Spectrum construction of differentially expressed circular RNAs in patients with leukoaraiosis and function analysis of differentially expressed genes

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Abstract. Circular RNAs (circRNAs) are class of endogenous RNAs that have a role in the regulation of gene expression. The present study aimed to investigate the diagnostic value and role of circRNA in the pathogenesis of leukoaraiosis (LA). The present study performed Arraystar Human circRNA Array analysis of 6 samples from LA cases and 6 samples from control cases. Differentially expressed (DE) circRNAs between two samples were identified through fold-change (>1.5-fold) screening. Afterwards, based on DE circRNAs, the gene ontology (GO) analysis of upregulated DE genes identified from DE circRNAs demonstrated that DE genes were primarily associated with cellular metabolic processes, membrane-bound organelles and binding. However, none were enriched in the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway. Downregulated DE genes were enriched in cellular localization, cytoplasm and kinase binding. For the KEGG pathways, the downregulated DE genes were primarily associated with the insulin signaling pathway. The results of the present study indicated that the DE genes from differently expressed circRNAs may have an important role in the pathogenesis of LA and may be a novel targfet for further research.

Introduction

Leukoaraiosis (LA), a common form of cerebral white matter lesion (WML), is characterized by punctuate or patchy hyperintensities in the periventricular or subcortical white matter observed via magnetic resonance imaging (MRI). Although it remains asymptomatic, LA is not considered to be benign, and has been demonstrated to be associated with poor clinical outcomes and increased risk of disability, dementia, depression, stroke, and the overall morbidity and mortality (1). LA is associated with increased age, hypertension, diabetes mellitus, history of stroke and chronic atherosclerotic diseases (2). As hypertension is reported to be one of the major determinants of WMLs, the present study only included subjects with a history of hypertension. Despite intensive research, an understanding of the pathological mechanism remains incomplete, and is likely to be multifactorial. Therefore, identifying biomarkers is essential to improve the early diagnosis of LA.

Circular RNAs (circRNAs) are a class of endogenous RNAs that have a stable structure (3), which is a clear advantage of using circRNAs as a diagnostic marker. Previous studies have demonstrated that circRNAs are involved in the development of several types of diseases, including nervous system disorders (4,5). However, limited information is available regarding the association of circRNAs with LA. The present study, based on the hypertension population, compared the circRNA expression profiles of LA sufferers and controls. By performing a circRNA array analysis and reverse transcription-quantitative polymerase chain reaction (RT-qPCR), the present study demonstrated that certain circRNAs, including has_circ_102533 and has_circ_103783, may have potential as a novel type of biomarker for the diagnosis of LA. In addition, the gene ontology and KEGG analysis of differentially expressed (DE) genes may contribute to an improved understanding of the pathogenesis of LA.

Materials and methods

Subjects and clinical specimens. The current study was performed in accordance with the guidelines of the Helsinki Declaration. Written informed consent was obtained from all subjects and the Ethics Committee of Shandong Provincial

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Hospital Affiliated to Shandong University (Jinan, China) approved all aspects of the present study. A total of 30 subjects were recruited between August 2014 and September 2015 at Shandong Provincial Hospital Affiliated to Shandong University. All cases had a history of hypertension for 5-15 years. The groups were as follows: Test (LA sufferers; n=20; 7 males and 13 females) and control (no LA; n=10; 3 males and 7 females). The mean age was 58±5.7 years. The subjects had a mean systolic pressure of 150±7.1 mmHg and a mean diastolic pressure of 84±4.2 mmHg. LA is defined as punctuate or patchy hyperintensities in the periventricular or subcortical white matter, observed by MRI. Hypertension is defined as a history of high blood pressure (≥140/90 mmHg) reported by the respondent, or by the current use of antihypertensive medication. None of the cases suffered from diabetes mellitus, heart diseases, dyslipidemia, hypotension, immune system diseases, hematological disorders, malnutrition, malignant tumors, serious liver and kidney diseases (including severe hepatitis or nephritis), liver or kidney failure, or had a history of heavy smoking (20 cigarettes daily) or alcoholism. Fresh fasting peripheral blood samples (3 ml) were collected early in the morning and subsequently stored at -80˚C.

