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Differential Expression Pattern of Exosome Long Non-Coding RNAs (lncRNAs) and MicroRNAs (miRNAs) in Vascular Endothelial Cells Under Heat Stroke

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Study Design A
Data Collection B
Statistical Analysis C
Data Interpretation D
Manuscript Preparation E
Literature Search F
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Background: Heat stroke is a life-threatening disease which is characterized by a high body temperature and multiple organ dysfunction syndrome. Vascular endothelial cell injury is a main feature of heat stroke. Little is known about the long noncoding RNA (lncRNA) and microRNA (miRNA) expression alternation in endothelial cell exosomes related to heat stroke. The aim of this study was to explore the changes of lncRNAs and miRNAs expression pattern in exosomes derived from vascular endothelial cells under heat stroke temperature conditions.

Material/Methods: Cultured medium exosomes from HUVECs (human vascular endothelial cells) either under normal temperature or heat stroke temperature conditions were harvested; then RNA was extracted and the lncRNAs and miRNAs were analyzed by high throughput sequencing.

Results: Ten significantly upregulated and 10 downregulated lncRNAs were identified in exosomes derived from heat stroke temperature treated cells. Furthermore, GO (Gene Ontology) and KEGG (Kyoto Encyclopedia of Genes and Genomes) analyses were used to evaluate the signaling pathway of differential expressions in lncRNAs. Finally, the interaction network of lncRNAs-miRNAs-mRNA was uncovered using ceRNA (competing endogenous RNA) principle via prediction software.

Conclusions: These results indicate that the identified lncRNAs and miRNAs in endothelial cell exosomes might serve as non-invasive biomarkers for heat stroke.

MeSH Keywords: Endothelial Cells • Exosomes • Heat Stroke • MicroRNAs • RNA, Long Noncoding

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Background

As global warming trends continue, more individuals are at increased risk of heat stroke (HS) [1,2]. HS is a life-threatening disease which is characterized by a high central body temperature (>40°C), high-grade hyperthermia, multiple organ dysfunction syndrome (MODS) with central nervous system dysfunction, and severe metabolic disturbances [3–5]. HS is considered one of the most common deadly natural hazards with a high morbidity and mortality rate during heat waves in some countries and regions [6]. Although some HS treatment strategies have been suggested and applied for the treatment and management of HS, such as rapid cooling, fluid resuscitation to stabilize organ function, and supportive treatments, the incidence and mortality of severe HS has continued to rise in recent decades. Recent epidemiological data has shown that the incidence of HS in urban areas in the United States during extreme heat is about 20 per 10 million with a potential high mortality rate of about 10–70%, while in the Saudi Arabia, it is about 50% [6]. Considering that proper diagnosis and treatment approaches are not as efficient as desired, new biomarkers and novel effective therapeutics should be developed.

Despite aggressive achievements obtained during the past decades, the pathology of HS is still poorly understood. Several mechanisms have been suggested to contribute to the process of HS. MODS and systemic inflammatory response syndrome have been reported to be involved into the pathology of HS [7,8]. In addition, heat stress can induce damage to the gut epithelial barrier, leading to bacteria and endotoxin leakage into the circulation system causing inflammation and cytokine secretion [9,10]. It is well-known that cytokines play significant roles in the development of systemic inflammatory response syndrome in HS [11]. Clinical investigation data have demonstrated that multiple types of cytokine expression are altered in patients with HS, such as TNF-α, IL-1β, IFN-γ, IL-6, IL-8, and IL-10 [12–14]. The serum concentrations of IL-1β, IL-6, and IL-10 were also elevated with an increasing core temperature in animal models for HS [13]. HS can cause MODS, including bleeding and coagulation activation [15,16].

Vascular endothelial cell injury is a main feature of HS; it is triggered by the initiation of coagulation disorders or even disseminated intravascular coagulation and participates in severe systemic inflammation response syndrome (SIRS), induces microthrombus formation leading to microcirculation, and drives the development of severe stroke with MODS [17,18]. The injury of vascular endothelial cells itself becomes an inflammatory effect which can initiate inflammatory cell adhesion, infiltration of tissues, and coagulation disorders that ultimately may result in the formation of disseminated intravascular coagulation and microthrombus [19]. With the deepening of scientific research, researchers have found that there is a vascular endothelial-mediated network between inflammation and thrombosis that jointly promotes the development of MODS [20].

Exosomes are membrane vesicles with a diameter of about 30–120 nm that are released by the multivesicular body after fusion with the cell membrane and then released to the extracellular matrix [21]. Previous studies have revealed that exosomes play pivotal roles in multiple biological process such as tumorigenesis and inflammation [22,23]. Meanwhile, exosomes can regulate biological function by transferring contents and information among cells, such as microRNAs (miRNAs) and long noncoding RNAs (lncRNAs). Metastatic tumor cells derived exosomes carrying special miRNA or lncRNA can promote tumor cell proliferation, apoptosis suppression, and chemoresistance that can lead to tumor metastasis and progression. Exosomes are also believed to contribute to the development of inflammation during sepsis [24]. However, little is known about lncRNA and miRNA expression alternation in endothelial cell exosomes during heat stroke.

