Circular Ribonucleic Acid Expression Profiles Differ Between the Plasma of Participants with Latent Autoimmune Diabetes in Adults and Healthy Individuals

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Research article

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Abstract

Background: To investigate the characteristic expression of circular RNAs (circRNA) in the peripheral blood of individuals with latent autoimmune diabetes in adults (LADA) and healthy individuals(NG) to identify dysregulated circRNAs that could be used to differentiate between LADA and healthy individuals.

Results: A total of 992 differentially expressed circRNAs were detected in the Group LA vs Group NG:209 circRNAs were significantly upregulated and 783 circRNAs downregulated.These circRNAs distributed on all chromosomes, including the sex chromosomes, and most of the differentially expressed circRNAs were derived from exons. Two circRNAs were identified as the most distinctive differentially expressed targets, hsa_circ_0097425 and newly-discovered circRNA-hsa_circ_62540520. We used qRT-PCR to verify the differentially expressed circRNAs, both circRNAs were statistically significant ($p < 0.05$). Further studies have found that the two circRNAs may be associated with metabolic syndrome , inflammation and cell cycle.

Conclusion: We determined that differentially expressed circRNAs could be used as new biomarkers for distinguishing LADA from healthy individuals. Further investigations may illustrate the partial pathogenesis of diabetes mellitus.

Trial registration: ChiCTR, ChiCTR1900020644. Registered 11 January 2019, http://www.chictr.org.cn/showproj.aspx?proj=30591

Background

Diabetes mellitus is a metabolic disease characterised by chronic hyperglycaemia that occurs due to a combination of genetic and environmental factors. However, the etiology and pathogenesis of diabetes has not yet been fully clarified [1]. So far, comprehensive diabetes classification is based on an understanding of the clinical manifestations, pathophysiology, and etiology of diabetes mellitus. Types of diabetes mellitus include type 1 and type 2 diabetes, gestational diabetes, genetic defects in β cell function and other special types [2]. Individuals that experience a slow start-up and inconspicuous early clinical manifestations could experience a long or short phase of insulin-free treatment. Early clinical manifestations in these individuals appear as type 2 diabetes mellitus, yet are characterised by islet β cell autoimmune damage seen in type 1 diabetes. This is considered as latent autoimmune diabetes in adults (LADA) [3]. The diagnostic criteria for LADA from the Immunology of Diabetes Society are: (1) adult age of onset > 30 years, (2) positive autoantibody to any islet cell, and (3) absence of insulin requirement for at least 6 months after diagnosis [4].

Circular RNA (circRNA) is a kind of non-coding RNA, which is derived from exonic circular RNAs (circRNAs), intronic circular RNAs (ciRNAs), or a combination of the two known as exon-intron circRNAs (ElciRNAs). Some circRNAs can be used as ‘microRNA (miRNA) sponges,’ playing a role in posttranscriptional regulation by engaging in competitive combination with miRNA [5]. CircRNA has a circular structure, is more stable *in vivo*, and is more abundant in tissues, which renders them to be

potential diagnostic molecular markers for various diseases [6]. For example, many recent studies on the roles and clinical significances of circRNAs in cancer are described below. It reported that increased expression levels of hsa_circ_0005273, spliced from the pre-mRNA PTK2, was involved in the metastasis of colorectal cancer via physical binding to phosphorylation sites Ser38, Ser55 and Ser82 of vimentin [7]. The over expression of circ-0051240 in ovarian cancer tissues enhanced tumor formation, cell growth, progression and invasion, in vivo and in vitro [8]. Besides, circRNAs are involved in the pathogenesis of chronic diseases. CircSlc8a1 can function as an endogenous sponge for miR-133a in cardiomyocytes, may serve as a novel therapeutic target for cardiac hypertrophy [9]. What's more, a present study speculated that hsa_circ_0046159 might be involved in the development of chronic thromboembolic pulmonary hypertension, participating in CTEPH development via regulating ATP2A2 expression by sponging miR-1226-3p [10]. As a classic disease in chronic diseases, there are many studies showing that circRNAs are closely related to the occurrence and development of diabetes.A study investigated the expression profiles of circRNAs in the peripheral blood of patients with T2DM and validated the utility of hsa_circ_0054633 as a diagnostic biomarker for pre-diabetes and T2DM [11]. A retrospective case–control study found that hsa_circRNA_0054633 was highly expressed in the blood during the second and third trimesters, and was highly correlated with GHBA1 and GHBA1c levels in maternal blood samples at various stages of the GDM group [12].

