WITHDRAWN: High-throughput Analysis of CircRNA in Cows with Naturally Infected Staphylococcus aureus Mammary Gland

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The full text of this preprint has been withdrawn by the authors while they make corrections to the work. Therefore, the authors do not wish this work to be cited as a reference. Questions should be directed to the corresponding author.

Abstract

Circular RNAs (CircRNA) is a special type of non-coding RNA molecule with a closed ring structure and is not affected by RNA exonucases. It has stable expression and is not easy to degrade, and exists in most eukaryotes. However, circRNA regulation of cow mastitis has not been widely recognized. Mammary epithelial tissues were collected from healthy Holstein cows (HCN) and mastitis Holstein cows (HCU). RNA sequencing (RNA SEQ) was performed for the differentially expressed circRNAs, and analysis results showed that 19 differentially expressed circRNAs were identified in HCN and HCU, among which 6 circRNAs were up-regulated and 13 circRNAs were down-regulated. We randomly selected 9 circRNAs for Q-PCR verification, and the results showed consistent expression. Three circRNAs: circRNA2860, circRNA5323 and circRNA4027 were confirmed to be significantly differentially expressed circRNAs in cow mastitis. Furthermore, RNA polymerase transcription factor binding and tight junction are most enriched in GO and KEGG pathways, respectively. In addition, the regulatory network of circRNA-miRNA has been inferred from a bioinformatics perspective, which may help to understand the underlying molecular mechanism of circRNAs involved in regulating mastitis in cows.

Introduction

Mastitis in dairy cattle and buffalo is a clinical condition that causes significant economic losses and is being considered one of the largest constraints to the dairy industry worldwide[1]. *Staphylococcus aureus* is recognized worldwide as one of the main contagious mastitis agents in cattle and can express a set of antimicrobial resistance genes and virulence-associated genes that explain the wide range of outcomes of intramammary infections[2]. *Staphylococcus aureus* is a pathogen that is the causative agent of several human and veterinary infections and plays a critical role in the clinical and subclinical mastitis of cattle. *Staphylococcus aureus* survival in cells is an important cause of chronic persistent mastitis infection. However, it is unclear whether *Staphylococcus aureus* can escape autophagy in innate immune cells[3]. This bacterium causes significant economic losses, including a severe decline in milk revenue, reproductive complications, and expenses incurred from the culling of infected animals, increased costs of veterinary medication, and replacing tainted milk[4, 5, 6]. Furthermore, numerous types of toxins and enzymes in the milk produced by *Staphylococcus aureus* can lead to severe food-borne diseases[7]. In addition, several important pathways have been demonstrated to be related to the formation of cow mastitis, for example, TLR4/NF-κB pathway[8], PI3K/Akt/mTOR signaling pathway[9],and other signaling pathways. However, there is no systematic study on the molecular regulation of cow mastitis in mammary epithelium.

CircRNAs are a class of long, non-coding RNA molecules that form covalently closed continuous rings with relatively stable framework and have high tissue-specific expression in eukaryotic transcriptome[10]. Earlier, circRNAs were found and considered to have no biological function[11]. However, with the development of RNA deep sequencing technology and bioinformatics analyses, it has been reported that parts of circRNAs are endogenous, abundant, conserved and jarless in mammalian cells and own physiological functions[12, 13, 14, 15]. CircRNAs are composed of at least a few hundred nucleotides that

regulate gene expression at the transcriptional or post-transcriptional level by binding to microRNAs (miRNAs) or other molecules[16, 17, 18, 19]. Many studies have demonstrated that circRNAs contribute to the generation of cancer[20, 21], regulate gene expression in many biological processes, and participate in the occurrence and development of various diseases[22].

In the present study, we aimed to study the expression of circRNA in dairy cow mastitis. RNA-seq identification of differentially expressed circRNA in HCN and HCU. Functional analysis of differentially expressed circRNA was performed by gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment. In addition, the circRNA-miRNA network diagram was constructed to further explore dairy mastitis, but the molecular mechanism of mastitis has not been fully determined. This study provides a useful reference for further understanding the relationship between circRNA and dairy cow mastitis and also helps to inhibit the development of dairy cow mastitis.

Materials And Methods

Ethics statement. All experiments in this study were approved and conducted according to the Animal Experimental Committee of Shenyang Agricultural University, Shenyang, China (201606005).

Sample preparation. We collected scapular skin samples from six Holstein cow(three HCN and three HCU). The cows we choose are 1 to 2 years old with 1 to 4 parities. The epithelial tissue of a cow's breast samples from healthy holstein cows (d = 19.4 m 19.5 m and 19.8 m) and unhealthy holstein cows (d = 13.8 m 14.0 m and 14.1 m) from three adult females were carefully collected. The animals we collected were based on all the same conditions, including sex, age, feeding and physiological status and other factors. To reduce pain to experimental animals, we used local anesthesia with procaine. One third of the right superior scapula, along the midline of the dorsal and midline of the abdomen, was taken from the lateral skin of 6 holstein cows, about 1 square centimeter, and disinfected with 75% ethanol. The skin samples were then washed three times with PBS and immediately stored in liquid nitrogen until the RNA was separated. In addition, three healthy (19.7 d = 19.5 m m and 20.2 m) and three (ft) holstein cows with mastitis (15.4 d = 15.3 m m and 15.6 m) samples were analyzed using the same method. All animals belong to Shenyang Agricultural University.

