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Hsa_circ_0086414 Might Be a Diagnostic Biomarker of Oral Squamous Cell Carcinoma

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Background: Circular RNAs (circRNAs), a newly-discovered class of non-coding RNAs, have a significant role in the progression of cancers, but the effect of hsa_circ_0086414 in human oral squamous cell carcinoma (OSCC) is still unclear.

Material/Methods: The circRNAs expression profile in OSCC tissue samples was assessed by high-throughput sequencing. The hsa_circ_0086414 expression level in 55 paired OSCC tissue samples and 2 kinds of OSCC cells was evaluated by quantitative real-time polymerase chain reaction (qRT-PCR). Additionally, the correlation between the hsa_circ_0086414 expression and clinicopathological characteristics of individuals with OSCC was studied. We used receiver operating characteristic (ROC) curves to observe the hsa_circ_0086414 value of diagnosis in OSCC. The network of hsa_circ_0086414-miRNAs-mRNAs was constructed. Gene Ontology (GO), Disease Oncology (DO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) analyses were carried out based on sequencing data and bioinformatics predictions.

Results: Hsa_circ_0086414 expression in OSCC tissue samples and OSCC cells was first discovered to be significantly downregulated compared with the adjacent healthy tissues (AHTs) and normal (HaCaT) cells, respectively. Moreover, its expression level was significantly correlated with stage in TNM, size of tumor, and lymph node metastasis. The area below the ROC curve was 0.749. Hsa_circ_0086414-miRNAs-mRNAs network analysis and GO, DO, and KEGG analyses all demonstrated that hsa_circ_0086414 is correlated with cancer progression to a certain extent.

Conclusions: We discovered that hsa_circ_0086414 might be an essential diagnostic biomarker in OSCC. Furthermore, hsa_circ_0086414 could be a target for OSCC therapy.

MeSH Keywords: Biological Markers • Diagnosis • Mouth Neoplasms

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Background

Oral squamous cell carcinoma (OSCC), ranking ninth among cancers worldwide, is one of the most prevalent malignancies in the oral and maxillofacial area, and an increasing trend has been observed in the incidence rate of OSCC [1]. Approximately 600 000 people are diagnosed with OSCC each year and the age of the population is becoming younger annually at the time of diagnosis [2]. Although the diagnosis and clinical treatments of cancers have improved, the 5-year survival rate of OSCC individuals is still lower than 50% [3,4]. In addition, a high proportion of patients were diagnosed in the late stage. This might be an important reason for the poor prognosis of OSCC patients [5]. Moreover, 5-year survival rate of tongue squamous cell carcinomas (TSCC) is 80% when cancer was diagnosed early at stage in T1, but the rate is as low as 20% if diagnosed at stage in T4 [6]. Accordingly, it is imperative to discover new biomarkers for early diagnosis and treatment of OSCC.

Circular RNAs (circRNAs) are a new class of non-coding RNAs that are formed from covalently closed loop RNAs by back-splicing without 3' and 5' structure [7]. Circular RNA was first found in Sendai viruses and then was clearly observed in the eukaryotic cell cytoplasm by electron microscopy 40 years ago [8,9]. In recent years, many circular RNAs have been found by high-throughput sequencing technology and biological technology, and it is reported that they are stable and conserved and they can regulate the expression of cell-specific and tissue-specific genes [10–14]. Recent research has revealed that circRNAs can act as miRNA sponges, transcriptional regulators, or competing endogenous RNAs [15–19]. To the best of our knowledge, only 1 study, reported by Wang et al. [20]. demonstrated the microarray circRNA expression profiles in OSCC acquired by high-throughput sequencing. Recent studies demonstrated that circular RNAs might be a new type of biomarker for diagnosis and treatment of cancers [21,22], which might provide an essential molecular biological basis for exploring the complex progress in cancers. However, the role of circular RNAs in OSCC remains unclear.

In the present study, we analyzed the expression profiles of circRNAs and mRNAs in OSCC tissues through high-throughput sequencing. Prediction of circRNA-miRNAs-mRNAs relationships was constructed according to the sequencing results and bioinformatics predictions. According to the high-throughput sequencing results, we selected several significantly differentially-expressed circRNAs and verified the expression of circRNAs in 10 pairs of OSCC tissues and AHTs. The circRNAs expression levels indicated that hsa_circ_0086414 is the most obviously differentially-expressed circular RNA. In addition, Liu et al. [23] found that the target circRNA was underexpressed in lung adenocarcinoma, indicating that hsa_circ_0086414 might be closely correlated with cancer. Furthermore, hsa_circ_0086414 has never been studied in oral squamous cell carcinoma. Therefore, hsa_circ_0086414 was selected as a targeted circular RNA. We first identified that hsa_circ_0086414, which is mapped on chr9: 16435552-16437522, was remarkably downregulated in OSCC tissues and OSCC cells by qRT-PCR. Moreover, we discovered that the differential expression was significantly correlated with major clinicopathological characteristics of populations with OSCC. Additionally, an ROC curve was created to analyze the diagnostic potential of hsa_circ_0086414 in OSCC. Our research indicated that hsa_circ_0086414 might be a new biomarker for use in OSCC diagnosis.

Material and Methods

Tissues

Tumor tissues and AHTs were obtained from 55 patients undergoing surgery after being diagnosed with OSCC at the department of Oral and Maxillofacial Surgery in the Affiliated Stomatological Hospital of China Medical University from June 2017 to May 2019. All tissues placed in liquid nitrogen immediately after removal from patients and were stored frozen until use. Every patient signed an informed consent before participation, and the Affiliated Stomatological Hospital of China Medical University Ethics Committee approved this study.