Current studies aim to explore the differential expression pattern of exosome lncRNAs and miRNAs derived from vascular endothelial cells under normal temperature or heat stroke temperature conditions. High throughput sequencing results revealed the dysregulation of lncRNAs and miRNAs in exosomes from heat stroke cells. Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis evaluate the signaling pathway of differential expression lncRNAs and the interaction network of lncRNAs-miRNAs-mRNA which were established according to competing endogenous RNA (ceRNA) principle. Our results suggest that the specific lncRNAs and miRNAs in human vascular endothelial cell (HUVEC) exosomes might serve as non-invasive biomarkers for HS.

Material and Methods

Cell culture and heat stroke cells

HUVECs were purchased from the American Type Culture Collection (USA) and cultured in DMEM (Invitrogen, USA) with 10% fetal bovine serum (FBS, Gibico, USA) and 1% penicillin/streptomycin antibiotics. The HUVECs were maintained in a humidified atmosphere at 37°C and 5% CO_2. The control cells were always placed in an incubator at 37°C, while the heat stroke cells were cultured in the incubator at 41°C for 2 hours, and then at 37°C for a further 6 hours.

Exosomes extraction

HUVECs were cultured in the medium without serum. To obtain exosome-free sera, the growth serum was centrifuged at 120 000g for 2 hours. Cells were cultured in an exosome-free

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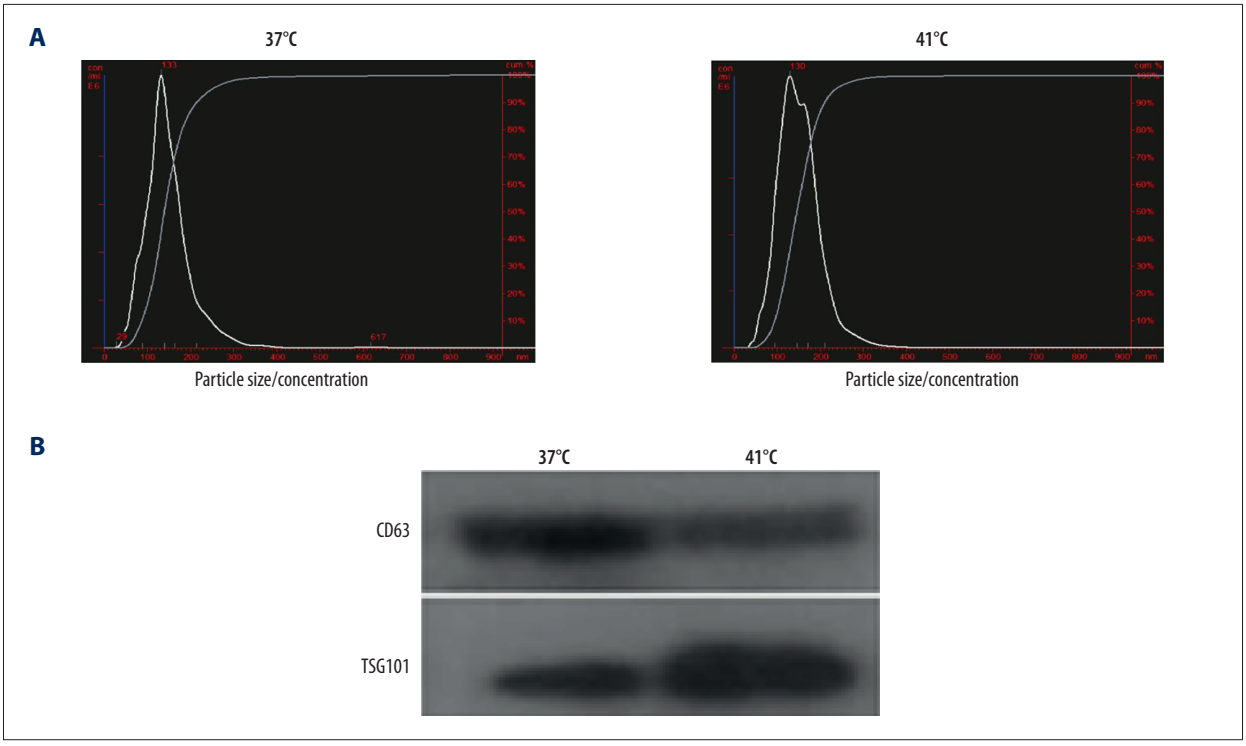

Figure 1. Characterization of exosomes from HUVECs with heat stroke treatment. (**A**) Representative particle tracking of a DU145 exosomes using the NanoSight Instrumentation. (**B**) Protein levels of exosome surface markers CD63 and TSG101 in HUVECs by western blot. HUVECs – human vascular endothelial cells.

medium for 24 hours. Exosomes from HUVECs medium were isolated using the Total Exosomes Isolation Kit (Invitrogen, Carlsbad, CA, USA) per the manufacturer's protocol.