Total RNA isolation, Library construction and Sequencing. The total RNA amount and purity of each sample was quantified by Nano Drop ND-1000 (Nano Drop, Wilmington, DE, USA). Approximately 5 ug of total RNA was used to deplete ribosomal RNA according to the manufacturer’s instructions for the Ribo-Zero rRNA Removal Kit (Illumina, San Diego, USA). In order to construct the cDNA library of circRNAs, were used Rnase R to remove linear RNA. The average insert size for the final cDNA library was 300 bp (± 50 bp), the library was purified and qualified by Agilent Bioanalyzer 2100 system.

Identification of circRNAs and analysis of differentially expressed circRNAs. The cDNA libraries were performed the paired-end sequencing on an Illumina Hiseq 4000 (LC Bio, China) following the vendor's recommended protocol. Firstly, low-quality reads and adapters were removed by Cutadapt v1.10, quality controlled by FastQC v0.10.1, and then obtained the high-quality clean reads. TopHat v2.0.4 was utilized

to map the clean reads to the reference genome from National Center for Biotechnology Information (NCBI) (https://www.ncbi.nlm.nih.gov/genome/?term=Capra+hircus)[58, 59]. Also, StringTie v1.3.0 was used to assemble and quantify expressed genes and transcripts (https://ccb.jhu.edu/software/stringtie/index.shtml)[60]. CIRCExplorer2 v2.2.6 software and the following criteria were used to identify candidate circRNAs: mismatch ≤ 2, back-spliced junction reads ≥ 1, and distances of two splice sites of less than 100 kb in the genome[61]. Then, the back-spliced reads with at least two supporting reads were annotated as circRNAs. The differential expression of circRNAs between the two groups was assessed using the Ballgown package. A *p*-value < 0.05 and |log2 (fc)| > 1 were set as the threshold for differential expression[62, 63], and R's phetmap package was used to draw the heatmap.

Gene Ontology (GO) analysis and KEGG analysis of host genes. GO analysis (http://www.geneontology.org) was applied to differentially expressed circRNA-hosting genes. Similarly, pathway analysis uncovered the significant pathways related to differentially expressed circRNAs according to the annotation of the Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.kegg.jp/kegg)[64]. A threshold of $p < 0.05$ was used as a criterion for the determination of whether the enrichment analysis was significant[65].

Network construction of the circRNAs-miRNAs interaction. The interaction of circRNAs-miRNAs was predicted with miRNA target prediction software miRanda (http://www.microrna.org/microrna/home.do) and TargetScan (http://www.targetscan.org/)[66], where the max free energy values of miRanda is < -10 and the score percentiles of TargetScan is ≥ 50. The differential expression circRNAs-miRNAs interaction and the network of circRNAs along with their target miRNAs were performed using cytoscape v3.5.1 software (https://cytoscape.org/, USA)[67].

Quantitative real-time PCR validation. We randomly detected 6 differentially expressed circrnas for QRT-PCR. To demonstrate the digestibility of circrnas to RNase R, we treated total RNA with RNase R prior to cDNA synthesis. To verify the differentially expressed circrnas, total RNA was synthesized directly into cDNA using RT-pcr kits. According to the manufacturer's instructions, real-time PCR was performed using SYBR Green (TaKaRa Biotech, Dalian). The glyceraldehyde – 3- phosphate dehydrogenase (GAPDH) gene was used as internal control to make circRNAs[68] expression level normal. Three independent experiments were conducted on HCN and HCU skin samples. Six pairs of primers were designed by primer 5 software (www.premierbiosoft.com) and are listed in supplementary table S1. Different analysis of the relative expression level circRNAs 2 - Δ Δ Ct method qPCR data[69]. Data were presented as mean ± standard deviation (n = 3). SPSS 22.0, Chicago, IL, USA was used for statistical analysis of the two groups of data. P < 0.05 was considered statistically significant. In addition, three ct-hcn and ft-hcu skin samples were verified by QPCR under the same experimental conditions to find the differential circrnas in HCN and HCU.

Results

Identification of circRNAs in HCN and HCU

A total of 13,679 circRNAs were identified from the RNA-seq data, including 705 circular intronic RNAs (ciRNAs) (Fig. 1a**)**. After deleting the low-quality original read data, the total read data was obtained. The mapping rates of HCN and HCU were 94.93% and 94.76% respectively. The HCN samples (45.87%) were compared with the HCU samples (50.6%), and the percentage of mapped sequence reads that could be aligned with the exon region were significantly lower (Fig. 1b). We analyzed the expression levels of HCN and HCU in circRNA, and the results showed that the expression of HCN was lower than that of HCN(Fig. 1C).