In this study, we investigated the characteristic expression of circRNAs in the peripheral blood of participants with latent autoimmune diabetes in adults and healthy individuals. The circRNA expression profile in participants with LADA and healthy individuals was systematically explored by comparison with matched control participants via RNA sequencing. Our findings provide important insights into the roles of circRNAs in individuals with diabetes.

Results

Differentially expressed circRNAs

A total of 992 differentially expressed circRNAs were detected in the Group LA vs Group NG:209 circRNAs were significantly upregulated and 783 circRNAs downregulated. These circRNAs were distributed in all chromosomes (Fig. 1). Most differentially expressed circRNAs were derived from exons (Fig. 2).

After screening and comparison, 2 dysregulated circRNAs are listed in Table 1, which were selected according to the following conditions: 1. Basic parameters: the difference multiple is greater than 1.5, and the p value is less than 0.05; 2. Exon source for subsequent functional research; 3. Combined with literature. One of which was recognized as unannotated, new circRNAs in the circBase or Circ2Traits database. According to the txStart genome coordinates of the circRNA, we named it hsa_circ_62540520.

Bioinformatics analysis

Compared to Group NG, 209 circRNAs were significantly upregulated and 783 circRNAs downregulated in the Group LA (Fig. 3a, b, and c). The functions of differentially expressed circRNAs were annotated and

hypothesised by gene ontology (GO) analysis of the host genes, including biological process (BP), cell component (CC), and molecular function (MF), as shown in Fig. 4(a-c) and Fig. 5(a-c). We found that the most significantly enriched GO terms in BP were organelle organization, mitotic cell cycle, cell cycle and cellular macromolecule metabolic process. GO terms in CC were intracellular part, intracellular, nucleoplasm and nuclear lumen. GO terms in MF were protein binding, binding, protein binding and protein serine/threonine kinase activity. The results of Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis are shown in Fig. 4(d) and Fig. 5(d). Differentially expressed circRNAs were mainly associated with the influenza A, herpes simplex infection, HTLV-I infection and cell cycle.

The source gene CDK1 of hsa_circ_62540520 was significantly enriched in the KEGG pathway, analysis of which was cell cycle (http://www.genome.jp/kegg-bin/show_pathway?hsa04110+996+983+1869+4998+7029, Fig. 6), and the difference was statistically significant (p value<0.05).

Differentially expressed circRNA validation by qRT-PCR

To verify the sequencing results, the top 2 most differentially expressed circRNAs were further confirmed by qRT-PCR (Fig. 7). We used qRT-PCR technology to draw the dissolution curve and amplification curve in real time. All primers (Table 2) were synthesised by Sangon Biotech (Shanghai, China). The results showed that the PCR amplification curve was smooth at baseline, and the exponential amplification period was obvious until the plateau period. The parallelism between the secondary pores was good, indicating that the PCR amplification was good. The dissolution curves were unimodal and no heterozygous peaks, which proved that the products amplified had specificity and no other non-specific products existed.

Prediction of circRNA-miRNA interaction and the circRNA network

We predicted the potential target miRNAs and coding genes of hsa_circ_62540520, hsa_circ_0097425 using MiRanda and TargetScan software (Table 3). The network map (Fig. 8) shows the top five miRNAs that potentially link to the circRNA, and the most likely target genes for each miRNA, clearly identifying the potential targets for the differentially expressed circRNAs.These findings lay the foundation for studying the specific mechanisms of these circRNAs in LADA.