Cell culture

CAL27 cells and SCC25 cells were obtained from the American Type Culture Collection (ATCC, USA) and the School of Stomatology, China Medical University (Shenyang, China), respectively. Normal (HaCaT) cells were also acquired from the School of Stomatology, China Medical University. Dulbecco's modified Eagle's medium (DMEM; ATCC, Manassas, VA, USA) with 10% fetal bovine serum (FBS; HyClone, USA) was used to culture CAL27 and SCC25 cells. HaCaT cells were maintained in DMEM with 10% FBS, 100 mg/mL streptomycin, and 100 U/mL penicillin. All cells were placed in a 37°C culture environment with 5% CO_2.

Sequencing analysis

The sequencing was performed by Gene Denovo Biotechnology Company (Guangzhou, China). After fresh specimens were obtained, samples (3 pairs of OSCC tissues and AHTs) were immediately frozen in liquid nitrogen. Total RNAs were obtained from samples and were digested by RNase R to remove the linear RNA, and then purified using the RNeasy MinElute Clean-up Kit. circRNAs were retained. After the circular RNAs were divided into small fragments with fragmentation buffer, random primers were used to produce cDNA through reverse transcription. dNTP (dUTP instead of dTTP), RNase H,

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DNA polymerase I, and buffer were used to synthesize second-strand cDNA. Subsequently, cDNA fragments were purified and the second-strand cDNA was digested using VAHTS™ DNA Clean Beads and UNG (Uracil-N-Glycosylase), respectively. The fragment size was selected by agarose gel electrophoresis and amplified by PCR. Then, we used Illumina HiSeq™ 2500 to perform PCR amplification and sequencing. Finally, differential expression of circRNA between OSCC tissues and AHTs was analyzed by edgeR software. Differential transcripts were screened by fold change and P values. Fold change >2.0 and P<0.05 were regarded as substantially different expression.

RNA isolation and qRT-PCR

Total RNAs of tissues and cells were obtained by use of Trizol reagent (Invitrogen, Carlsbad, CA, USA). The PrimeScript™ RT reagent Kit with gDNA Eraser (Takara Bio, Nojihigashi, Kusatsu, Japan) was used for reverse transcription. qRT-PCR was conducted by SYBR Green (Takara, Japan) in a Light Cycler 480 II Real-Time PCR system based on the product manuals. GAPDH expression was used as an internal control. The primer sequences of hsa_circ_0086414 were as follows:
Forward: 5'-CCGAAGCCGAGACAGGATG-3';
Reverse: 5'-CGCAGAAACTGCTGAAGGGT-3'.
The primer sequences of GAPDH are as follows:
Forward: 5'-CAGGAGGCATTGCTGATGAT-3';
Reverse: 5'-GAAGGCTGGGGCTCATTT-3'.
The $2^{-\Delta\Delta Cq}$ method was used to assess the relative expression.

Functional enrichment analyses of hsa_circ_0086414 gene and integrated analyses of hsa_circ_0086414-miRNAs-mRNAs

To further understand the functions, we conducted GO, DO, and KEGG pathway analyses for hsa_circ_0086414, and P value <0.05 was considered to show significant enrichment results. The targeting relationship between miRNAs and hsa_circ_0086414 was found through use of the StarBase (v2.0) database. Subsequently, MirTarBase (v6.1) was used to predict the targeting correlation between microRNAs and mRNAs. Then, Pearson correlation analysis was performed based on the hsa_circ_0086414 expression and mRNA, and relationship pairs with correlation coefficient >0.96 were screened. The targeting relationship of hsa_circ_0086414-miRNAs-mRNAs was visualized by Cytoscape 3.01.

Statistical analyses

Statistical analyses were carried out using SPSS 23.0 software (IBM Corp) and GraphPad Prism 7.0. Differences between OSCC tissue samples and paired AHTs were measured by *t* test. The relationship between hsa_circ_0086414 expression and clinicopathological characteristics was evaluated by the nonparametric test.

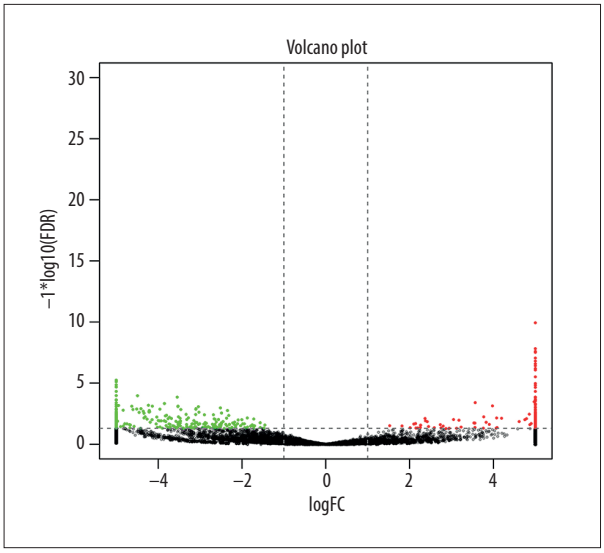

Figure 1. Volcano plots were used for evaluating the differential expression of circRNA between OSCC and AHT samples. The horizontal ordinate represents the logarithmic value of the difference multiple of the 2 samples, the longitudinal coordinate indicates the negative log10 value of FDR in the 2 samples. Red (OSCC samples upregulated relative to AHTs) and green (OSCC samples downregulated relative to AHTs) points indicate the differential expression of circRNA with statistical significance (P value <0.05, and fold change >2.0).

The hsa_circ_0086414 diagnostic value was assessed by the ROC curve. Every experiment was conducted at least in triplicate, and data are displayed as mean±standard deviation. P value <0.05 was considered as a statistically significant difference.