Identificaton of differentially expressed **circRNAs in HCN and HCU**

We identified 19 expressed circRNAs that different significantly in HCN and HCU. Of these, 6 and 13 proteins were up- and down-regulated in HCN and HCU, respectively(Table 1). Volcano plots representing these circRNAs were prepared (Fig. 2a), with differentially expressed circRNA (≥ 1.2-fold, $P < 0.05$) being located in the upper quadrant. Hierarchical clustering analysis was performed for the differentially expressed circRNAs to better display circRNAs abundance differences between groups(Fig. 2b).

Table 1
Up-regulated and down-regulated circRNAs in HCN and HCU

circRNA ID	Gene name	HCN FPKM	HCU FPKM	log2FC	p-value	regulation
circRNA8743	GALNT7	2	12	2.930768087	0.0296	up
circRNA7780	FBXO42	4	18	2.56065763	0.0114	up
circRNA5323	SLC12A2	4	20	2.711812883	0.0073	up
circRNA3780	B4GALT6	9	24	1.831701616	0.0491	up
circRNA2860	TRPS1	7	20	1.926049836	0.048	up
circRNA206	MBTPS2	2	12	2.930768087	0.0296	up
circRNA8726	AFDN	12	1	-3.02316057	0.028	down
circRNA7085	USP33	21	3	-2.33379349	0.0457	down
circRNA6352	EPS8	17	1	-3.52063365	0.0074	down
circRNA628	ZC3H13	32	6	-1.96301944	0.0323	down
circRNA6086	LARP4	17	1	-3.52063365	0.0074	down
circRNA4638	LIFR	107	25	-1.66029547	0.0255	down
circRNA4513	CREBRF	38	6	-2.20992877	0.0148	down
circRNA4027	MYH11	38	3	-3.18501273	0.0023	down
circRNA3689	TACC1	17	1	-3.52063365	0.0074	down
circRNA2584	TEAD1	12	1	-3.02316057	0.028	down
circRNA1407	DMGDH	14	1	-3.24311337	0.0198	down
circRNA1330	EHMT1	54	7	-2.49648070	0.0036	down
circRNA1119	ENSBTAG00000010619	39	5	-2.07968379	0.0335	down

Functional analysis of differentially expressed circRNAs

We performed GO and KEGG enrichment analysis for differentially expressed circRNAs. A total of 19 circRNAs were enriched in GO terms, and the top 25, top 15 and top 10 in biological processes, cellular components and molecular functions, respectively (Fig. 3a). The RNA polymerase transcription factor binding is the most enriched in GO terms (Fig. 3b), and the tight junction is the most enriched in KEGG pathway (Fig. 3c). They are closely related to cow mammary tissue epithelial tissue and could be involved in the regulation of cow mastitis.

Analysis of interactions between circRNAs and miRNAs.

It is generally accepted that circRNA is an adsorbed miRNA sponge and interacts with miRNA. We predicted the potential circRNAs-miRNAs interactions for these differential circRNAs, and the results indicated that the co-expression networks included 9 differentially expressed circRNAs, their 121 miRNAs (Fig. 5). The results suggested that circRNA4027 may function as a sponge for these miRNAs, such as miR-4297, miR-4530, miR-5581-5p, miR-4703-3p and miR-6844.CircRNA5323 has an interaction with miR-4778-5p,miR-4762-3p miR-4309 miR-5008-5p miR-6070 miR-3198 miR-3615 miR-34b-3p miR-6769b-3p miR-4723-3p and miR-3183. In addition, the interactions among the nine differentially expressed candidate circRNAs and their target miRNAs are shown in Fig. 4.

Validation of circRNAs by qRT-PCR

To investigate the expression of circRNA and determine that circRNA may play an important role in the regulation of cow mastitis, we used qPCR to verify the differential expression of certain circRNAs in HCN and HCU. Nine differentially expressed circRNAs were selected and specific qPCR primers were designed in the circRNA junction region. RNA-SEQ results showed that circRNA2860 had the highest expression level in the up-regulated circRNA, while circRNA4027 had the highest expression level in the down-regulated circRNA. The qPCR experimental results of HCN and HCU were shown in Fig. 5. It was proven that these circRNAs really existed and showed similar expression patterns in the epithelial tissue of a cow's breast, with the majority exhibiting a higher expression level in cow mastitis. The results of circRNA2860, circRNA5323 and circRNA4027 are significantly differentially expressed in RNA-seq and qPCR, which suggests that they might play a key role in cow mastitis.

