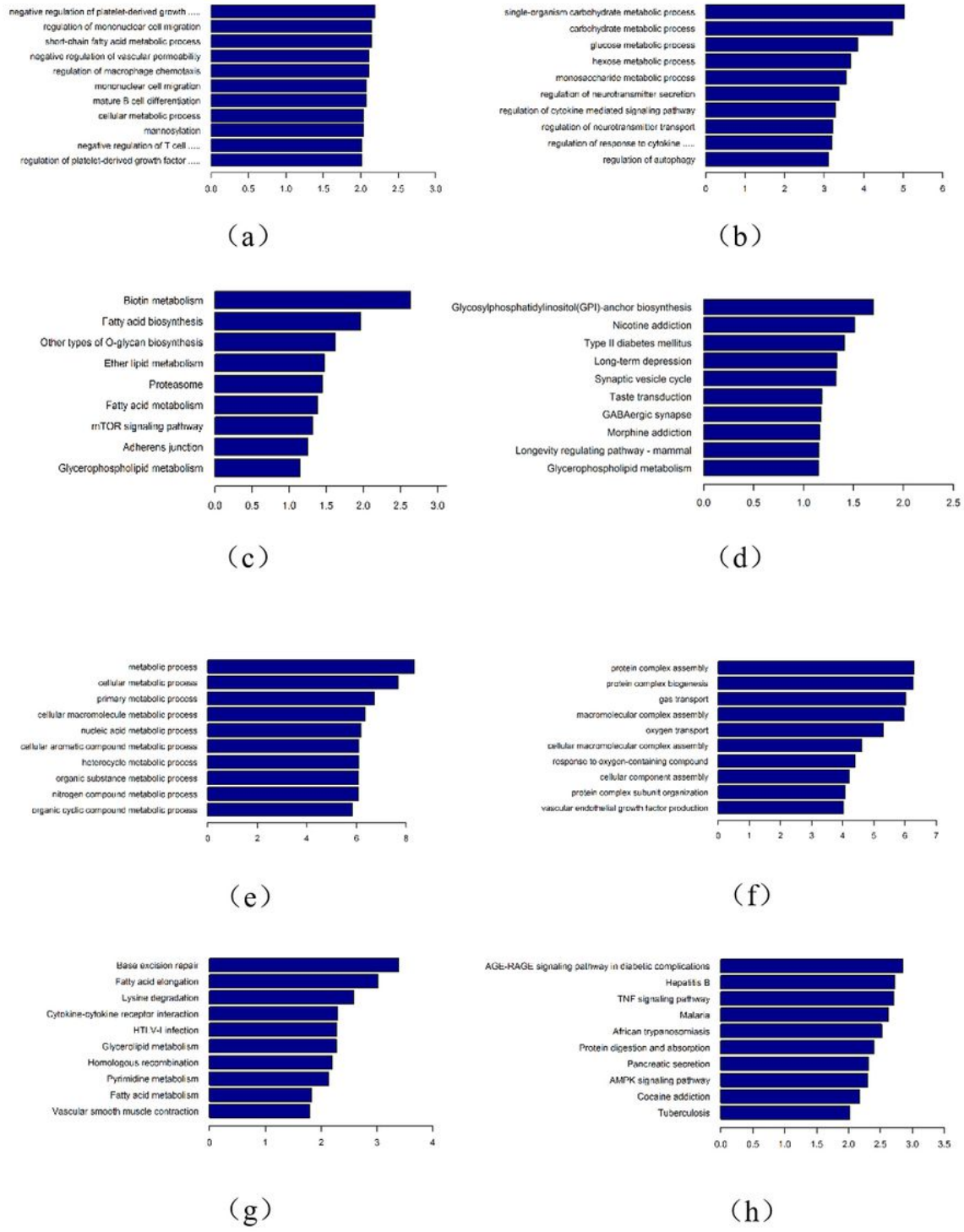

Figure 4

Down-regulated lncRNA GO analysis (a); up-regulated lncRNA GO analysis (b); down-regulated lncRNA KEGG analysis (c); up-regulated lncRNA KEGG analysis (d); down-regulated mRNA GO analysis (e); up-regulated mRNA GO analysis (f); down-regulated mRNA KEGG analysis (g); up-regulated mRNA KEGG analysis (h). the Enrichment Score value of the Pathway ID, it equals -log10 (Pvalue).