Results

CircRNAs expression profile in OSCC

The high-throughput sequencing results showed that 407 differentially-expressed circular RNAs were identified. Of these, 134 circRNAs were significantly underexpressed, and 273 circular RNAs were significantly overexpressed by more than 2-fold higher in OSCC tissues compared with AHTs on volcano plots (Figure 1) and cluster heatmap (Supplementary Figure 1). The hsa_circ_0086414 expression in OSCC was significantly underexpressed. To verify the sequencing results, qRT-PCR was carried out on OSCC tissue samples and paired AHTs from 55 patients with OSCC to assess the expression of hsa_circ_0086414. The significant downregulation of hsa_circ_0086414 in OSCC tissues (83.64%, n=46) was shown by qRT-PCR compared with AHTs (Figure 2A, 2B). We further compared hsa_circ_0086414 expression between OSCC cell lines (CAL27 and SCC25) and normal (HaCaT) cells, and similar results were found in 2 OSCC cell lines (Figure 2B).

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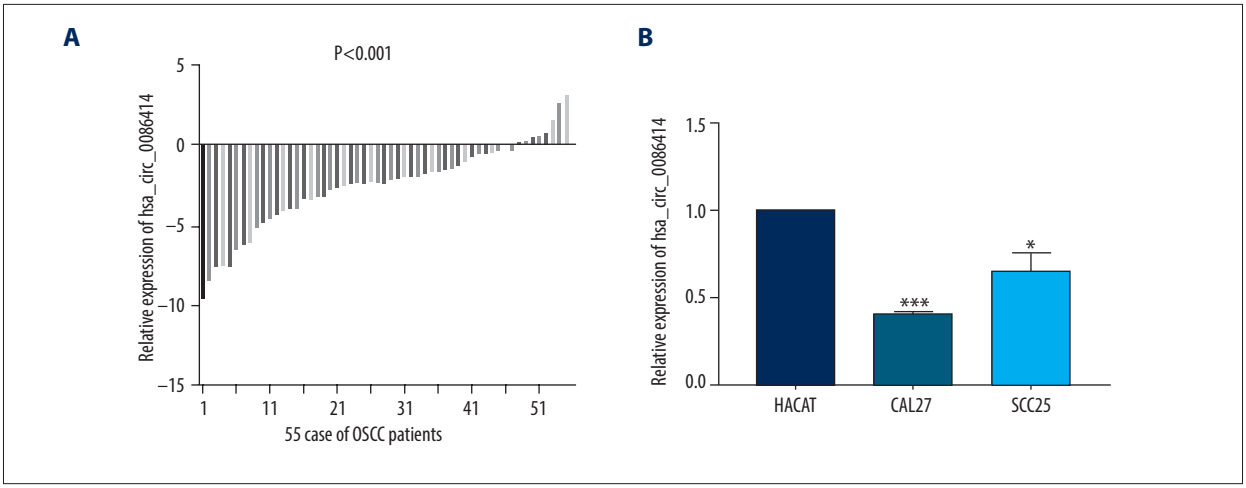

Figure 2. Decreased expression of hsa_circ_0086414 in OSCC tissues and human OSCC cells. (**A**) Relative expression of hsa_circ_0086414 in OSCC tissues and AHTs was analyzed by qRT-PCR (n=55). (**B**) Relative expression of hsa_circ_0086414 in 2 OSCC cells and normal (HaCat) cells was analyzed by qRT-PCR. *** P<0.001, ** P<0.01, * P<0.05.

Table 1. Relationship between clinicopathological features and the expression of hsa_circ_0086414 in 55 OSCC patients.

Features	Number of patients	Mean±SD	P value
Sex			0.082
Male	41	0.572±1.002	
Female	14	0.938±2.427	
Age (year)			0.784
≤60	36	0.666±1.532	
<60	19	0.664±1.413	
Alcohol			0.373
Yes	24	0.677±1.260	
No	31	0.657±1.649	
Smoking			0.797
Yes	33	0.557±1.096	
No	22	0.828±1.937	
Tumor location			0.646
Gingiva	15	0.304±0.409	
Tongue	20	0.950±2.016	
Others	20	0.652±1.357	
Tumor size			0.012*
<4 mm	22	1.255±2.200	
≥4 mm	33	0.272±0.344	
TNM stage			0.047*
I–II	24	1.107±2.084	
III–IV	31	0.323±0.574	
Differentiation			0.585
Low	10	0.191±0.145	
Middle	40	0.800±1.702	
High	5	0.544±0.698	
Lymph node metastasis			0.016*
Yes	30	0.318±0.586	
No	25	1.083±2.043	

Mean – mean of relative expression; SD – standard deviation; TNM – tumor node metastasis. * Indicates statistical significance (P<0.05).

Clinicopathological analysis of hsa_circ_0086414

Clinicopathological analysis showed that the target circular RNA was strongly correlated with stage in TNM (P=0.047), size of tumor (P=0.012), and lymph node metastasis (P=0.016). The results indicated that patients with lower hsa_circ_0086414 expression tended to have a higher TNM stage and positive lymph node metastasis. Additionally, the reduced hsa_circ_0086414 expression was positively correlated with larger tumor size. Collectively, these results show that decreased hsa_circ_0086414 can promote the processes of growth and metastasis in OSCC. However, no relationships were observed between hsa_circ_0086414 and

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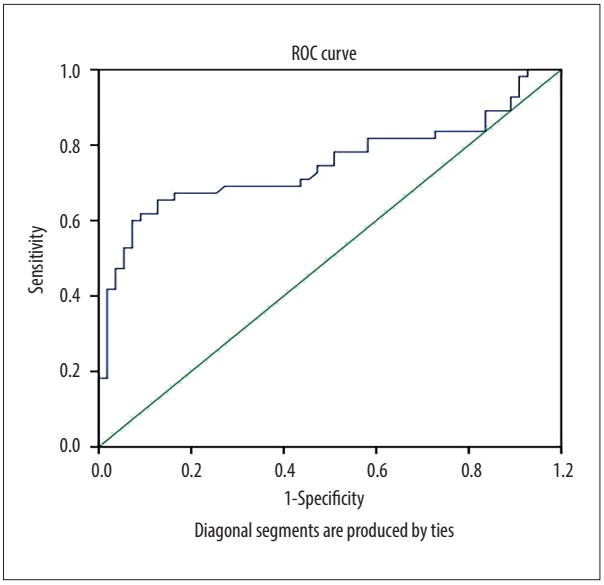

Figure 3. The receiver operating characteristic (ROC) curve of 55 OSCC patients according to the expression of hsa_circ_0086414.

sex, age, alcohol drinking, smoking, tumor location, or cancer differentiation (Table 1).