Discussion

According to the Diabetes Atlas of International Diabetes Federation, 10 million people are newly diagnosed with diabetes every year [13]. A Chinese LADA study found that rate of LADA in individuals over 30 years old newly diagnosed with type 2 diabetes mellitus was 5.9% [14]. The development of diabetes has become an urgent public health threat problem, which has a great impact on social economy and needs to be solved. In fact, diabetes has become a global epidemic associated with the leading cause of hospitalization and death in older populations.

LADA is currently classified as T1DM, but its clinical manifestations are similar to T2DM. The autoantibodies of the patients with LADA indicate the pathogenesis of autoimmune diseases. However, the autoimmune process of LADA seems to be milder, and the procession of islet β cell failure is slower and consistently shows a higher level of C-peptide as an indicator of insulin secretion, indicating that other mechanisms also play an important role in the pathogenesis of LADA [15]. Studies have shown that the number of natural killer cells in individuals who were newly diagnosed with LADA is similar to that in individuals with type 1 diabetes, but lower than that in individuals with type 2 diabetes [16]. The degree of insulin resistance in individuals with LADA is similar to that in individuals with T2DM [17]. Another Chinese LADA study found that the prevalence of metabolic syndrome in individuals with LADA is slightly lower in those with type 2 diabetes mellitus, but higher in those with type 1 diabetes and healthy individuals [18]. To summarise, while some of the relationships and differences among individuals with type 1 diabetes, type 2 diabetes, and LADA have been explored, the nature of these connections has not yet been fully elucidated.

Circular RNA was first discovered by scientists using electron microscopy to observe the virus in the 1970s. However, due to technical limitations, circRNAs were initially considered as by-products of mal-splicing of mRNA, with extremely low levels in cells.With the development of scientific and technological advances, especially high-throughput sequencing and bioinformatics, we gradually uncover the mystery of circRNAs. Most of the circRNAs currently studied are produced by antisplicing of the messenger RNA precursor (pre-messenger RNA, pre-mRNA) of the exon, where the downstream 5' splicing site is connected to the upstream 3' splicing site, and the 3'-5' phosphodiester bond is connected to produce a circular structure. The characteristics of circRNAs are also worthy of mention due to their circular structure, which is different from other linear non-coding RNAs:1. Universality: circRNA has been found in a variety of eukaryotic cells, including human, fruit fly and yeast [19]. 2. Stability: circRNA has a covalent closed-loop structure, unlike linear non-coding RNA, which has a polar terminal of 3'/5' or a polynucleotide tail. Therefore, it is not affected by ribonuclease R and RNA nucleic acid exonuclease, and has strong stability.3. High abundance: due to its strong stability, its abundance can be more than 10 times that of corresponding linear RNA, and it can exist not only in cells but also in extracellular fluids [20]. Therefore, real-time fluorescence quantitative PCR can be used to detect the expression level of circRNA in tissues.4. Specificity: specific expression of circRNA in tissue or developmental stage can be observed in multiple times in the same organism, and such specificity also exists in different species [21]. Because of this specificity, we can compare the circRNA in the normal dynamic equilibrium state with the disease state and use it as a target for disease diagnosis and treatment.

Metabolic syndrome refers to the pathological status of metabolic disorders of proteins, fats, carbohydrates and other substances in the human body. It is a group of complex metabolic disorders, which is a risk factor for dyslipidaemia, hyperglycaemia, and cerebrovascular diseases [22]. Individuals with metabolic syndrome often show a state of pro-inflammation. The change in cytokine expression may be one of the mechanisms of low inflammation accompanying disorder of lipid and glucose metabolism [23]. The hsa_circ_0097425 gene, *HECTD4,* has been reported to be a pleiotropic gene that regulates metabolic syndrome and inflammation [24]. In our results, hsa_circ_0097425 was obviously

downregulated in participants with T2DM and LADA relative to the control group, especially in the LADA group. We speculate that hsa_circ_0097425 regulates metabolic syndrome and inflammation via *HECTD4*, and acts as a protective circRNA; this is consistent with the results of GO and KEGG analysis.