Exosomes characterize validation

The presence of exosomes was confirmed by Nanoparticle Tracking Analysis (NTA) assay and western blot assay. NTA analysis was performed using the NanoSight NS300 instrument following the standard procedures (Malvern, Worcestershire, UK). Western blot was performed to verify the presence of exosomes. The protein was extracted, and equal amounts of protein extractions were separated using 12% polyacrylamide SDS gels (SDS-PAGE) and transferred onto polyvinylidene fluoride (PVDF) membranes (Amersham Pharmacia Biotech). The membranes were then probed with primary antibody against CD81 (ab79559, Abcam, 1: 1000) and TSG101 (ab30871, Abcam, 1: 1000). Primary antibodies were detected with secondary peroxidase-conjugated antibody (1: 3000, Proteintech) and the signals were visualized by enhanced chemiluminescence kit (GE, Fairfield, CT, USA) according to the manufacturer's instructions.

RNA extraction of exosome from HUVECs

The total of RNA exosomes was extracted using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) and the concentration and quality of RNA was determined by Nanodrop 2000 (Thermo, USA). RNA samples were stored at –80°C for further analysis.

Library construction and RNA sequencing

Total RNA from each sample was used to construct lncRNA and miRNA library using the TruSeq Small RNA Sample Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's instruction.

For lncRNA libraries construction, the ribosomal RNA was removed by Epicentre Ribo-zero™ rRNA Removal Kit (Epicentre, USA), and rRNA free residue was cleaned up via ethanol precipitation. Sequencing libraries were then generated using the rRNA-depleted RNA by NEBNext® Ultra™ Directional RNA Library Prep Kit for Illumina (NEB, USA) following manufacturer's recommendations. The library quality was assessed on the Agilent Bioanalyzer 2100 system and sequenced on an Illumina Hiseq 2500 platform.

For miRNA libraries construction, we used the NEB Next Multiplex Small RNA Library Prep Kit (NEB, USA) following the manufacturer's protocol. In brief, the RNA 3' adaptor was specifically ligated to miRNA with the excess adaptor removed by hybridization. The 5' ends of miRNA were then ligated to the 5' adaptor, followed by reverse transcription to convert

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Table 1. The top 10 upregulated and 10 downregulated lncRNAs ranked by fold changes in sequencing data.

GeneID	Genomic coordinates(hg38)	Fold change	Up/Down	P-value
LINC01005: 2	chr7: 64028065-64030105	14.74587498	Up	2.86E-13
EDRF1-AS1: 12	chr10: 125745230-125752065	12.33641828	Up	1.49E-15
MIF-AS1: 2	chr22: 23914032-23926468	8.288531026	Up	1.76E-09
LINC01499: 3	chr11: 41720443-41734754	8.095774496	Up	1.57E-10
LINC01411: 11	chr5: 174517759-174532457	7.90301796	Up	6.35E-09
LINC00589: 8	chr8: 29673922-29735223	7.76533472	Up	5.62E-10
LINC01016: 6	chr6: 33867506-33893653	6.939235282	Up	1.73E-06
FBXO22-AS1: 1	chr15: 75932683-75933130	6.360965675	Up	7.10E-05
FALEC: 2	chr1: 150515757-150518032	6.113135844	Up	2.83E-07
APCDD1L-AS1: 10	chr20: 58515727-58573970	5.782696069	Up	3.74E-05
LINC00273: 1	chr16: 34160034-34161707	13.57201704	Down	0
PSMB8-AS1: 9	chr6: 32844134-32845695	6.052540127	Down	9.63E-09
LINC00582: 1	chr1: 231591292-231612090	6.052540126	Down	6.86E-06
LINC00115: 16	chr1: 701936-720150	5.014961819	Down	0.000129078
LINC01346: 3	chr1: 3940613-3949351	4.842032102	Down	0.000207712
TRG-AS1: 1	chr7: 38308140-38345162	4.621397634	Down	2.03E-18
PVT1: 29	chr8: 127984020-127997041	4.539405095	Down	7.90E-06
SNHG3: 7	chr1: 28509390-28510869	4.496172667	Down	6.75E-07
EVX1-AS: 5	chr7: 27239243-27241228	4.431324021	Down	1.25E-05
NADK2-AS1: 1	chr5: 36221055-36221902	4.323242948	Down	1.97E-05

the ligated small RNA into cDNA, which was then uniquely indexed by PCR to generate the sequencing library. The quantified libraries were clustered to a flow cell and sequenced using TruSeq SBS Kit on a HiSeq system (Illumina).

Target gene prediction and functional analysis

The function analysis of identified lncRNAs was performed as followed. Target genes of selected miRNAs were commonly predicted by TargetScan and miRanda. Only the overlapping genes that were in all 3 databases were considered as the target genes of the differentially expressed miRNAs. The predicted genes subsequently underwent GO analysis for functional annotation analysis and KEGG database analysis to identify the involved enriched pathways. Fisher's exact and chi-square tests were used to determine the significance of the GO terms and pathways, and the false discovery rate (FDR) was calculated to correct the *P*-value. Only the GO terms and pathways with an adjusted *P* value of <0.05 and an FDR of <0.05 were selected.

Statistical analysis

All quantitative data are presented as mean ± standard deviation. Comparisons between the 2 groups were performed using the Student's *t*-test. Comparisons of multiple group data were performed using one-way analysis of variance ANOVA) followed by Turkey's post hoc test. *P* values <0.05 were considered statistically significant. Statistical values were calculated using SPSS software, version 20.0 (IBM Corp., USA).