The possible diagnostic effect of hsa_circ_0086414 in OSCC

The possible diagnostic value of hsa_circ_0086414 in OSCC was assessed by ROC curve. The area below the ROC curve was 0.749 (P<0.0001), with a specificity of 87.3% and sensitivity of 65.5% (Figure 3), which indicated that the target circular RNA expression level was closely related to OSCC.

Functional enrichment analyses of hsa_circ_0086414 gene and construction of hsa_circ_0086414-miRNAs-mRNAs network

To explore the potential functions of hsa_circ_0086414, we performed GO, DO, and KEGG enrichment analyses for the targeted gene. The top 20 enriched, cellular components (CCs), molecular functions (MFs), and biological processes (BPs) are shown in Figure 4A–4F, respectively. We discovered that the most strongly enriched terms of GO in the CCs, MFs, and BPs were cytoplasm (GO: 0005737; P value=0.00035), protein binding (GO: 0005515; P value=0.000014), and developmental process (GO: 0032502; P value=0.000031), respectively. The results of DO analysis indicated that the target circRNA was significantly associated with cancers, such as clear cell sarcoma, thoracic cancer, and breast carcinoma (Figure 5A, 5B). KEGG results suggested that the top 20 of KEGG enrichment terms are correlated with cancer development (Figure 6A, 6B). Among these items, were tumor-correlated, such as the cAMP signaling pathway, proteoglycans in cancer, and the AMPK signaling pathway [24–27]. Furthermore, AMPK signaling pathway has been discovered to be correlated with OSCC progression [28]. It has been reported that mTOR, SIRT1, Akt, and CaMKK2 are the important signaling molecules in the AMPK signaling pathway, and RHEB is one of the important components in the cAMP signaling pathway [29–36]. The relative expression levels of these important signaling molecules were determined by qRT-PCR in 10 paired OSCC tissue samples that were selected randomly. The results of qRT-PCR demonstrated that the expression levels of mTOR, SIRT1, Akt, CaMKK2, and RHEB in OSCC tissue samples were significantly upregulated compared with the adjacent healthy tissues (P<0.05), indicating that hsa_circ_0086414 might be correlated with the AMPK and cAMP signaling pathway in OSCC patients by influencing the expression level of mTOR, SIRT1, Akt, CaMKK2, and RHEB. To determine the molecular mechanism of hsa_circ_0086414, we constructed a hsa_circ_0086414-miRNAs-mRNAs network using Cytoscape 3.01 to visualize their interactions according to our circRNA sequencing data and mRNA sequencing data (Supplementary Figure 2). In the network, there are 168 miRNAs and mRNAs related to hsa_circ_0086414 in the hsa_circ_0086414-miRNAs-mRNAs network. Among them, the 2 most relevant mRNAs are ENSG00000189241 and ENSG00000164161.

Discussion

Early diagnosis and treatment are of great importance for the prognosis of patients with OSCC. Recently, an increasing number of articles have reported that miRNAs and lncRNAs might play an essential role in OSCC [37–42]. However, whether circRNAs serve as diagnostic markers in the progress of OSCC remains unclear. circRNA is more stable than linear RNA since the 3- and 5-end of circRNA was discovered to be joined together to shape covalently closed loop structures [7]. In recent years, several studies have found that circRNAs could serve as diagnostic biomarkers for cancers [43–45]. Nevertheless, to the best of our knowledge, only 1 study, reported by Wang et al. [20], analyzed the profiles of differentially-expressed circRNAs in OSCC patients. In addition, fewer than 20 articles have demonstrated that circRNA is related to the progression of OSCC [46–53]. For instance, Wang et al. reported that circDOCK1 can inhibit OSCC cell apoptosis by regulating BIRC3 expression [46]. Dou et al. discovered hsa_circ_0072387 expression was reduced in OSCC samples and was related to the TNM stage in OSCC [51]. Sun et al. found hsa_circ_001242 expression in OSCC was substantially downregulated compared to AHTs [52]. Accordingly, the role of circRNAs in OSCCs remains unclear. Therefore, studying the circRNAs expression profile in OSCC and identifying the possible clinical significance of circRNAs in OSCC are particularly important.