Microarray expression analysis. Based on random sampling, Arraystar Human circRNA Microarray analysis version 2.0 (Arraystar, Inc., Rockville, MD, USA) of 6 samples from LA cases and 6 samples from control cases was performed. The sample preparation and microarray hybridization were performed based on the Arraystar's standard protocols. Total RNA from each sample was quantified using the NanoDrop® ND-1000 (NanoDrop Technologies; Thermo Fisher Scientific, Inc., Wilmington, DE, USA). RNA Integrity and genomic DNA contamination test was performed by denaturing agarose gel electrophoresis. Sample labeling and array hybridization were performed according to the manufacturer's protocol (Arraystar Inc.). Briefly, 2,600 ng/µl total RNA (including 28s and 18s ribosomal RNA from each sample was treated with RNase R (Epicentre, Inc.) to enrich circRNA. The enriched RNA was subsequently amplified and transcribed into fluorescent complementary RNA (cRNA) utilizing random primers according to the Arraystar Super RNA Labeling kit protocol (Arraystar, Inc.). The labeled cRNAs were hybridized onto the Arraystar Human circRNA Mircoarrays (8x15K; Arraystar, Inc.) and incubated for 17 h at 65˚C in an Agilent hybridization oven. After washing four times in hybridization buffer the labeled cRNAs, slides were scanned with an Agilent G2505C Microarray Scanner System (Agilent Technologies, Inc., Santa Clara, CA, USA).

Data collection and analysis. Scanned images were imported into Agilent Feature Extraction software version 11.0.1.1 (Agilent Technologies, Inc.) for raw data extraction. Quantile normalization of raw data and subsequent data processing were performed using the R software package (R Project for Statistical Computing, Vienna, Austria). After quantile normalization of the raw data, low intensity filtering was performed, and the circRNAs that were flagged as 'P' or 'M' ('All Targets Value') in at least 3 out of 6 samples were retained for further analyses. When comparing profile differences between two groups (including disease vs. control), the 'fold change' (i.e., the ratio of the group mean averages) between the groups for each circRNA was computed. The statistical significance of the difference was determined by a t-test. circRNAs with fold changes >1.5 and P<0.05 were selected as significantly differentially expressed circRNAs. circRNA sequences were predicted by bioinformatics methods, as described previously (6).

RT-qPCR detection of hsa_circ_102470, hsa_circ_101396, hsa_circ_102533 and hsa_circ_103783. Total RNA in plasma was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and was quantified using the NanoDrop® ND-1000, according to the manufacturer's protocol. cDNA was synthesized by RT (Arraystar Super RNA Labeling kit; Arraystar, Inc., Rockville, MD, USA) 3 times using random primers and the Gene Amp PCR system 9700, according to the manufacturer's protocol. A total of 1 µg RNA, 1 µl random (N9), 1.6 µl dNTP mix (2.5 mM dATP, dGTP, dCTP, and dTTP, provided by HyTest Ltd) was combined to make the annealing mixture. This mixture was incubated in a 65˚C water bath for 5 min and then put on ice for 2 min. After brief centrifugation at 12,000 x g or 15 min at 4˚C, the RT reactant solution (4 µl 5X First-Strand Buffer, 1 µl 0.1 M DTT, 0.3 µl RNase inhibitor and 0.2 µl SuperScript III RT, provided by Invitrogen; Thermo Fisher Scientific, Inc.) was added to a centrifuge tube and incubated at 37˚C in a water bath for 1 min. The tube was then incubated at 50˚C in a water bath for 60 min, followed by 70˚C for 15 min. Subsequently, cDNA was placed on ice. qPCR was performed using Arraystar SYBR®-Green qPCR Master Mix (ROX-), 5 ml on the ViiA™ 7 Real-time PCR system. Cycling conditions were as follows: An initial predenaturation step at 94˚C for 20 sec, followed by 40 cycles of denaturation at 95˚C for 10 sec, annealing at 60˚C for 60 sec and extension at 72˚C for 15 sec. The primers used to quantify levels of the 4 circRNAs (hsa_circ_102470, hsa_circ_101396, hsa_circ_102533 and hsa_circ_103783) and GAPDH are presented in Table I. The data were analyzed using the relative values following internal calibration, presented as hsa_circ_102470/GAPDH, hsa_circ_101396/GAPDH, hsa_circ_102533/GAPDH and hsa_circ_103783/GAPDH. Subsequently, the ratios (average relative values in test group divided by the average relative values in control group) of the 4 circRNAs were further analyzed. The $2^{-\Delta\Delta Cq}$ method was used for quantification (5).