Results

Verification of HUVECs exosomes

Exosomes from either normal or heat treated HUVECs were isolated using the extraction kit and verified by nanoparticle tracking assay and western blot. Nanoparticle tracking results implied that the size of the major particles from the 2 groups was 130 nm (Figure 1A). We then tested by western blot the

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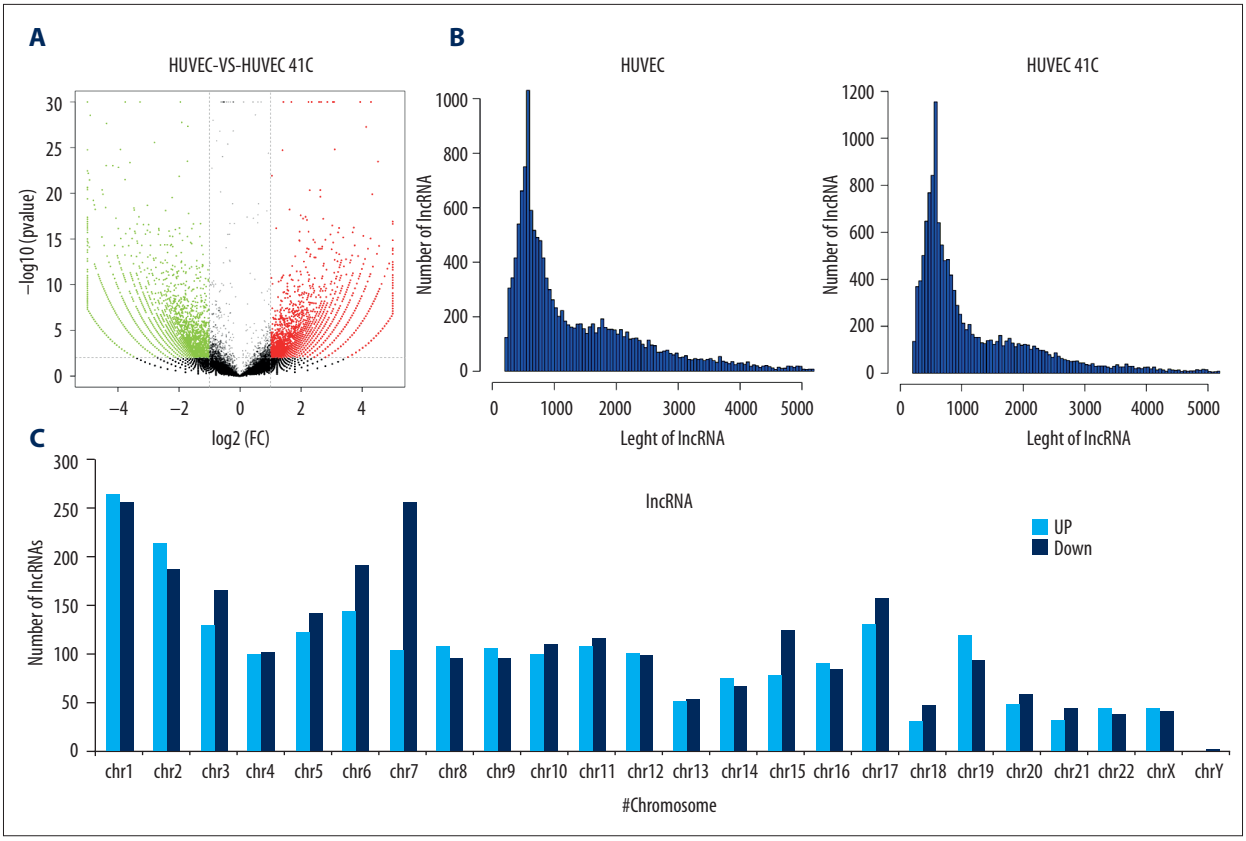

Figure 2. The lncRNAs were differentially expressed in exosomes from HUVECs after heat treatment. (**A**) Volcano plots showed all of the detected lncRNAs in exosomes from HUVECs after heat treatment. (**B**) The number and length of lncRNAs were analyzed in exosomes from HUVECs after heat treatment. (**C**) The chromosome distribution of exosome lncRNAs from HUVECs after heat treatment was presented. HUVECs – human vascular endothelial cells; lncRNAs – long noncoding RNAs.

levels of the 2 proteins CD63 and TGS101, which are commonly enriched and located in exosome membranes. The results showed that both of the exosome markers could be detected in exosomes from control and heat treated HUVECs (Figure 1B).

The lncRNAs were differentially expressed in exosomes derived from heat treated HUVECs

The lncRNA expression profiles were then performed by high throughput lncRNA sequencing. The results showed that thousands of lncRNAs are found to be significantly differentially expressed in exosomes released from HUVECs. A total of two, 370 lncRNAs were upregulated while two, 604 lncRNAs were downregulated (fold change >2, *P* value <0.05) in exosomes from heat treated HUVECs, compared to that in the normal HUVECs. The top 10 upregulated and 10 downregulated lncRNAs are listed in Table 1. Their volcano plots and heat map are displayed in Figures 2A and 3A. The length and number of all lncRNAs from the 2 groups are shown in Figure 2B. The distribution of all differential expression lncRNAs were also examined and were found to be originated from all chromosomes (Figure 2C).

