CircRNA-associated ceRNA network reveals ErbB and Hippo signaling pathways in hypopharyngeal cancer

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Received April 11, 2018; Accepted October 12, 2018

DOI: 10.3892/ijmm.2018.3942

Abstract. Accumulating evidence has suggested that circular RNAs (circRNAs), a novel class of non-coding RNAs, have crucial roles in tumor progression. However, the significance of circRNAs in hypopharyngeal cancer (Hca) remains to be investigated. The present study has identified aberrantly expressed circRNAs by performing circRNA sequencing analyses of three pairs of tumor and adjacent normal samples from patients with Hca. The results demonstrated that 173 circRNAs were differentially expressed (dE), including 71 upregulated and 102 downregulated circRNAs (FdR<0.05 and fold changes of ≥2 or ≤0.5 by Mann-Whitney U test followed by Benjamini-Hochberg correction for multiple testing). Pathway analyses of the genes producing dE circRNAs revealed that many of them were involved in cancer-related pathways. To further illustrate the roles of circRNAs in Hca progression, a competing endogenous RNA (ceRNAs) network was constructed, consisting of circRNAs, miRNA, and miRNA targeted genes. The results demonstrated that multiple cancer-related pathways were affected by performing enrichment analyses of the targeted genes. Of note, a ceRNA subnetwork was isolated, consisting of two circRNAs (hsa_circ_0008287 and hsa_circ_0005027) and one miRNA (hsa-miR-548c-3p), which significantly affect both ErbB and Hippo signaling pathways. In conclusion, the present study identified a set of circRNAs that are potentially implicated in the tumorigenesis of Hca and may serve as potential biomarkers for the diagnosis of Hca.

Introduction

Hypopharyngeal carcinoma is a primary malignant tumor of the hypopharynx, accounting for 3-5% of the malignancies in the upper aerodigestive tract. Early diagnosis of hypopharyngeal cancer is hard because the early stages of hypopharyngeal carcinoma have no specific symptoms. Studies have reported that 60-80% of these patients had ipsilateral lymph node metastases and ≤40% of these patients have contralateral occult lymph node tumor deposits (1-3). Thus, the majority of patients with hypopharyngeal cancer have a poor prognosis and low survival rate (4). Therefore, identifying early stage indicators or biomarkers to improve patient survival is urgent.

Unlike normal linear RNA, the 3' and 5' ends of circular RNAs (circRNAs) are linked by covalent bonds and lack polarities or polyadenylated tails, thereby rendering them stable in tissues, serum and urine (5). Owing to this characteristic, the potential of circRNAs as biomarkers for human cancer has attracted significant focus. In addition, circRNAs are widely involved in cancer; ciRS-7 in HeLa cells (6), Hsa_circ_001569 in colorectal cancer (7), circHIPK3 in several types of cancer (8), f-circM9, f-circPR in hematological malignancy (9), and circTcF25 in urinary bladder carcinoma (10). Previous studies have demonstrated that the main function of circRNAs is that they can function as a microRNA (miRNA) sponge, binding to miRNAs and regulating them and their...
downstream gene targets, through a competing endogenous (ce) RNA mechanism (11).

The present study comprehensively investigated the expression profile of circRNAs in HCa patients. The results identified a circRNA signature in HCa and suggested that a core miRNA-ceRNA network, regulating both the ErbB and Hippo signaling pathways, may have important roles in HCa progression.

Materials and methods

Patients and specimens. The study included three patients with HCa who underwent partial or radical cystectomies at the First Affiliated Hospital of Kunming Medical University (Kunming, China); samples were collected from March 2017 to October 2017. All three patients were male and their ages were 44, 54 and 56. Following surgery, the matched specimens were immediately preserved in liquid nitrogen until use. All patient samples were confirmed by pathological examination and none of the patients received neoadjuvant therapy. The study was approved by the Second Department of Otolaryngology Head and Neck Surgery of the First Affiliated Hospital of Kunming Medical University (Kunming, China). Written informed consent was obtained from all the participants in the study.