Recent studies have shown that circRNAs are derived from the exons or introns of their host genes, and may regulate the expression of these host genes [25]. The GO analysis results of our study showed that the circRNA host genes were related to diabetes development. Moreover, the results of KEGG analysis revealed that the host genes of significantly dysregulated circRNAs were involved in many important pathways, among which, the source gene CDK1 of hsa_circ_62540520 was significantly enriched in the KEGG pathway. The analysis of its KEGG pathway was cell cycle, which is highly consistent with the results of GO analysis, further validates that circRNA may be involved in the development of diabetes by regulating the cell cycle, and also provides ideas for us to select signaling pathways in further cell and animal experiments. Therefore, GO and KEGG analyses showed that the dysfunctional circRNAs might be involved in the pathogenesis of different types of diabetes. Nevertheless, we need further studies to confirm these findings.

Conclusions

Our study is the first to analyse circRNAs of Chinese participants with LADA and healthy people. Additional research is needed to verify circRNAs as a diagnosis and treatment target for individuals with LADA in the future. Our findings need to be further validated in larger, more diverse samples.

Methods

Study design

This study was approved and supervised by the Ethics Committee of The Second Hospital of Jilin University, Changchun, China (No. [2018]137). All experiments were performed in accordance with the Code of Ethics of the World Medical Association. All participants provided written, informed consent. This study conformed to the ethical guidelines of the 1975 Declaration of Helsinki, is registered with the Chinese Clinical Trial Registry, number Chictr1900020644, and was closed to accrual.

Study participants were categorised into two groups including LADA participants (Group LA) and healthy participants (Group NG). Experimental group participants were enrolled from the department of endocrinology outpatients and inpatients, The Second Hospital of Jilin University from February to May of 2019 and healthy participants were included from the population who underwent physical examination in the hospital during the same period. Table 4 shows the characteristics of the Group LA and Group NG.

LADA definition and whole blood sample collection

The LADA group contained participants who were 30–55 years at the time of diabetes diagnosis, who did not require insulin treatment for at least 6 months after diagnosis, and who had glutamic acid

decarboxylase autoantibody or other islet cell autoantibody (islet cell antibody-IgG, zinc transporter-8 Antibody, insulin autoantibody, protein tyrosine phosphatase-like IA-2 autoantibody) positivity. Exclusion criteria included other special types of diabetes or gestational diabetes, malignancies, serious liver and kidney dysfunction, any other acute or chronic inflammatory diseases, untreated essential hypertension (≥ 160/100 mmHg), any endocrine diseases other than diabetes, any acute and chronic diabetic complications, and pregnant or lactating women.

Study process

Each participant was tested using the American Diabetes Association diagnostic criteria to determine whether an individual was healthy or had diabetes. Five autoantibodies for diabetes (glutamic acid decarboxylase antibody, islet cell antibody-IgG, zinc transporter -8 antibody, insulin autoantibody, and protein tyrosine phosphatase-like IA-2 autoantibody) were used to determine whether the individual with diabetes was a LADA participant. Venous blood samples were collected from all control individuals and LADA participants with matched clinical features, and total RNA was extracted for further analysis.

RNA extraction and quality control

Blood samples from LADA and healthy participents were collected and centrifuged for 10 min at 3000 rpm and 4 ℃ to separate and collect serum. Samples were subsequently refrigerated at -80 °C prior to analysis. Total RNA of each specimen was quantified and quality assurance was confirmed using NanoDrop ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA). Standard denaturing agarose gel electrophoresis was performed to assess RNA integrity.

RNA library sequencing and subsequent bioinformatics analysis

For each sample we pooled random 3 blood samples in each group, in order to limit variation due to differences in vector injection. Cloud-Seq Biotech (Shanghai, China) performed transcriptome high throughput sequencing and subsequent bioinformatics analysis. Ribo-Zero® rRNA Removal Kit (MRZG12324;Illumina, USA) was used for the removal of rRNAs with total RNA. Subsequently, samples were quality controlled by BioAnalyzer 2100 system (SK-07017; Agilent Technologies, USA) and RNA libraries were constructed using rRNA-depleted RNAs with TruSeq Stranded Total RNA Library Prep Kits (batch No. 20020596; Illumina, USA).