The mRNAs were differentially expressed in exosomes from heat treated HUVECs

The sequencing also showed the changes of the exosome mRNA expression pattern in heat treated HUVECs. The expression profile is displayed by volcano plots and heat map in Figures 4A and 3C. Then, the length and number of mRNAs from the 2 groups are also compared in Figure 4B. The distribution of mRNAs showed that mRNAs were originated from all chromosomes (Figure 4C).

The miRNAs were differentially expressed in exosomes from heat treated HUVECs

We furthered examined the miRNA expression in both exosome groups by high throughput miRNA sequencing. The results showed that hundreds of miRNAs are significantly differentially expressed in exosomes released from HUVECs. A total of 111 miRNAs were upregulated while 103 were downregulated (>2 folds, *P* value <0.05) in exosomes from heat treated HUVECs compared to those in the normal HUVECs. The heat

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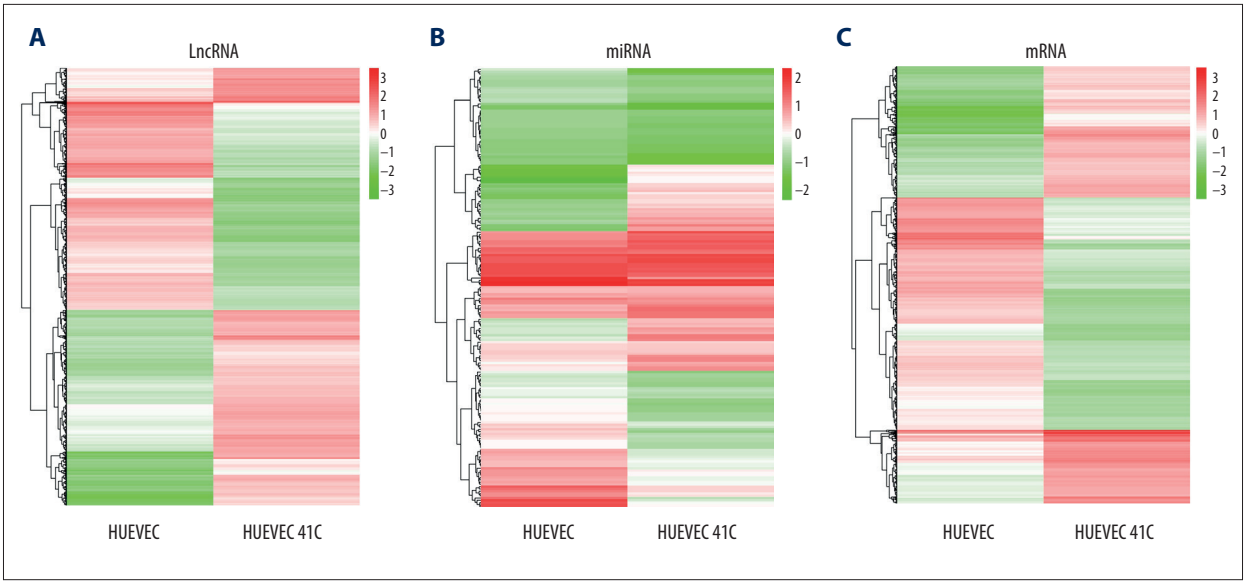

Figure 3. The lncRNA/mRNA/miRNA differential expression profiles. (**A**) The heat map shows lncRNA expression profiles. (**B**) The heat map shows miRNA expression profiles. (**C**) The heat map shows mRNA expression profiles. lncRNA – long noncoding RNA; mRNA – messenger RNA; miRNA – microRNA.