Discussion

For decades, mastitis has caused large-scale economic losses worldwide in dairy farming due to treatment costs, discarded milk, reduced milk yield, and increased culling rates[23–28]. A recent study from Canada estimated costs on typical dairy farms to be 662 Canadian Dollars per milking cow per year, in which nearly half of the costs were associated with subclinical mastitis[29]. Circular RNAs are a novel class of endogenous RNAs with covalently closed loop structures. They are generated during RNA splicing and arise from exons (exonic circular RNAs or circRNAs), introns (intronic circular RNAs or ciRNAs), or a combination of both (ElciRNAs)[30]. With the advent of high-throughput sequencing and novel computational approaches for non-polyadenylated RNA transcripts, thousands of circular RNAs have been successfully identified in various species[31]. Furthermore, circRNAs have been implicated in tissue and organ development and may play a role in various disease processes, such as neurodegeneration and cancer development[32, 33]. Circular RNA CircKIAA plays a regulatory role in cow mastitis epithelial cells. circRNAs are connected with hepatocellular carcinoma (HCC) according to the latest research. It is found that circ_0001649 expression is lower in HCC than in the adjacent tissues, and its expression is related to tumor size and tumor embolus[34]. Zhao et al. investigated the expression profile of circRNAs in early-stage lung adenocarcinoma tissues versus the normal tissues[35]. They identified a total of 357 circRNAs that were dysregulated in the early-stage lung adenocarcinoma, including 204 up-regulated circRNAs and 152 down-regulated circRNAs. CircRNA_404833 and

circRNA_406483 displayed significant differences in their expression between LC tissues and normal tissue[35].

The function of circRNAs remains largely unknown. A handful of circRNAs are involved in post-transcriptional regulation by functioning as "sponges" of miRNAs, reducing their ability to target mRNAs[36, 37]. Pu test results show hsa_circ_0000092 impaired miR-338-3p-mediated HN1 inhibition to aggravate the development of HCC, indicating that hsa_circ_0000092 is a potential candidate marker and therapeutic target for HCC[38]. In Zhong's experiment, it was concluded that circ_PUM1 is capable of binding to miR-136 and up-regulating its target gene NOTCH3, which can be reversed by overexpression of miR-136. Circ_PUM1 can compete with miR-136, leading to up-regulation of NOTCH3, and thereby promote the development of endometrial cancer[39]. According to his experiments, He found that circFUT8 functions as a tumor suppressor in BCa cells by targeting the miR-570-3p/KLF10 axis and may serve as a potential biomarker and therapeutic target for the management of BCa patients with LN metastasis[40]. In Hu's study of the mammary glands,he showed that circRNA-0001283 positively regulated HIPK3 expression by sponging miR-187. The results reveal a new functional circRNA-0001283 in breast cancer and may provide targets for developing novel therapeutic strategies for breast cancer[41]. In Hu's study of the mammary glands he showed that circRNA-0001283 positively regulated HIPK3 expression by sponging miR-187. The results reveal a new functional circRNA-0001283 in breast cancer and may provide targets for developing novel therapeutic strategies for breast cancer[42]. Cao's findings illustrate the critical role of circRNF20 / mir-487 a/hif-1 / HK2 axis in breast cancer progression and Warburg effect, providing an interesting insight into BC (breast cancer) neogenesis[43]. In Jia's experiments, circ_0007255 inhibited tumor growth in vivo. Circ_0007255 is the sponge of mir-335-5p regulating SIX2 expression in the BC process. Circ_0007255 functioned as a will oncogene in the progression of BC by regulating miR – 335-5 p/SIX2 axis and took a be a promising biomarker for BC treatment[44]. Circ-TFF1 is a facilitator in breast cancer relying on TFF1 by absorbing miR-326, providing a novel promising target for BC treatment[45]. However, the majority of functions and mechanisms of circRNAs remain unknown, which suggests that circRNAs may be a promising avenue to explore in medical research[46].

We obtained 217 terms from GO enrichment analysis, including 106 biological processes, 62 molecular functions, and 49 cellular components. Transepithelial ammonium transport, transcriptional activator activity, RNA polymerase II trans, RNA polymerase II transcription factor binding, regulation of actin filament length, protein localization to cell junction, positive regulation of prolactin signaling pathway, NMDA selective glutamate receptor complex, negative regulation of glucocorticoid-mediated signaling pathway, lactosylceramide biosynthetic process, histone methyltransferase activity H3-K9 specific, elastic fiber assembly, dimethylglycine dehydrogenase activity, choline catabolic process, cerebral cortex development, cation: chloride symporter activity, C2H2 zinc finger domain binding, ATF6-mediated unfolded protein response, ammonium transport, ammonium transmembrane transporter activity and adherence junction maintenance pathways are very significant for cow mastitis. Among them, the RNA polymerase II transcription factor binding pathway had the most significant effect on cow mastitis. Sodium houttuyfonate (SH) has been indicated to play an important anti-inflammatory role[47]. SH

significantly inhibited LPS-induced TLR4 expression and NF-κB activation. In summary, these results suggested that SH inhibited LPS-induced inflammatory response by inhibiting TLR4/NF-κB signaling pathway. SH is a potential agent for the treatment of mastitis[48]. The nuclear factor-kappa B (NF-κB) pathway proteins are key players in controlling both innate and adaptive immunity. However, the information on NF-κB pathway genes is very limited in mastitis resistance and milk production of Chinese Holstein cows[49]. Our data also enriched these pathways, it further illustrates the importance of these circRNAs in cow mastitis.