Prediction of DE genes and the function analysis of DE genes. GO covers three domains, including biological process, cellular component and molecular function. Fisher's exact test is used by professionals to establish whether there is a higher overlap between the DE list and the GO annotation list than expected by chance. The P-value denotes the significance of GO term enrichment in the DE genes. The lower the P-value, the higher the significance of the GO Term (P<0.05 is recommended). Pathway analysis is a functional analysis that maps genes to KEGG pathways. The P-value (EASE-score, Fisher-P-value or Hypergeometric-P-value) denotes the significance of the pathway associated with the conditions; the lower the P-value, the higher the significance of the pathway (The recommended P-value cut-off is 0.05).

Table I. Primers for reverse transcription-quantitative polymerase chain reaction.

Gene	Sequence		Annealing temperature (˚C)	Product length (bp)
	Forward	Reverse		
GAPDH	5'GGGAAACTGTGGCGTGAT3'	5'GAGTGGGTGTCGCTGTTGA3'	60	299
hsa_circRNA_102533	5'GCTGCCAAAAGCATAACCAA3'	5'CCCCTTTTCTGCTAAATGAACTCT3'	60	198
hsa_circRNA_103783	5'AAGCTGTTAGCATGATCCCACC3'	5'GATGAAACTTTTCCAAGTGTGGC3'	60	133
hsa_circRNA_101396	5'AAAGGTCCACTTCGTATGCTG3'	5'ACTCTGTCATTGGAGCAACTGTAT3'	60	221
hsa_circRNA_102470	5'CCTAAATTTCACGACACCAG3'	5'ATTCAGATTGCTCAAGGTAACT3'	60	144

circRNA, circular RNA.

Table II. Subject characteristics.

Characteristic	Total	LA	Control	P-value
Age, years	58±5.7	59±6.3	56±2.4	0.074
Number of males (%)	10 (33.3)	7 (35)	3 (30)	0.784
Systolic pressure, mmHg	150 (±7.1)	148 (±3.2)	153 (±11.0)	0.059
Diastolic pressure, mmHg	84 (±4.2)	82 (±3.9)	85 (±4.6)	0.162

Data are presented as the mean ± standard deviation (continuous variables) or as a proportion (discrete variables). LA, leukoaraiosis.

Statistical analysis. Data are presented as the mean ± standard deviation (continuous variables) or as a proportion (discrete variables). The differences between groups were analyzed by Student's t-test (continuous variables) or chi-squared test (discrete variables). SPSS 17.0 software (SPSS, Inc., Chicago, IL, USA) was used for statistical analysis.