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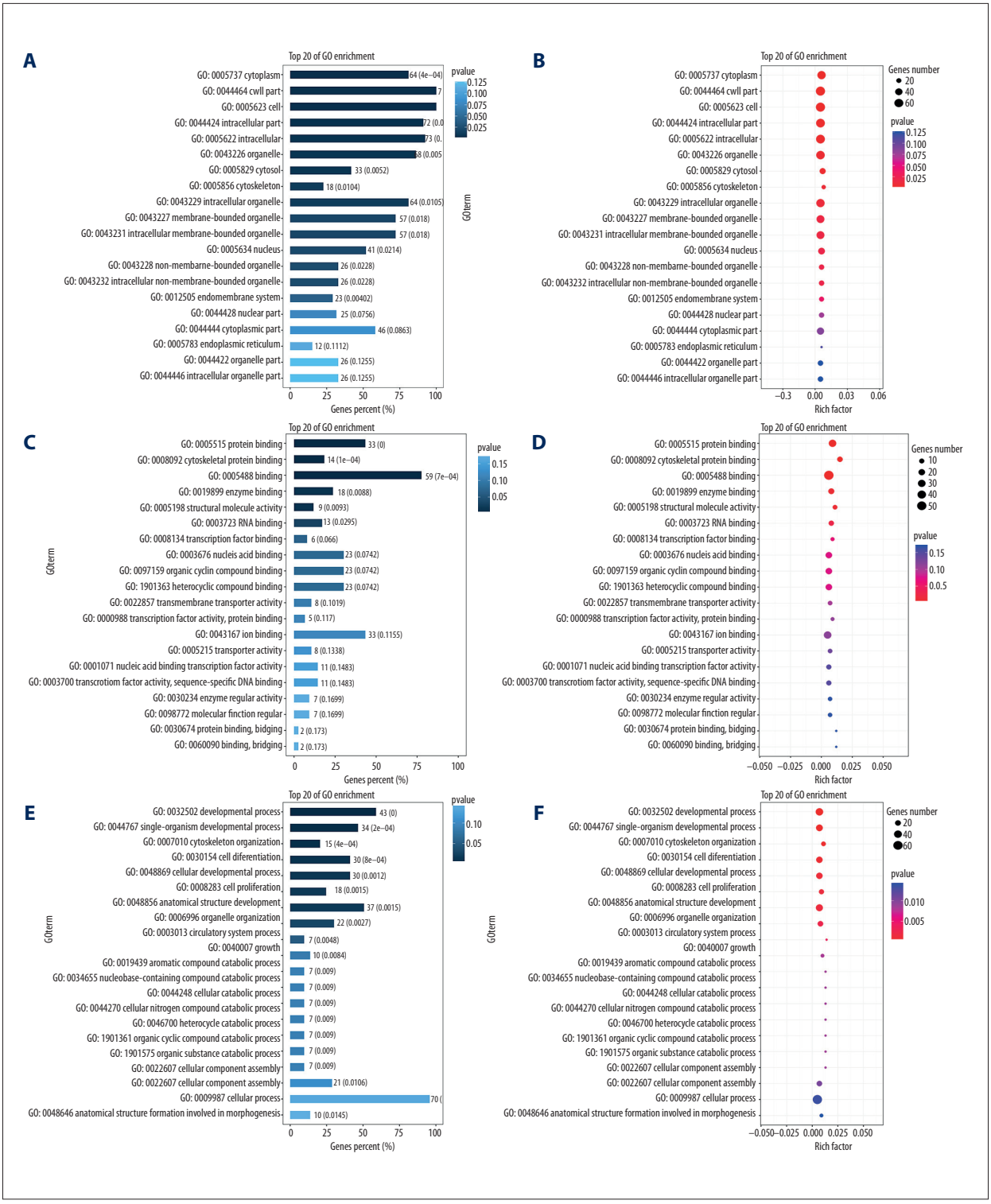

Figure 4. Top 20 of hsa_circ_0086414 GO function analysis. (**A, B**) GO analysis to determine the hsa_circ_0086414 cellular components (CCs). (**C, D**) GO analysis to determine the hsa_circ_0086414 molecular functions (MFs). (**E, F**) GO analysis to determine the hsa_circ_0086414 biological processes (BPs).

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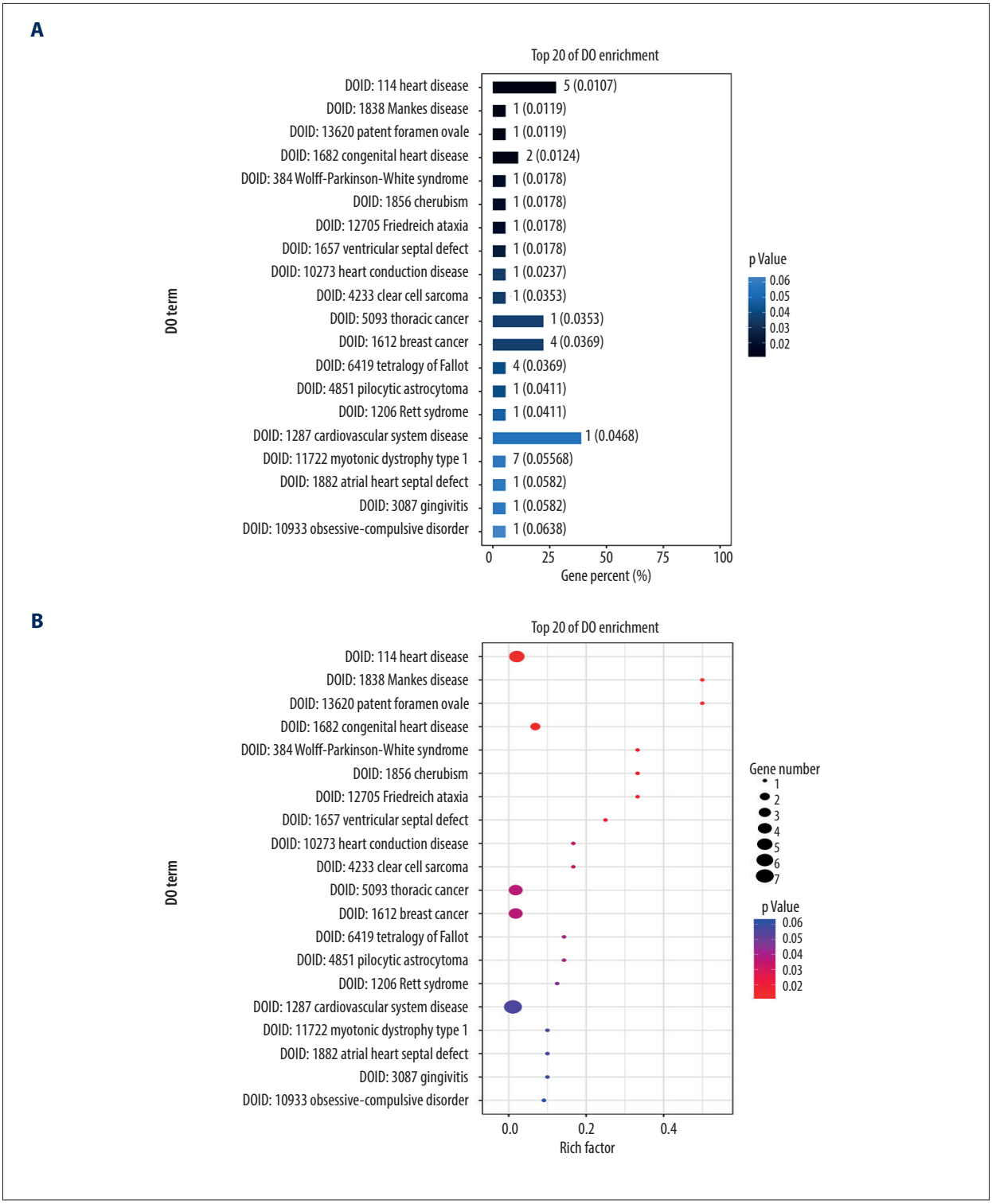