The expression of circRNAs has been appropriately correlated with an abundance of genes in different animal tissues[50–53]. PCR-SSCP was applied to analyze the polymorphisms of CXCR2 gene and its relationships with milk quality and mastitis in 160 cattle samples including Holstein dairy cow, Simmental dairy cow and Tongjiang cattle. The results showed that the gene had a significant effect on cow mastitis[54]. The authors examined a possible association of polymorphism of the ATP1A1 gene with somatic cell score and 305-day milk yields. Individuals with genotype CC in ATP1A1 had significantly lower somatic cell scores and 305-day milk yields than those with genotype CA. We also examined changes in Na(+), K(+)-ATPase activity of red cell membranes. The Na(+), K(+)-ATPase activity was significantly higher in dairy cows with genotype CC compared to the other two genotypes, and the Na(+), K(+)-ATPase activity of the resistant group was significantly higher than that of the susceptible group in dairy cows. We conclude that this polymorphism has potential as a marker for mastitis resistance in dairy cattle[55]. To test the possibility of gene therapy for treating dairy cow mastitis, two eukaryotic expression vectors harboring human lysozyme(hLYZ) cDNA, called pTLYZ and p205C3LYZ, were injected into milk pools of the mammary gland with mastitis, and their effectiveness was demonstrated by CMT assay. After the treatments, total bacterial numbers, as well as representative causative bacterial numbers(staphylococcus, streptococcus and E.coli), of the milk samples decreased and the milk yields increased[56]. There are MBL1 and MBL2 genes that encode the MBL-A and MBL-C proteins, respectively. This study was carried out to investigate the relationship between the variants of the bovine MBL2 gene and milk production traits, mastitis, serum MBL-C levels, and hemolytic complement activity in both classical pathways (CH50) and alternative pathway (ACH50) in Chinese Holstein cattle. Four single-nucleotide polymorphisms (SNPs) in exon 1 of the MBL2 gene in Chinese Holstein cattle and Luxi yellow cattle were identified by the direct sequencing method. MBL2 gene has a significant effect on cow mastitis[57].

Conclusions

In this work, we performed RNA-seq analysis that identified 13,678 circRNAs in HCN and HCU, of which 19 circRNAs were found to be differential expression. Functional analysis of differentially expressed circRNAs by GO and KEGG, RNA polymerase transcription factor binding and tight junction are the most enriched pathways. The result of qRT-PCR confirmed that three circRNAs (circRNA2860, circRNA5323 and circRNA4027) were significantly differentially expressed in HCN and HCU.The study of circRNA-miRNA regulatory network from the perspective of bioinformatics is helpful to understand the molecular mechanism of potential circRNA involved in the regulation of cow mastitis. Our findings will provide

meaningful resources for further research on the regulatory function of circRNAs in cow mastitis. This study provides a theoretical basis for circRNAs to prevent and control mastitis in cows.

Declarations

Ethics approval and consent to participate: All animals experiments used in this study were conducted in accordance with the guidelines of the Laboratory Animal Management Committee of Shenyang Agricultural University.

Consent for publication: The author agrees to publish.

Availability of data and material: The data that support the findings of this study are available from the corresponding author upon reasonable request.

Competing interests: The authors state that there are no competing interests.

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Figures

Figure 1

Information on circRNAs from RNA-seq in Holstein cow normal (HCN) and I Holstein cow unsound (HCU) skin tissue. (a) The types of circRNAs. (b) Distribution of exons, introns, and intergenic circRNAs. (c) The expression of circRNA