Results

Differential expression of circRNAs. The characteristics of age- and sex-matched test and control group subjects are presented in Table II. Compared with the controls, certain circRNAs exhibited >1.5-fold change (P<0.05) in the LA group; 32 were upregulated and 132 were downregulated. Among the upregulated circRNAs, hsa_circ_103783, hsa_circ_101396 and hsa_circ_102533 exhibited the highest statistically significant difference in expression, while of the downregulated circRNAs, hsa_circ_102470 was identified to exhibit the largest difference in expression between the LA and control groups. The results of the RT-qPCR detection of hsa_circ_102470, hsa_circ_101396, hsa_circ_102533 and hsa_circ_103783 are presented in Fig. 1. The expression of hsa_circ_103783 and hsa_circ_102533 in the test group was significantly higher compared with the control group, with fold increases in expression of 3.67 (P<0.001) and 3.52 (P<0.05), respectively. The expression of hsa_circ_101396 was 1.25-fold higher in the test group compared with the control; however, this was not statistically significant (P=0.05). Comparing the expression of hsa_circ_102470 between the two groups, a 0.8-fold downregulation, which was not statistically significant, was observed in the test group (P=0.39).

GO analysis of DE genes. The top 10 most significant enrichment GO terms of the upregulated DE genes are presented in Tables III-V. The biological process was primarily enriched in metabolic processes, including nucleobase-containing compounds, and heterocycle and cellular aromatic compound metabolic processes. The major cellular components were organelles, membrane-bounded organelles, intracellular membrane-bounded organelles and intracellular organelles. The molecular function predominantly enriched in the activity of histone acetyltransferase and acetyl-CoA were L-lysine, N6-acetyltransferase and ion channel activity. The top 10 most significantly enriched GO terms among the downregulated DE genes are presented in Tables VI-VIII. The present study

Table III. Top ten gene ontology BP terms that upregulated genes were enriched in.

BP term, metabolic process	Enrichment score	P-value
Nucleobase-containing compound	2.66	0.002
Heterocycle	2.51	0.003
Cellular aromatic compound	2.50	0.003
Organic cyclic compound	2.33	0.004
Cellular nitrogen compound	2.33	0.004
Regulation of nucleobase-containing compound	2.33	0.004
Regulation of nitrogen compound	0.52	0.005
Nitrogen compound	2.24	0.009
Single organismal cell-cell adhesion	2.03	0.013
Nucleic acid	1.86	0.014

BP, biological process.

Table IV. Top ten gene ontology CC terms that upregulated genes were enriched in.

CC term	Enrichment score	P-value
Organelle	4.48	<0.001
Membrane-bounded organelle	4.24	<0.001
Intracellular membrane-bounded organelle	3.68	<0.001
Intracellular organelle	3.46	<0.001
SAGA-type complex	2.97	0.001
Nucleus	2.83	0.001
Nuclear part	2.18	0.007
Nuclear lumen	2.16	0.007
Histone acetyltransferase complex	2.12	0.008
Intracellular organelle part	2.11	0.008

CC, cellular component.

demonstrated that cellular localization was the most enriched biological process of DE genes, and the major cellular components involved were the cytoplasm and cytosol. The molecular function was predominantly enriched in kinase, enzyme and protein kinase binding.

KEGG analysis of DE genes. KEGG enrichment was identified for downregulated DE genes; however, not for upregulated DE genes. The top 10 most significant enrichment KEGG terms are listed in Table IX. The major KEGG item was the insulin signaling pathway, with 10.3% of downregulated DE genes involved in it (Fig. 2; http://www.genome.jp/kegg-bin/show_pathway?scale=1.0&query=&map=hsa04910&scale=0.67&auto_image=&show_description=hide&multi_query= and http://www.kegg.jp/kegg/legal.html).

Table V. Top ten MF terms that upregulated genes were enriched in.

MF term	Enrichment score	P-value
Histone acetyltransferase activity	2.46	0.003
Acetyl-CoA:L-lysine N6-acetyltransferase	2.46	0.003
Ion channel activity	2.46	0.003
Substrate-specific channel activity	2.42	0.004
Channel activity	2.34	0.005
Passive transmembrane transporter activity	2.34	0.005
Enzyme activator activity	2.29	0.005
Anion channel activity	2.12	0.008
N-acetyltransferase activity	2.11	0.008
GTPase activator activity	2.09	0.008

MF, molecular function.