Scatter plots, volcanic maps, and hierarchical clustering maps were used to evaluate circRNA expression variation. Differential circRNA identification, with standardised read numbers, was utilised to calculate the statistically significant differential expression of circRNAs among different groups of samples (fold change ≥ 2.0 and $P \leq 0.05$). Differentially expressed circRNA genes were annotated, and the functions of the screened circRNAs were hypothesised using gene ontology (GO) analysis. Molecular data was integrated from genomics, transcription, proteomics, and metabonomics to infer the pathways and biological functions involved in differentially expressed circRNA using KEGG pathway analysis.

Differential circRNA-miRNA interactions were predicted to explore the functional role of circRNAs used as ‘miRNA sponges.’ The circRNA-miRNA network was constructed and displayed using Cytoscape software.

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) validation assay

Complementary DNA was produced by reverse transcription using a PrimeScript™ RT reagent kit (Takara Bio, Nojihigashi, Kusatsu, Japan) according to the manufacturer's instructions. CircRNA expression was measured by qPCR using SYBR® Green Master Mix (Takara, Tokyo, Japan) in a ViiA™ 7 Real-time PCR System (Applied Biosystems Inc., Foster City, CA, USA). All indexes were performed according to the following procedures: 95℃, 10min;40 PCR cycles (95℃, 10 seconds;60℃, 60 seconds (fluorescence collection).In order to establish the melting curve of PCR products, after the amplification reaction was completed, the reaction was conducted at (95℃, 10 seconds;60℃, 60 seconds;95℃, 15 seconds);It was slowly heated from 60℃ to 99℃ (the automatic -ramp Rate of the instrument was 0.05℃/ second).RNA relative expression was calculated by the $2^{-\Delta\Delta Ct}$ method.

All qPCR reactions were repeated three times. A single peak in the melting curve indicates the specificity of the PCR product.

Statistical analysis

We used SPSS Statistics software (version 22.0; IBM, Armonk, NY, USA) for statistical analysis and figures were generated using GraphPad Prism 5.0 (GraphPad Software, Inc. La Jolla, CA, USA). Categorical data were analysed by Chi-square test. Data were presented as mean ± SD of at least three independent experiments. $P < 0.05$ was considered statistically significant. The most representative circular RNAs were annotated using circBase or Circ2Traits databases [26]. These circular RNAs were selected on the basis of differences among groups identified by data analysis and the significance of their corresponding genes.

Declarations

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent

Informed consent was obtained from all individual participants included in the study.

Availability of data and materials

The statistics data used to support the findings of this study are available from the corresponding author upon request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

LN drafted the initial manuscript. YY and XF analysed the data; YC, ML have interpreted the results; XZ, HS, MY, LW have contributed to the literature search and bioinformatic analysis, HC has conceived the idea of the study and edited the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1 Top two dysregulated circRNAs

c	chrom	CircBase ID	Gene Name	*P* value	LogFC
62540521-62544619+	chr10	hsa_circ_62540520	*CDK1*	0.0152	5.3456
112707508-112717190-	chr2	hsa_circ_0097425	*HECTD4*	0.0050	-5.9020

Table 2 Primers used for qRT-PCR analysis of circRNA and mRNA levels

circRNA	Primer sequences, 5'-3'	AT (°C)
hsa_circ_62540520	F: AGAAAGTGAAGAGGAAGGGGT	60
	R: TTCTGCTGGAGTCGAGGAAC	
hsa_circ_0097425	F: GGTCGGGATTGCTTCTTTGG	60
	R: CCAAGTAGGTGCCACAGGTA	
GAPDH	F: GGCCTCCAAGGAGTAAGACC	60
	R: AGGGGAGATTCAGTGTGGTG	

AT, annealing temperature; F, Forward; R, Reverse

Table 3 Top 5 predicted miRNA binding to circRNAs

circRNA	miRNA	miRNA	miRNA	miRNA	miRNA
hsa_circ_62540520	hsa-miR-3609	hsa-miR-297	hsa-miR-4802-5p	hsa-miR-3658	hsa-miR-548aa
hsa_circ_0097425	hsa-miR-338-3p	hsa-miR-8070	hsa-miR-6500-3p	hsa-miR-4699-5p	hsa-miR-8066