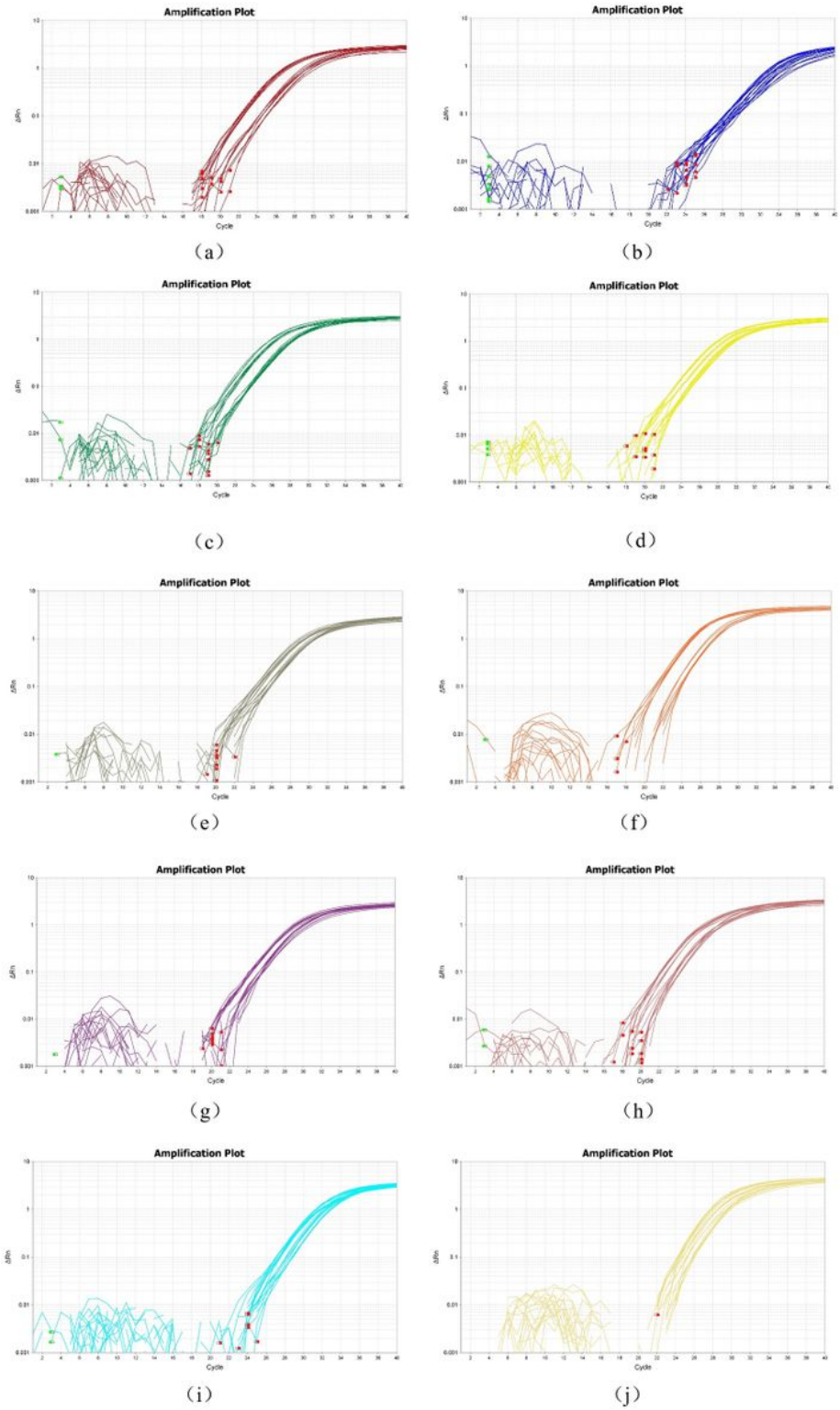

Figure 5

ENSRNOT00000067908 (a) ; XR_590093 (b) ; XR_351404 (c) ; XR_591426 (d) ; XR_591455 (e) ; XR_353833 (f) ; XR_360081 (g) ; XR_346933 (h) ; XR_590076 (i) ; XR_590719 (j).