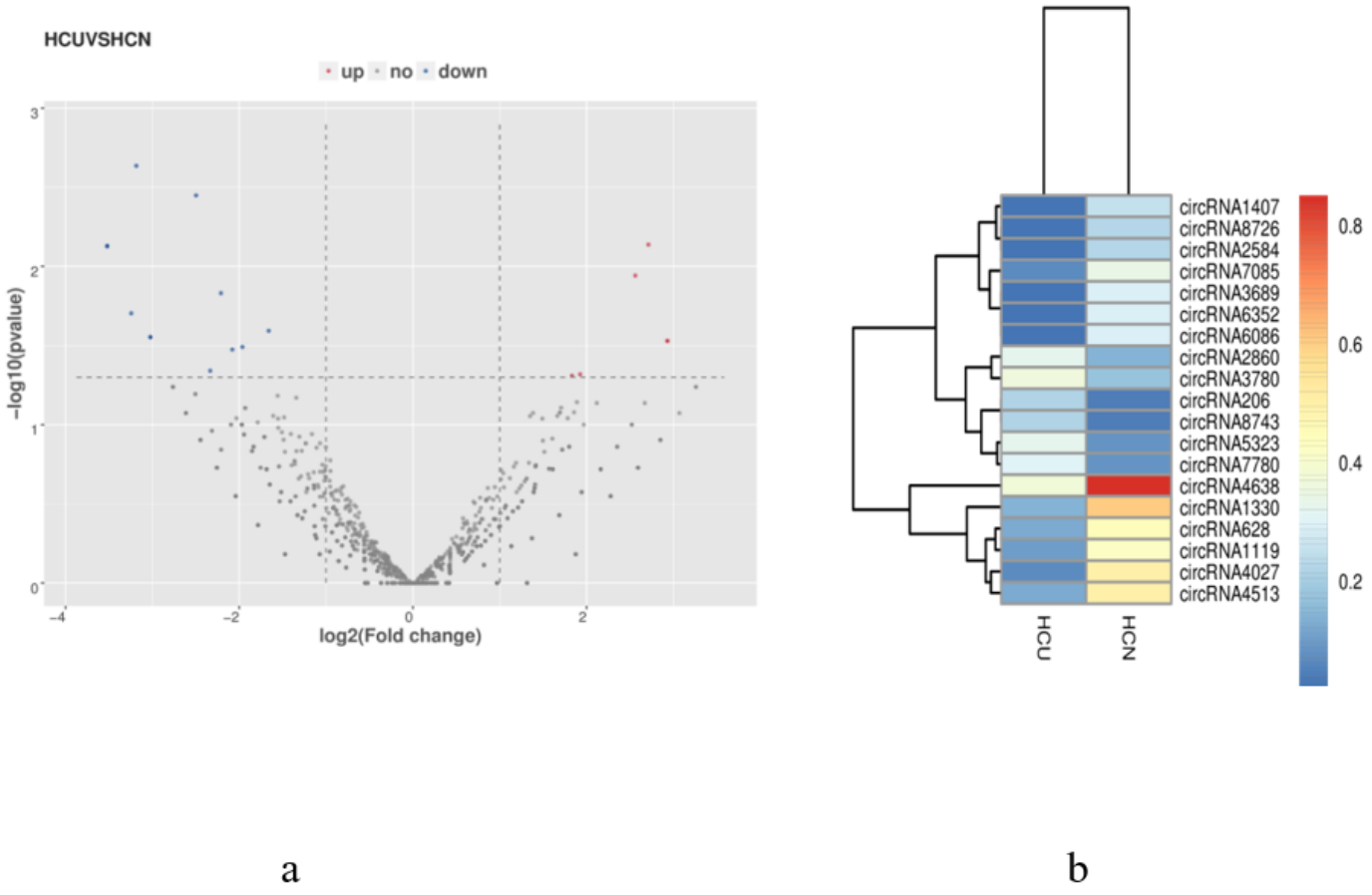

Figure 2

Differentially expressed circRNAs in HCN and HCU. (a) Volcano map of differentially expressed circRNAs. Red dots indicate up-regulation and blue dots indicate down-regulation. (b) Cluster heatmap of differentially expressed circRNAs. The sample is represented by the abscissa and the log value of circRNA expression is regarded by the ordinate, which means that the heatmap is drawn from log10 of circRNA expression. The highly expressed circRNA is indicated by red, meanwhile, the lowly expressed circRNA is presented by blue.