Table 4 The clinical characteristics of the study population

Characteristic	Group LA (n=12)	Group NG (n=12)	*P* value
Male gender	6 (50%)	6 (50%)	1
Age	43.33±3.17	42.75±2.89	0.656
Hypertension	2	2	1
Smoker	5	4	0.689
BMI (kg/m^2)	21.80±1.53	22.99±1.19	0.053
TC (mmol/L)	4.26±0.63	3.47±0.72	0.012
TG (mmol/L)	2.74±0.62	1.98±0.54	0.007
HDL (mmol/L)	1.53±0.35	1.55±0.18	0.872
ALT (mmol/L)	17.04±4.42	15.08±4.07	0.421
AST (mmol/L)	22.06±2.40	19.69±3.29	0.067
HbA1c (%)	9.83±0.91	5.08±0.17	<< 0.001
GLU (mmol/L)	8.58±0.82	4.58±0.23	<< 0.001

Figures

Figure 1

CircRNA distribution by location in human chromosomes.

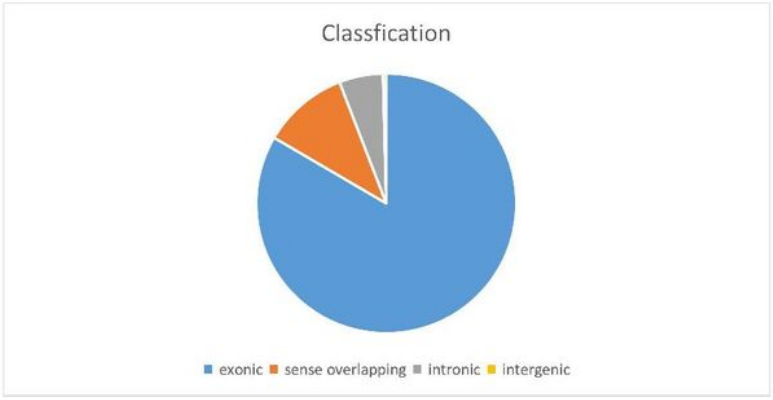
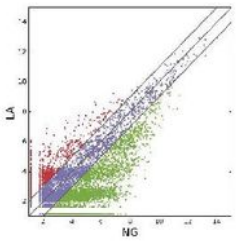
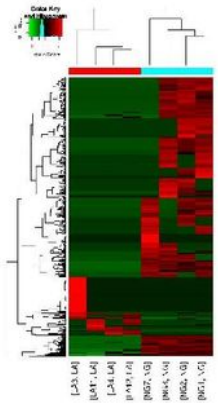


Figure 2

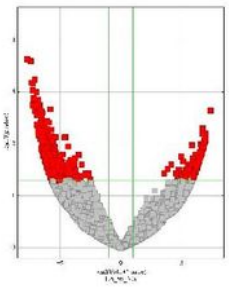
Classification of significantly dysregulated circRNAs based on genomic origin.



a



b



c

Figure 3

Changes in the expression profiles of circRNAs in the Group LA and Group NG. (a) The scatter plot reveals that a significant difference existed in the distribution of circRNAs between the Group LA and Group NG. (b) The heat map depicts the hierarchical clustering of altered circRNAs in the Group LA and Group NG. Red represents upregulation while green represents downregulation. (c) The volcanic map displays the upregulated and downregulated circRNAs in the Group LA and Group NG.