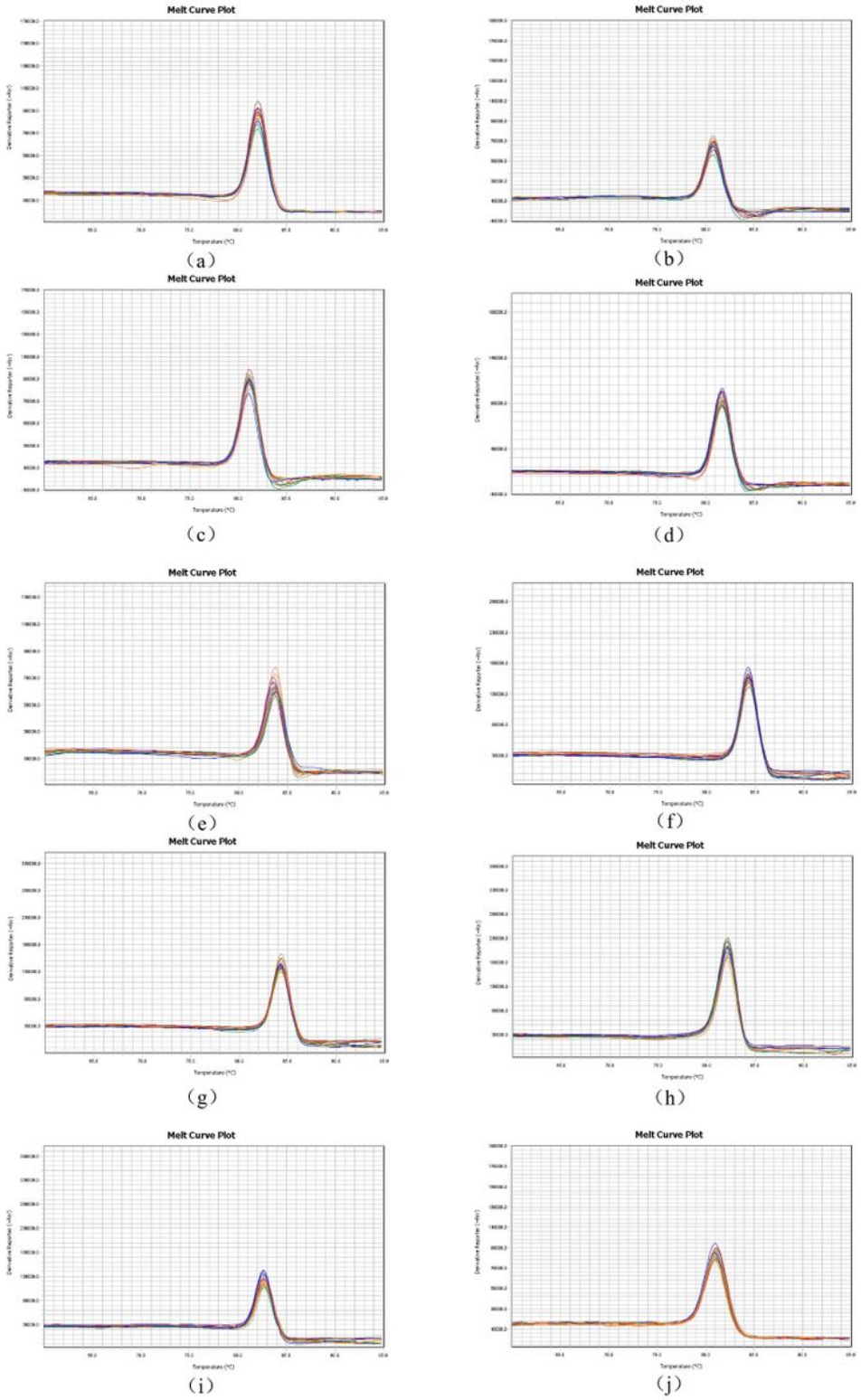

Figure 6

ENSRNOT00000067908 (a) ; XR_590093 (b) ; XR_351404 (c) ; XR_591426 (d) ; XR_591455 (e) ; XR_353833 (f) ; XR_360081 (g) ; XR_346933 (h) ; XR_590076 (i) ; XR_590719 (j).