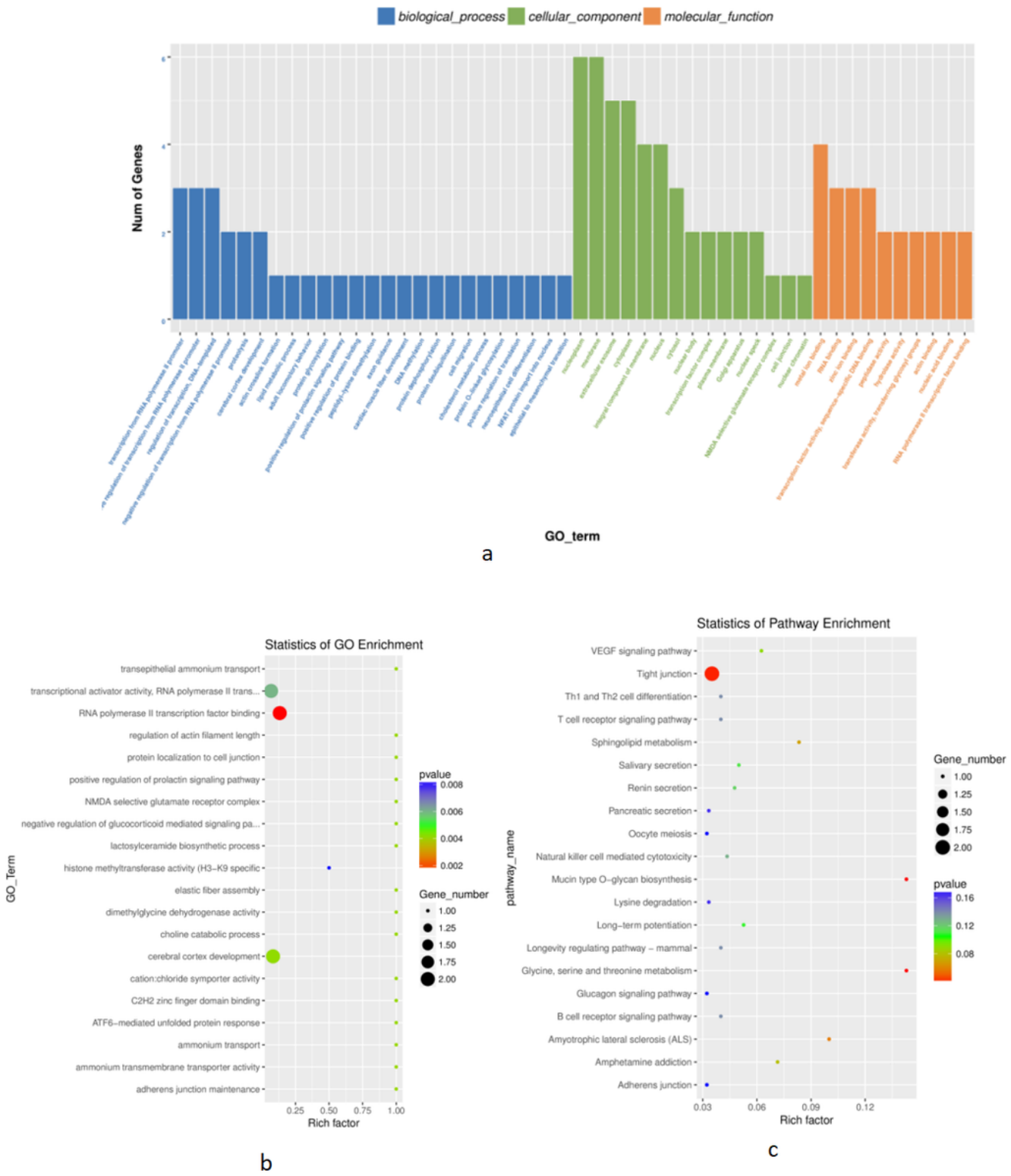

Figure 3

Functional analysis of differentially expressed circRNAs. (a) Classification of GO terms. (b) GO analysis of differentially expressed circRNAs. The color of the dot corresponds to different p-value ranges, and the size of the dot indicates the number of genes in the pathway. Rich factor denotes the number of differentially expressed circRNAs in the GO/ the total number of circRNAs in the GO. (c) KEGG pathways of differentially expressed circRNAs. The color of the dot corresponds to different p-value ranges, and the

size of the dot indicates the number of genes in the pathway. Rich factor denotes the number of differentially expressed circRNAs in the KEGG/ the total number of circRNAs in the KEGG.