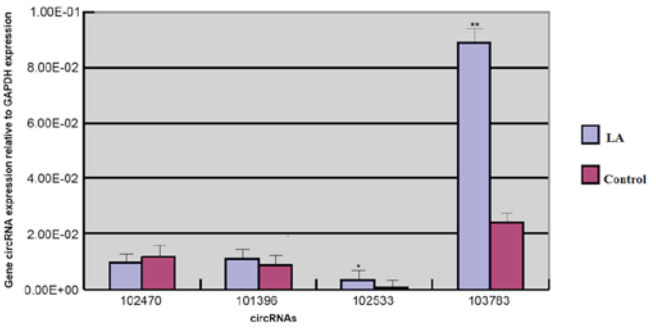

Figure 1. Mean relative expression of 4 circRNAs in LA and control groups. Mean relative expression of hsa_circ_102470, hsa_circ_101396, hsa_circ_102533 and hsa_circ_103783 was determined in LA and control groups. circRNAs, circular RNAs; LA, leukoaraiosis. *P<0.05 and **P<0.001.

Discussion

At present, understanding of the pathogenesis of LA remains to be fully elucidated, and there no reliable indicator for the early diagnosis of LA in the hypertension population. Previous studies have eported that LA may be associated with various factors, including age, sex (7), hypertension (2), small vessel disease and atherosclerosis (8). Hypertension is one of the most important established risk factors for LA (9), and pulse pressure was previously demonstrated to be independently associated with LA, regardless of classical cardiovascular risk factors in elderly men (10). However, patients with hypertension do not necessarily suffer from LA. To predict the potential for development of LA among patients with hypertension, the present study investigated potential predictors at the circRNA level.

Previously, circRNA was demonstrated to be a highly prevalent RNA species in the human transcriptome (6), and they may have important roles in the regulation of gene expression (11). circRNA expression levels can be >10-fold

Table VI. Top ten BP terms that downregulated genes were enriched in.

BP term	Enrichment score	P-value
Cellular localization	4.97	<0.001
Establishment of localization in cell	4.92	<0.001
Intracellular transport	4.39	<0.001
Macromolecular complex subunit organization	4.26	<0.001
Cytoplasmic transport	4.18	<0.001
Modification-dependent macromolecule catabolic process	4.10	<0.001
Organic substance catabolic process	3.98	<0.001
Catabolic process	3.97	<0.001
Cytoskeleton organization	3.84	<0.001
Organelle localization	3.83	<0.001

BP, biological process.

Table VII. Top ten CC terms that downregulated genes were enriched in.

CC term	Enrichment score	P-value
Cytoplasm	8.17	<0.001
Cytosol	8.16	<0.001
Membrane-bounded organelle	6.11	<0.001
Cytoplasmic part	5.46	<0.001
Intracellular part	5.42	<0.001
Organelle	5.33	<0.001
Intracellular	5.12	<0.001
Intracellular organelle	4.20	<0.001
Intracellular membrane-bounded organelle	4.18	<0.001
Macromolecular complex	3.36	<0.001

CC, cellular component.

Table VIII. Top ten MF terms that downregulated genes were enriched in.

MF term	Enrichment score	P-value
Kinase binding	6.17	<0.001
Enzyme binding	5.05	<0.001
Protein kinase binding	4.53	<0.001
Protein binding	3.74	<0.001
Transferase activity	3.48	<0.001
Nucleotide binding	3.01	<0.001
Nucleoside phosphate binding	3.01	<0.001
Protein homodimerization activity	2.89	0.001
ATP binding	2.82	0.002
Protein domain specific binding	2.81	0.002

MF, molecular function.

Table IX. Top ten KEGG pathways that downregulated genes were enriched in.