Figure 5. (**A, B**) Top 20 of hsa_circ_0086414 DO function analysis.

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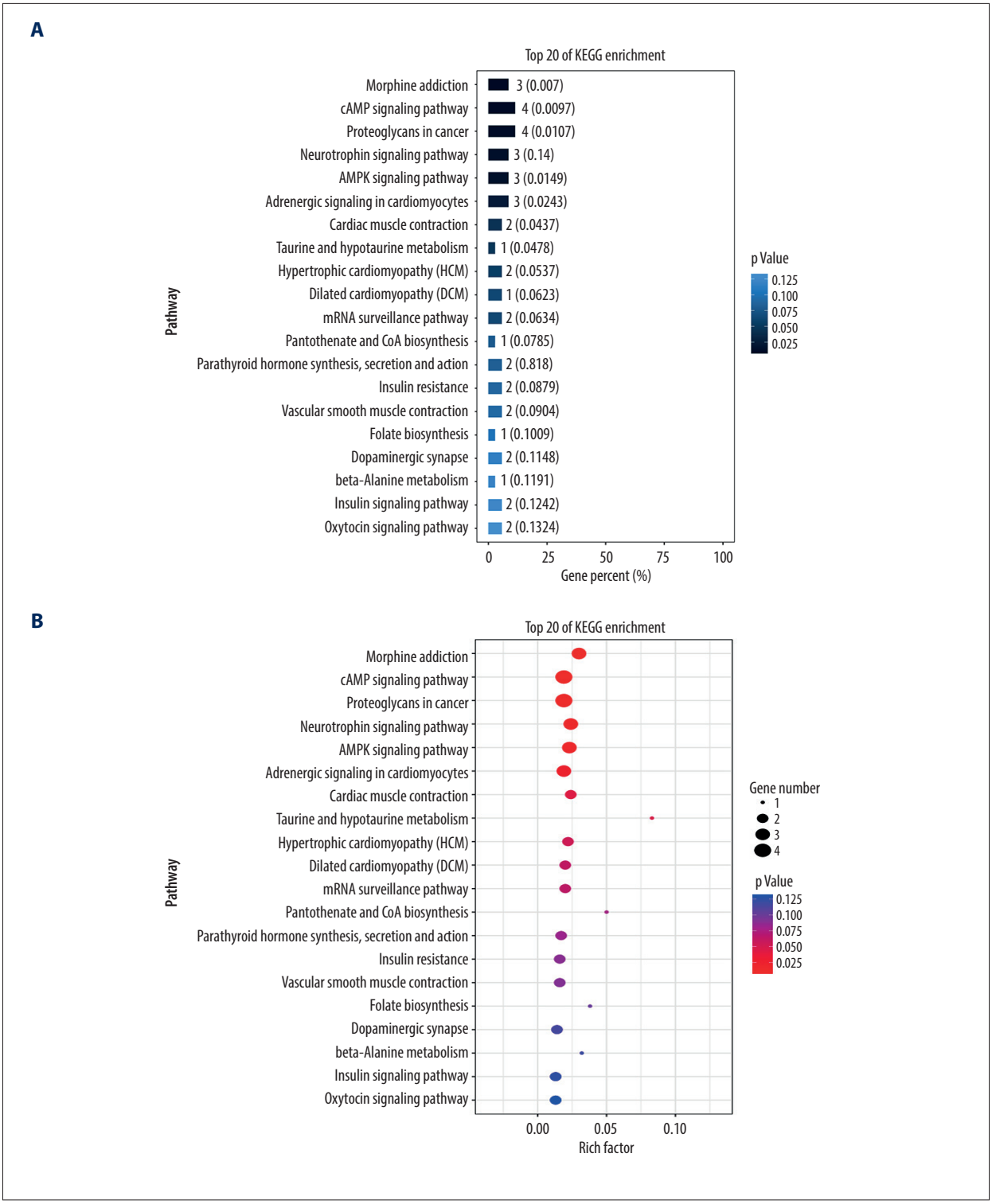

Figure 6. (**A, B**) Top 20 of hsa_circ_0086414 KEGG pathway analysis.