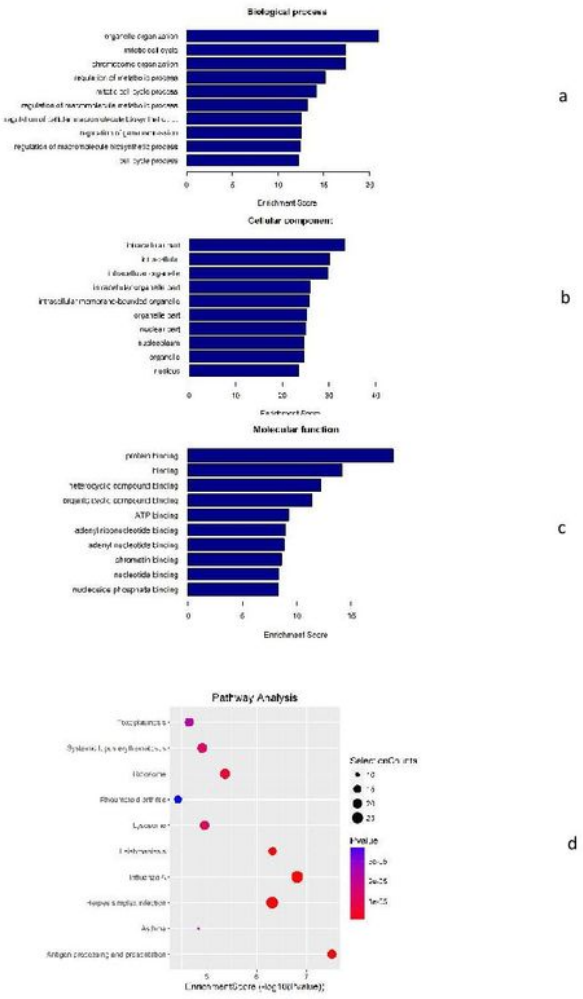


Figure 4

GO and KEGG signaling pathways for the circRNAs that are differentially downregulated in the Group NG and Group LA. (a) Biological process: organelle organization, mitotic cell cycle. (b) Cellular component:intracellular part, intracelluar. (c) Molecular function:protein binding, binding. (d) Analysis of the KEGG signaling pathways of circRNAs:influenza A, herpes simplex infection.

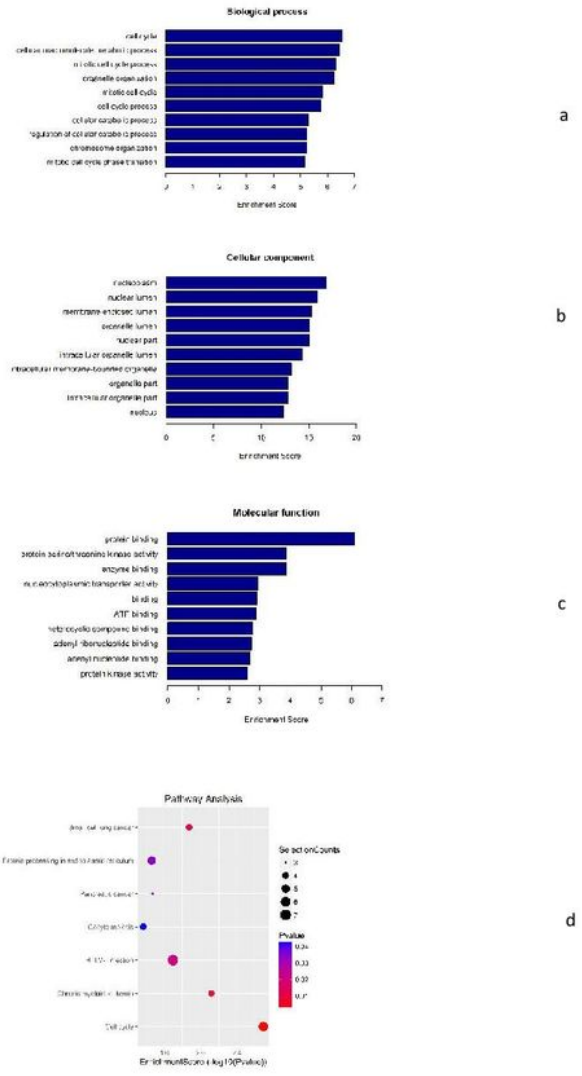


Figure 5

GO and KEGG signaling pathways for the circRNAs that are differentially upregulated in the Group NG and Group LA. (a) Biological process:cell cycle, cellular macromolecule metabolic process. (b) Cellular component:nucleoplasm, nuclear lumen. (c) Molecular function:protein binding, protein serine/threonine kinase activity. (d) Analysis of the KEGG signaling pathways of circRNAs:HTLV-I infection, cell cycle.