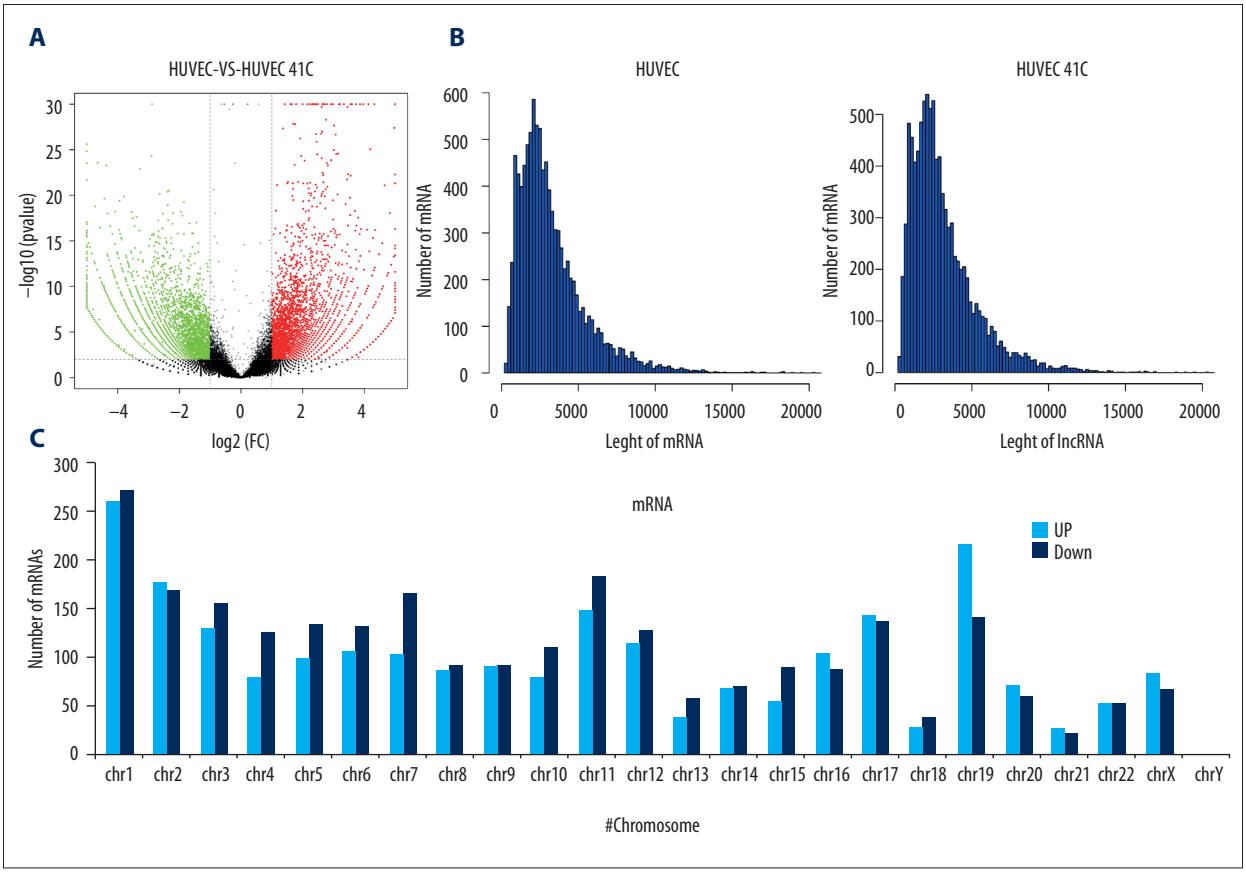

Figure 4. The mRNAs were differentially expressed in exosomes from HUVECs after heat treatment. (**A**) Volcano plots showed all of the detected mRNAs in exosomes from HUVECs after heat treatment. (**B**) The number and length of mRNAs were analyzed in exosomes from HUVECs after heat treatment. (**C**) The distribution of mRNAs in chromosomes were analyzed in exosomes from HUVECs after heat treatment. HUVECs – human vascular endothelial cells; mRNA – messenger RNA.

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Table 2. The top 10 upregulated and 10 downregulated miRNAs ranked by fold changes in sequencing data.

miRNAID	HUVEC	HUVEC-41C	Fold change	Up/Down	P-value
hsa-miR-1-3p	0.981937126	159.076074	162.0023012	Up	4.04E-93
hsa-miR-1255b-2-3p	0.140276732	11.38064123	81.12992845	Up	0.000000814
hsa-miR-6742-5p	0.140276732	11.00128652	78.42559748	Up	0.00000141
hsa-miR-3922-5p	0.841660394	61.32901105	72.86669479	Up	2.38E-32
hsa-miR-4788	1.402767323	86.49287332	61.65874547	Up	1.76E-43
hsa-miR-3944-5p	0.981937126	58.6735281	59.75283605	Up	1.26E-29
hsa-miR-3190-5p	2.945811379	146.9367234	49.8798818	Up	6.82E-69
hsa-miR-582-5p	0.280553465	11.75999593	41.91712952	Up	0.00000417
hsa-miR-6769a-5p	0.420830197	15.67999458	37.25967075	Up	0.000000141
hsa-miR-675-5p	1.262490591	44.89030706	35.55694385	Up	1.58E-19
hsa-miR-1225-5p	104.3658889	11.38064123	9.170475265	Down	0
hsa-miR-1202	120.3574363	11.75999593	10.23447942	Down	0
hsa-miR-6748-3p	15.71099402	1.51741883	10.3537624	Down	1.3E-70
hsa-miR-4458	1.402767323	0.126451569	11.09331687	Down	9.71E-08
hsa-miR-624-3p	2.805534647	0.252903138	11.09331687	Down	4.65E-14
hsa-miR-6741-5p	11.78324552	1.011612553	11.6479827	Down	2.4E-54
hsa-miR-6776-5p	115.0269205	8.851609842	12.99502831	Down	0
hsa-miR-7159-5p	3.927748505	0.252903138	15.53064361	Down	7.6E-20
hsa-miR-451a	74.90777506	2.908386091	25.75578782	Down	0
hsa-miR-4730	149.1141665	4.678708059	31.87079951	Down	0

map is displayed in Figures 3B and the top 10 upregulated and 10 downregulated miRNAs are listed in Table 2.