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To the best of our knowledge, the present study is the first to find decreased expression of hsa_circ_0086414 in OSCC tissue samples and OSCC cells, based on the sequencing data and the qRT-PCR results. According to the clinical information, the expression level of hsa_circ_0086414 was remarkably associated with stage in TNM, tumor size, and lymph node metastasis, which demonstrated that patients with lower expression of hsa_circ_0086414 tend to have higher TNM stage and more positive lymph node metastasis. Additionally, the reduced expression of hsa_circ_0086414 was positively associated with increased tumor size. These results suggest that the decreased hsa_circ_0086414 is involved in carcinogenesis and progression of OSCC. Furthermore, the results of ROC analysis showed that the expression of hsa_circ_0086414 was associated with OSCC diagnosis. Collectively, our results suggest that hsa_circ_0086414 is a valuable new biomarker for early diagnosis and treatment of individuals with OSCC.

To further observe the relationship between the target circRNA and OSCC development, we conducted KEGG pathway analysis using sequencing data and bioinformatics technology. Pathway analysis results showed that many items corresponded to cancer, such as the cAMP signaling pathway, proteoglycans in cancer, and the AMPK signaling pathway [24–27]. The AMPK pathway has been found to be related to OSCC progression [28]. The relative expression levels of some important signaling molecules related to cancers in the AMPK signaling pathway and cAMP signaling pathway, such as mTOR, SIRT1, Akt, CaMKK2, and RHEB [29–36], were detected by qRT-PCR, and the results demonstrated that the expression levels of these signaling molecules were remarkably overexpressed in OSCC tissue samples. In addition, a previous study reported that hsa_circ_0086414 was underexpressed in lung adenocarcinoma and was associated with EGFR mutations [23]. All these results suggest that the target circRNA is associated with cancer development. Accordingly, we hypothesized that hsa_circ_0086414 affects OSCC development and progression via certain signaling pathways.

It has been discovered that circRNAs can regulate the expression of mRNAs by competing with miRNAs [54]. Therefore, we constructed a hsa_circ_0086414-miRNAs-mRNAs network to predict their interactions according to our sequencing data and the StarBase and MirTarBase databases. We discovered that hsa_circ_0086414 contains many binding sites for miRNAs, indicating that hsa_circ_0086414 can promote cancer progression by miRNA sponging effects (Supplementary Figure 2). However, the hsa_circ_0086414 regulation mechanism remains unclear in OSCC, and this is a topic that warrants further research.

Conclusions

In conclusion, to the best of our knowledge, the present study is the first to show that hsa_circ_0086414 is remarkably downregulated in OSCC tissue samples and OSCC cells compared with AHTs and the normal cell line (HaCat), respectively. Additionally, the level of hsa_circ_0081414 expression is remarkably correlated with stage in TNM, tumor size, and lymph node metastasis. Our study may provide new ideas for researchers in analyzing the mechanisms involved in OSCC development, and our results suggest that hsa_circ_0086414 could be as a novel diagnostic biomarker for OSCC.

Acknowledgements

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Conflict of interests

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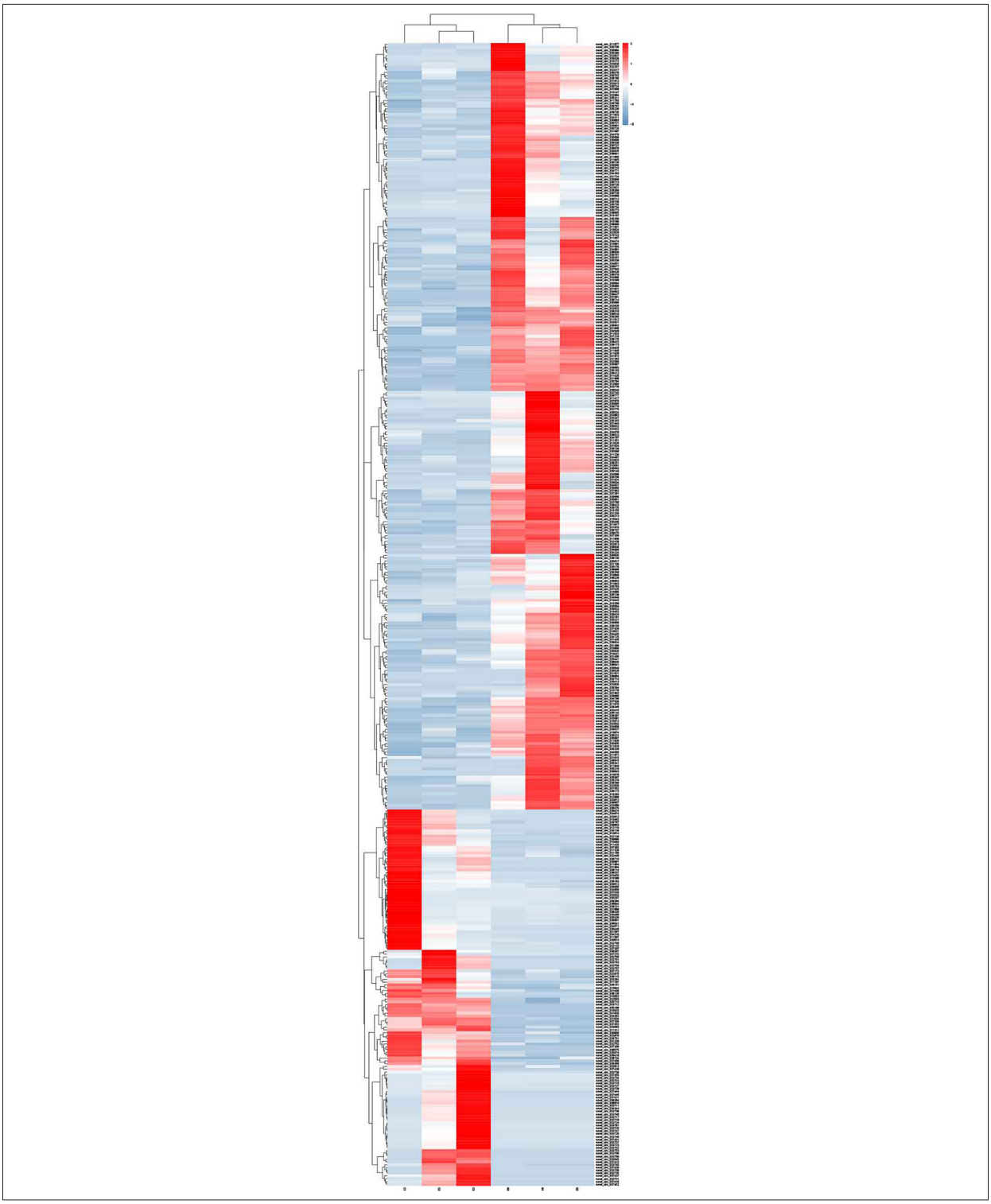
Supplementary Data