KEGG pathway	Enrichment score	P-value
Insulin signaling pathway, *Homo sapiens*	2.97	0.001
B cell receptor signaling	2.52	0.003
Thyroid hormone signaling	2.51	0.003
Axon guidance	2.38	0.004
mTOR signaling	1.86	0.014
Transcriptional misregulation in cancer	1.77	0.017
Chemokine signaling	1.68	0.02
Glyoxylate and dicarboxylate metabolism	1.64	0.02
Propanoate metabolism	1.64	0.02
Platelet activation	1.62	0.02

KEGG, Kyoto Encyclopedia of Genes and Genomes.

higher compared with their linear isomers (6,12). The two most important properties of circRNAs are highly conserved sequences and have a high degree of stability in mammalian cells (13). Certain circRNAs regulate gene expression by serving as competing endogenous RNAs (14). circRNAs block the inhibitory effect of microRNAs (miRNAs) on the target RNA by combining with miRNAs, so as to regulate the expression level of the target RNA (15). Currently, the circRNA with the most compelling evidence for a biological function is the miRNA-7 sponge, CDR1as (16). miRNA-7 was previously demonstrated to serve a key role in Parkinson's and Alzheimer's diseases (17,18).

Although an increasing number of studies have investigated the potential functions of circRNAs in the brain, to the best of our knowledge, the present study is the first to investigate the association between circRNAs and LA. The current study was the first to construct a spectrum of differentially expressed circRNAs in patients with LA, and to identify potential biomarkers for the diagnosis of LA and its pathogenesis. At present, there is no circRNA database for LA. The present study aimed to search a circRNA database for patients with LA and hypertension, via an Arraystar Human circRNA Microarray analysis of 6 test samples and 6 control samples. Subsequently, 32 upregulated and 132 downregulated circRNAs were screened out. Furthermore, 3 upregulated circRNAs (hsa_circ_101396, hsa_circ_102533 and hsa_circ_103783) and 1 downregulated circRNA (hsa_circ_102470) were selected based on a >1.5 fold-change (P<0.05), which were further