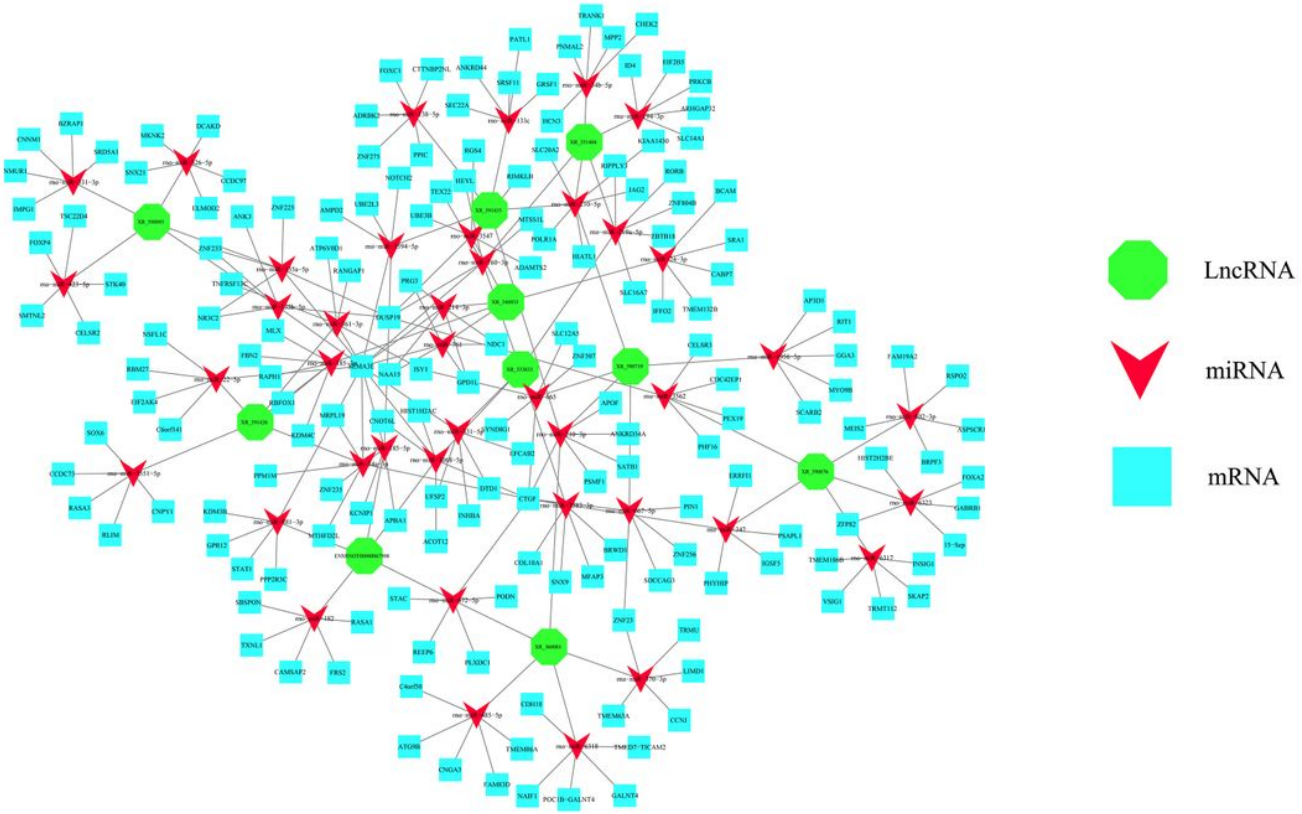

Figure 7

lncRNA-miRNA-mRNA network. lncRNAs: green, polygon; miRNAs: red, triangular. mRNAs: blue, square.