The lncRNAs function analysis and annotation by GO analysis and KEGG pathway

To further evaluate the function of differential expression lncRNAs, bioinformatics analyses were performed to analyze lncRNA function based on their target genes. GO analysis was used to assess biological processes, molecular functions, and cellular components (Figure 5A). The classification showed the top 20 GO terms (Figure 5B). KEGG pathway analysis identified associations with 17 pathways ($P<0.05$) ranked by target-gene count, with some highly enriched pathways related to PI3K-AKT and transcriptional dysregulation in cancer pathway (Figure 5C).

Construction of the lncRNAs-mRNAs-miRNAs interaction network

To determine the function of the identified lncRNAs, interactions between lncRNAs and their target miRNAs were theoretically predicted by conserved seed-matching sequence using the software for miRNA target prediction such as the TargetScan and miRanda database. The most differentially expressed 10 lncRNAs were predicted according to the complementary miRNA matching sequence. The data displayed each lncRNA and its potential complementary binding miRNAs (Figure 5D).

Discussion

Recently, more studies have focused on high temperature-related diseases, as the total temperature-related fatality rate has increased rapidly [7]. Epidemiological survey data found that high temperature can induce increased heart disease, respiratory disease, stroke, and other mortalities [8]. Therefore,

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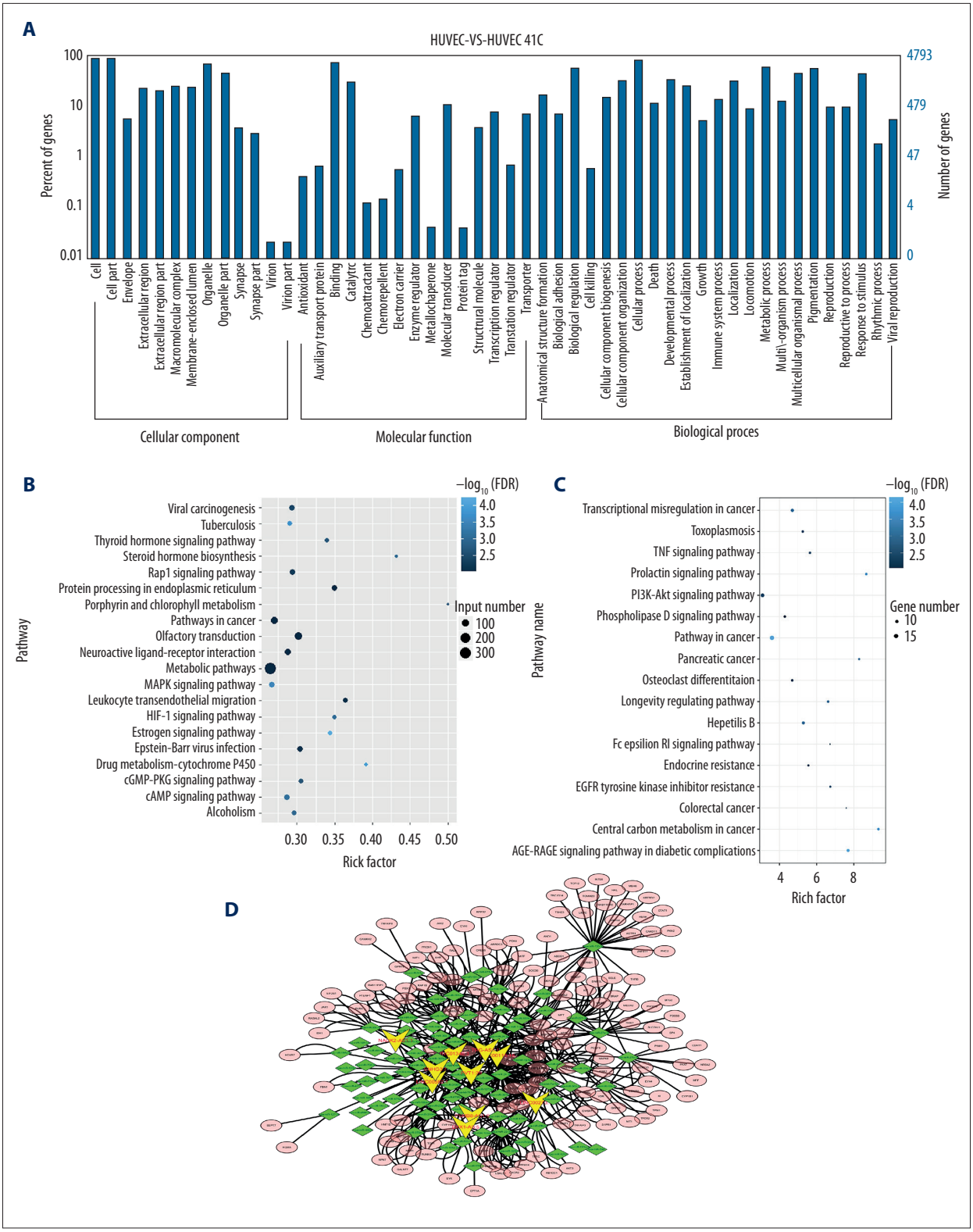

Figure 5. Functional analysis and network analysis of lncRNAs. (**A**) GO analysis for all lncRNA genes. (**B**) GO enrichment histogram for differentially expressed lncRNA genes. (**C**) Pathway analysis based on lncRNA-target genes. (**D**) Construction of lncRNA-miRNA-mRNA co-expression interaction network. GO – Gene Ontology; lncRNA – long noncoding RNAs; mRNA – messenger RNA; miRNA – microRNA.