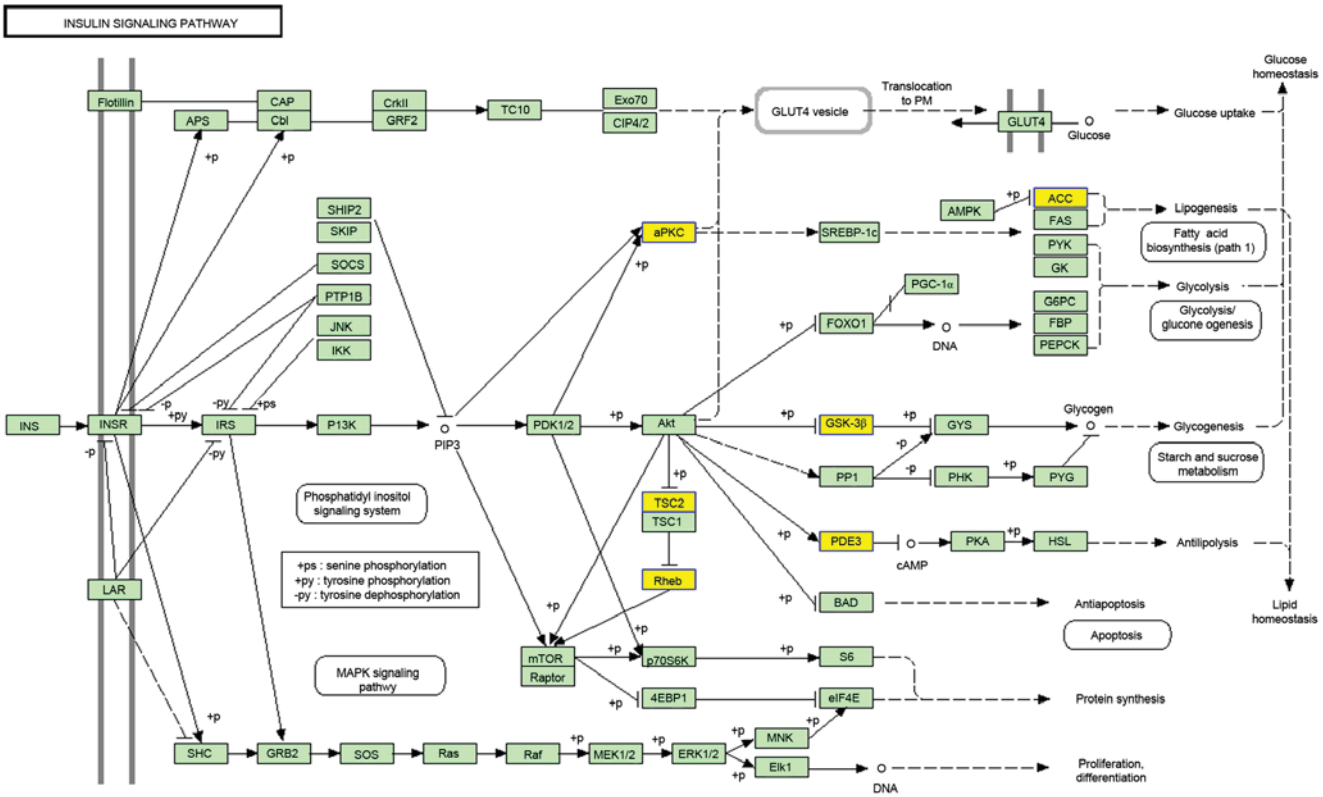

Figure 2. Insulin signaling pathway. KEGG enrichment analysis identified that genes that were significantly downregulated in patients with leukoaraiosis compared with controls were enriched in the insulin signaling pathway. The yellow boxes indicate the main compounds in the insulin signaling pathway.

assessed by RT-qPCR. The results of the current study demonstrated that the exonic RNAs, including hsa_circ_102533 (3.52-fold; P<0.05) and hsa_circ_103783 (3.67-fold; P<0.001), were significantly upregulated in the LA patients compared with the control group, indicating the potential diagnostic value of certain circRNAs for LA.

GO enrichment analysis of the upregulated DE genes indicated that DE genes were primarily associated with metabolic processes, organelles and the activity of certain transferases. However, it remains to be elucidated whether such gene products have the potential to be novel detectors for LA. In addition, GO and KEGG analysis of downregulated DE genes indicated that DE genes were primarily enriched in the cytoplasm, kinase binding and the insulin signaling pathway. The insulin signaling pathway was revealed to be pleiotropic, and included c-Cbl-associated protein, insulin receptor substrate-1, mitogen activated protein kinase, plasma cell glycoprotein-1, phosphoinositide-dependent kinase, protein kinase C and phosphatidylinositol 3-kinase (PI3-K). The involvement of phosphatidylinositol 3-kinase/protein kinase B (PI3-K/Akt) -dependent signaling pathways were found in normal cellular functions. The dysfunction of this signaling pathway has a pivotal role in atherosclerosis (19) and hypertension (20). Therefore, it was hypothesized that the PI3-K/Akt-dependent signaling pathway may contribute to the pathological changes of LA. However, this requires further investigation.

In conclusion, DE circRNAs were identified between patients with LA and controls among a hypertension population. The present study indicated that the upregulated circRNAs, hsa_circ_102533 and hsa_circ_103783, may have potential as biomarkers for the diagnosis of LA. GO and KEGG enrichment analyses of DE genes indicated that the DE genes were associated with the pathogenesis of LA. However, due to the limited number of plasma samples of LA, the present study only analyzed the expression of 4 circRNAs in 20 plasma samples with LA and 10 control samples. Future studies should increase the sample size to further confirm the diagnostic value of hsa_circ_102533 and hsa_circ_103783 for LA. Importantly, the conclusions were based on a large number of early experimental results; therefore, the specific mechanisms requires further investigation.

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