Supplementary Figure 1. Based on the amount of gene expression, we clustered the relationship between samples and genes hierarchically, and used the cluster heat map to present the clustering results. The circRNAs expression in different samples is marked as different colors. Red represents high relative expression and blue indicates low relative expression.

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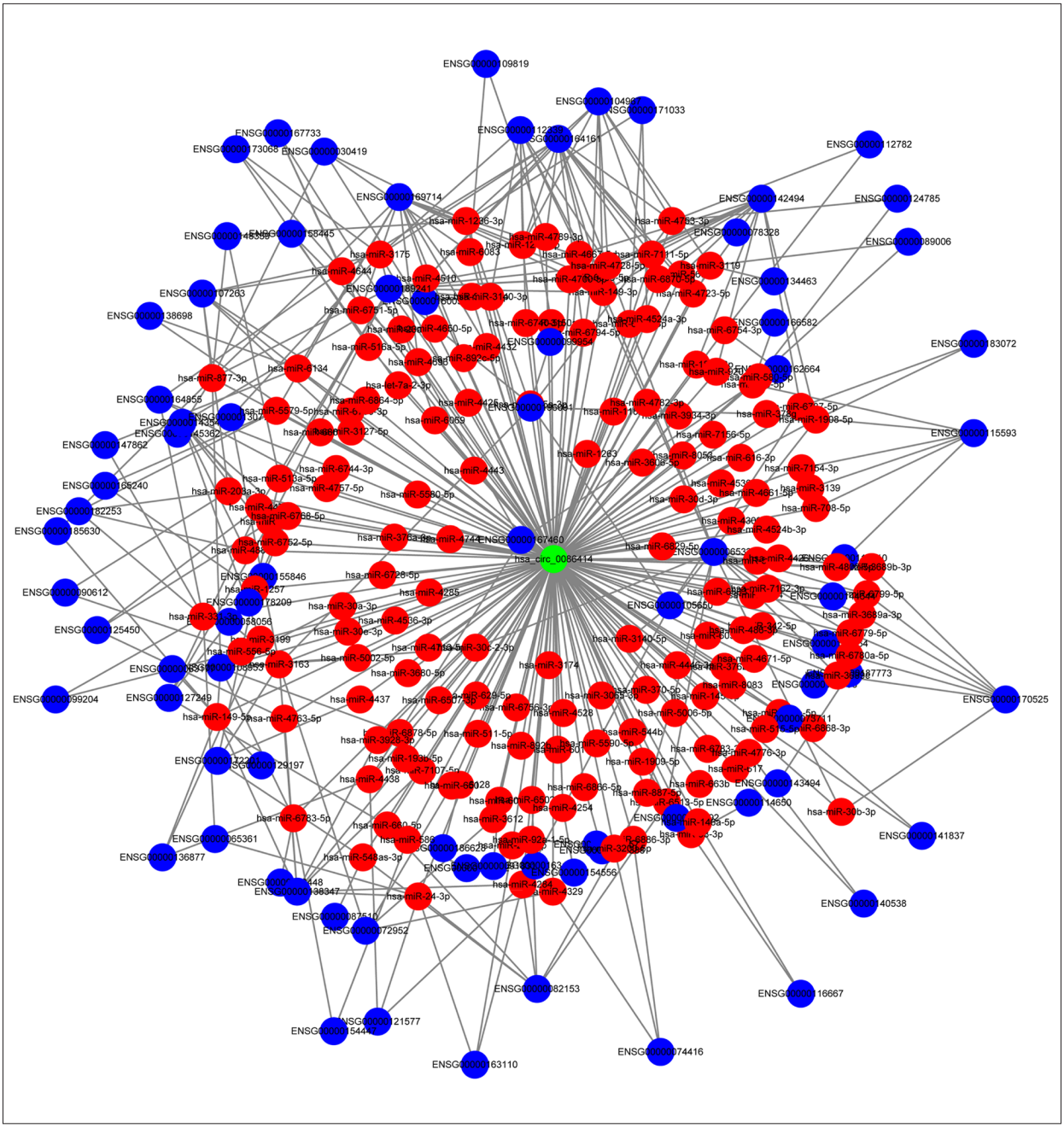

Supplementary Figure 2. Integrated analyses of hsa_circ_0086414-miRNAs-mRNAs. Cytoscape was performed to visualize circ_0086414-miRNAs-mRNAs interactions based on the circRNA sequencing and mRNA sequencing data. There are 168 miRNAs and mRNAs related to hsa_circ_0086414 in the hsa_circ_0086414-miRNAs-mRNAs network. Among them, the 2 most relevant mRNAs are ENSG00000189241 and ENSG00000164161. Hsa_circ_0086414 has 14 binding sites to bind with ENSG00000189241 or ENSG00000164161. Green circle represents hsa_circ_0086414; Red circle represents miRNAs; Blue circle represents mRNAs.

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