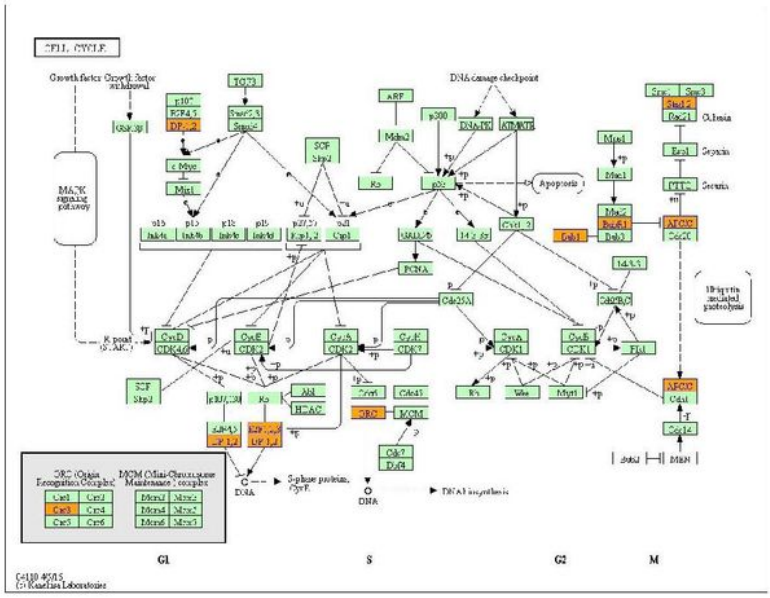


Figure 6

KEGG pathway of the source gene CDK1 of hsa_circ_62540520.

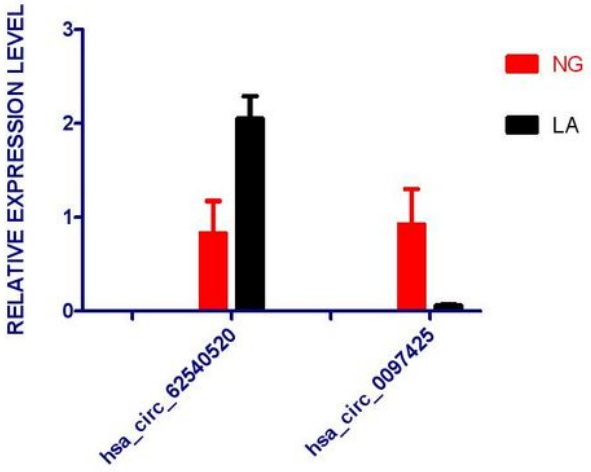


Figure 7

Validation of the top 2 dysregulated circRNAs validated by qRT-PCR. The results show the mean ± SD.

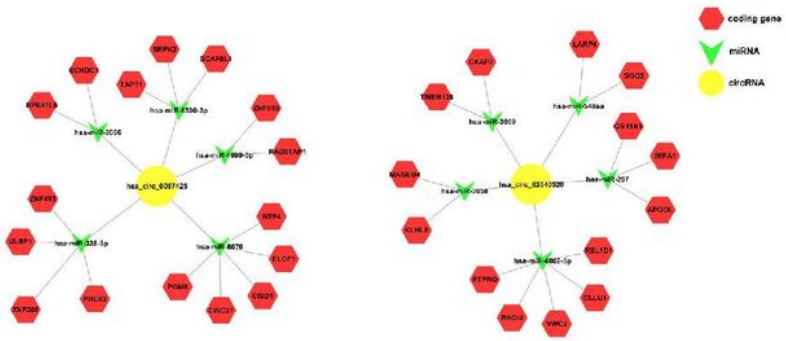


Figure 8

The network diagram of the top five miRNAs that may bind to circRNAs and several of the most likely target genes for each miRNA.