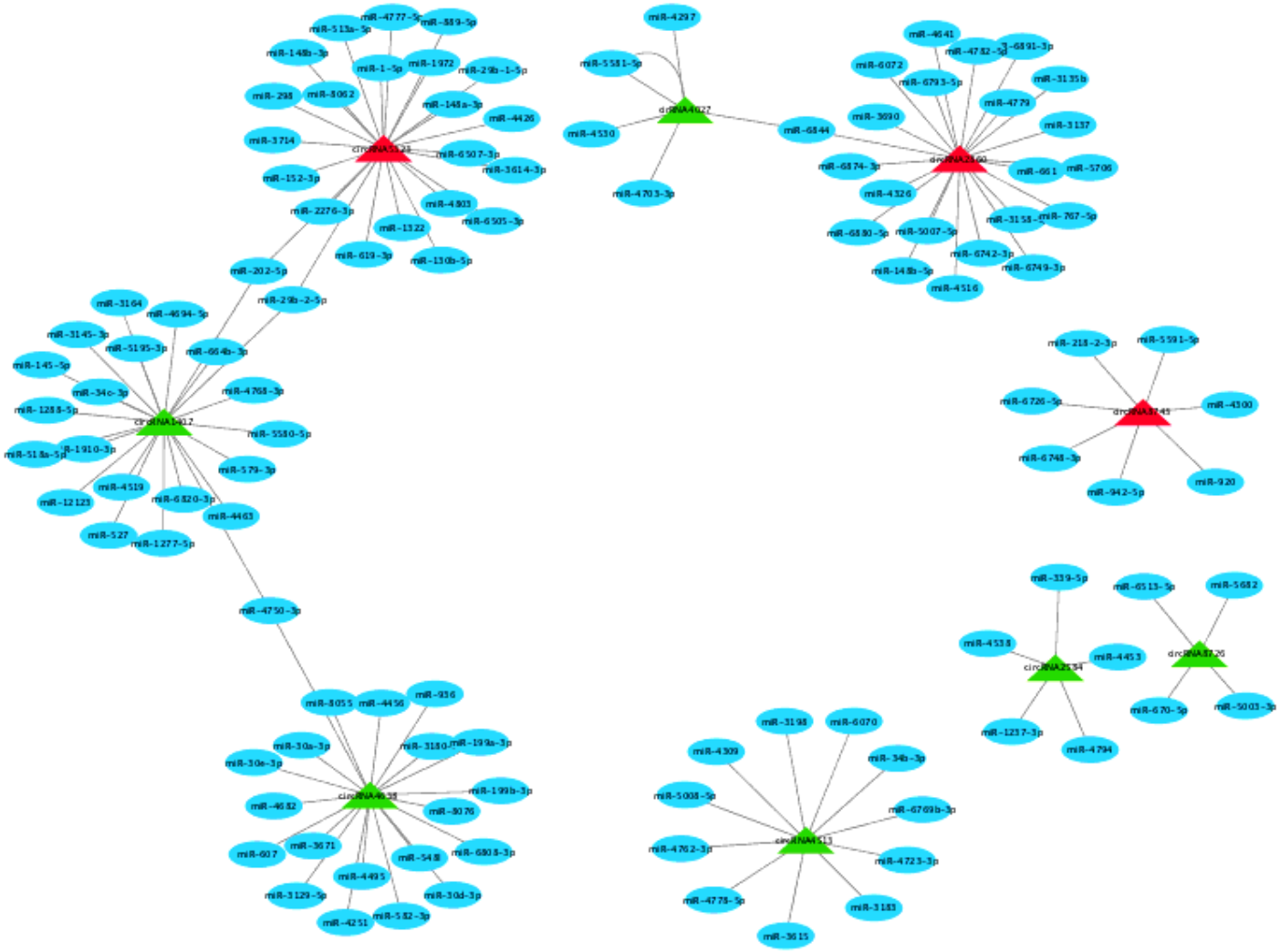

Figure 4

The circRNAs-miRNAs network. Network of 9 differentially expressed circRNAs. Red and green represent up and down regulation, and blue represents target miRNA.

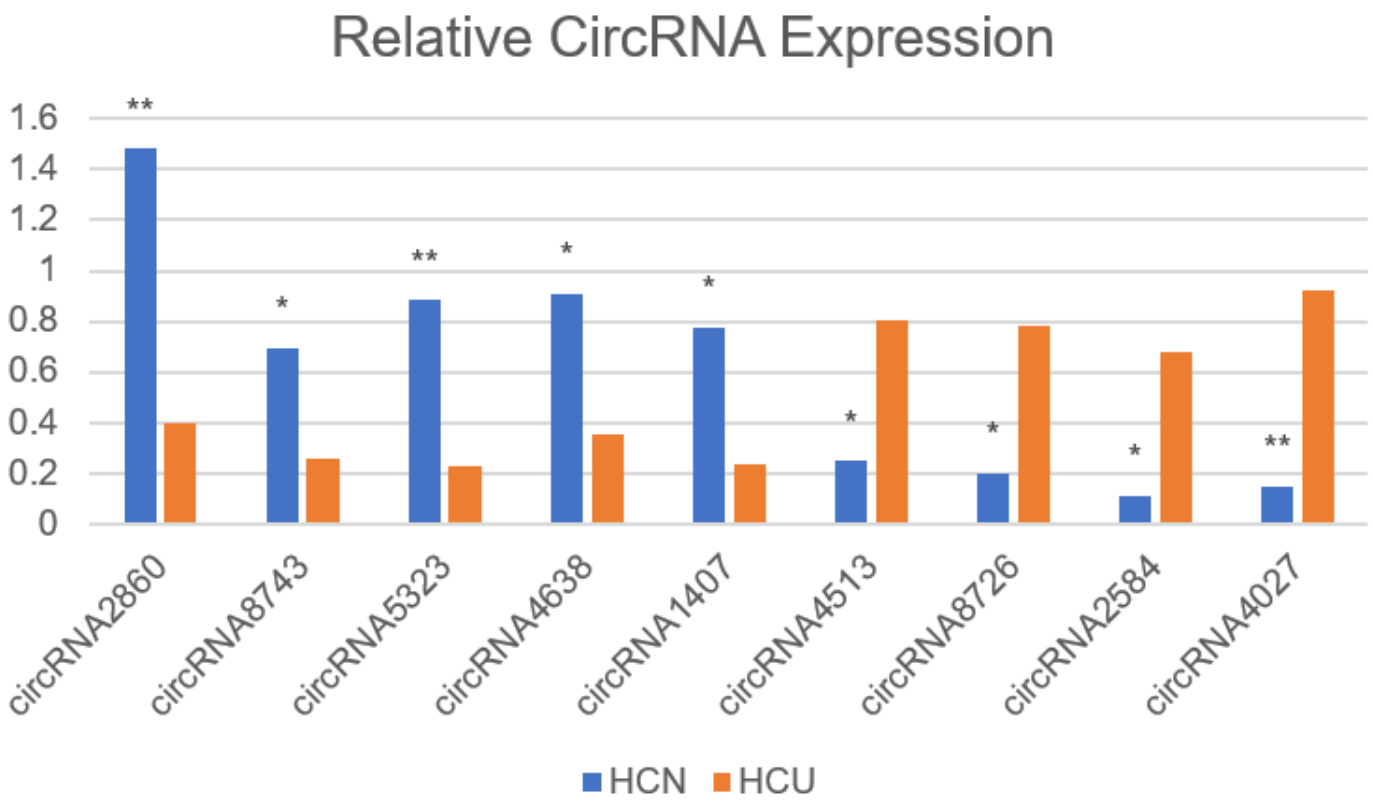

Figure 5

Quantitative real-time PCR results of circRNAs expression. Blue: HCN; Red: HCU. Error bars represents standard deviations within the group, the “*” indicates the significant difference $p < 0.05$, “**” indicates $p < 0.01$.