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more attention should be paid to high temperature-related diseases. The exposure of the body to a high temperature environment causes the central body temperature to increase above 40°C and that is the main cause of severe HS. Endotoxin, cytokines, and other immune regulatory factors co-stimulus can directly cause tissue damage, coagulation disorders, and SIRS, resulting in the occurrence of MODS [7]. More novel diagnostic biomarkers are needed for the clinical diagnosis of HS.

In recent decades, emerging evidence indicates that lncRNAs play a critical role in embryonic development, tumorigenesis, and inflammation progress [25]. Indeed, a series of lncRNAs have been identified and verified as the potential biomarkers in a variety of disease models [26]. It is believed that cells can release exosomes (~100 nm) to regulate downstream signal pathways through carrying the content inside particles such as miRNA, lncRNA, and protein [27]. Through transmitting genetic information inside the original organs to target spaces, exosomes can change the micro-environment to be more beneficial surrounding [28]. Exosomes from metastatic tumor cells can release specific miRNA to interfere with the target gene expression, leading to tumor proliferation and apoptosis suppression in the brain [29]. Exosomes can also alter sunitinib-sensitive clear renal carcinoma cells to sunitinib-resistant clear renal carcinoma cells by carrying specific lncRNA, leading to clear renal carcinoma metastasis and progression [30]. One study found that exosomes from gastric cancer cells can promote tumor liver metastasis by transmitting estimated glomerular filtration rate (EGFR) [31]. In the present study, by RNA sequencing, we identified a series of lncRNAs and miRNAs that were differentially expressed in exosomes from HUVECs between 37°C (normal) and 41°C (heat stroke treatment). Heat stress might destroy the vascular structure integrity and stability, which in turn, induces the inflammation response and systemic multiple organ failure. Since vascular endothelial cell apoptosis and inflammation are the main clinical features, thousands of lncRNAs and hundreds of miRNAs have been found, and in our study, they were suggested to be associated with vascular endothelial cell apoptosis and inflammation.

Our results showed that exosomes released by HUVEC contained a variety of miRNAs after heat stroke temperature exposure and the targeted genes of these upregulated miRNAs included several apoptosis-related genes such as TP53, SIRT1, and MAPK. The SIRT1 agonist can improve and promote the expression of p53, inhibit the apoptosis of vascular endothelial cells induced by advanced glycation end products, and inhibit its signal molecules that promote apoptosis. In contrast, SIRT1 inhibitors suppress this effect and lead to apoptosis [32]. MAPK family members, such as MAPK9 and MAPK8, can inhibit p53 ubiquitination and stabilize p53 to induce apoptosis [33]. The miRNAs predicted to suppress apoptosis are similarly upregulated, indicating that apoptosis-inducing effect was activated by heat stroke. PI3K/Akt signal system is also involved in the regulation of apoptosis of vascular endothelial cells after heat stroke. On the one hand, PI3K/Akt can directly regulate the Bcl2 family by phosphorylating of BAD protein and inhibiting apoptosis [34,35]. On the other hand, PI3K/Akt signal can also indirectly regulate mouse double microsomal 2 (MDM2), inhibiting p53, enhancing the activity of CREB and the function of Bcl2 [36,37]. Moreover, activation of Akt can suppress caspase-3 and inhibit GSK-3β activity, resulting in anti-apoptotic effect [38,39].

It is well-known that lncRNAs play critical roles in gene regulation and perform their functions at various levels including transcriptional regulation, epigenetic modification, post-transcriptional regulation, and post-translational modulation [40]. Their underlying mechanisms are attributed to epigenetic silencing, lncRNA-miRNA interaction, lncRNA-protein interaction, and lncRNA-mRNA interaction [41]. Accumulating evidence indicates that lncRNAs regulate gene expression by functioning as ceRNAs [42]. The lncRNAs act as miRNA sponges or inhibitors to suppress the interactions between miRNAs and target mRNAs [43].

Our current study provided evidence that lncRNAs were differentially expressed in exosomes secreted from HUVECs after exposure to heat stroke temperatures. Meanwhile, numerous miRNAs and mRNAs were also found to be altered simultaneously. Through ceRNA principle, we predicted the potential target genes and constructed the co-expression network, which indicated that several lncRNAs may be involved in the heat stroke response. Our study shortcoming was that we did not carry out a validation process. In the future, studies should be conducted to further reveal the function and regulatory mechanisms underlying the lncRNAs-miRNA network.

Conclusions

This study confirmed the feasibility of high throughput sequencing analysis of exosome RNA and shows that RNA sequencing is a reliable and valid method to screen and identify novel lncRNAs that can serve as biomarkers of HS. We have shown that there are some significant differentially expressed lncRNAs and miRNAs that may have a diagnostic potential for HS. The function and underlying mechanisms of these lncRNAs and miRNAs should be further studied in order to determine their specific value regarding clinical application.

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