Total RNA isolation and quality control. Total RNA was isolated from samples using TRIzol reagent (Thermo Fisher Scientific, Inc., Waltham, MA, USA) following the manufacturer's protocol. The quantity and quality of total RNA samples were measured using NanoDrop ND-1000 (Thermo Fisher Scientific, Inc.). RNA integrity was assessed and confirmed via electrophoresis using denaturing agarose gels. Isolated RNA samples were stored at -80°C prior to use.

Library preparation and sequencing. Total RNA from three matched HCa samples and adjacent normal tissues were treated with Epicenter Ribo-Zero rRNA Removal kit (Illumina, Inc., San Diego, CA, USA) and RNase R (Epicenter; Illumina, Inc.) to remove ribosomal and linear RNA. Then, the RNA-seq libraries were constructed using TruSeq Stranded Total RNA HT/LT Sample Prep kit (Illumina, Inc.). Sequencing was determined on Illumina Hiseq 2500 instrument with 2x150 bp paired reads.

Computational analysis of circRNAs. The clean reads were obtained after the raw reads were preprocessed with the FastQC quality control tool (12). CircRNAs were identified using CIRI (v.1.2) pipeline with default parameters (13). Genomic circRNAs were mapped to the human reference genome (GRCh37) by BWA (14). All circRNAs were annotated for circRNA-hosting genes with the application of GENCODE v24 (15). The identified circRNAs were converted to circRNA ID with web server circBase (16).

Principal component analysis (PCA). PCA was performed as previously described (17). A total of 4,634 distinct circRNAs with non-zero raw counts across the six samples were isolated and expressions of circRNAs were normalized with the reads per Million mapped reads (RPM) method and the expression matrix (each row represented a gene, each column represented a sample) were used for PCA. The prcomp package from R was used to perform PCA and the default parameters were used (18). The ggplot2 package from R was used to draw the scatter plot (19).

Normalization and differential expression analysis of circRNAs. Two steps were performed to normalize circRNA expression for depth. Firstly, the total back-spliced reads in a sample were counted and that number was divided by 1,000,000. This resulted in the 'per million' scaling factor. Secondly, the read counts were divided by the 'per million' scaling factor. This method normalized for sequencing depth, giving RPM. CircRNAs were isolated with RPM>0 across 6 samples and Mann-Whitney U test (20) (paired=T) followed by Benjamini-Hochberg multiple testing correction (21) were applied to identify the differentially expressed (DE) circRNAs. FDR<0.05 and a fold change of >2.0 or <0.5 were the selection criteria for significant DE circRNAs.

Functional enrichment analysis. Gene ontology (GO) term enrichment analysis and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis were conducted with web server DAVID 6.8 (22). P<0.05 was considered as statistically significant.

CeRNA network. The top 20 upregulated circRNAs and the top 20 downregulated circRNAs were used to survey miRNA targets with the web tool CircInteractome (23). Specifically, CircInteractome downloads the mature sequences of circRNAs from the UCSC browser mirror (http://genome.ucsc.edu) (24) and predicts miRNAs that target circRNA by surveying for 7-mer or 8-mer complementarity to the mature sequence of the circRNA. mirPath 3.0 (26) was also used for miRNA KEGG pathway analysis. The ceRNA network was displayed by Cytoscape (v3.5.1) (27).

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from pooled normal and tumor tissue samples using TRIzol (Thermo Fisher Scientific, Inc.), and 1 µg of total RNA was reverse transcribed into first-strand cDNA using a PrimeScript RT Reagent kit (Takara Bio, Inc., Otsu, Japan), according to the manufacturer's protocol. qPCR was performed with a SYBR-Green real-time PCR kit (Thermo Fisher Scientific, Inc.) using the ABI StepOnePlus Real-Time PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.). CircRNAs were analyzed with 18s rRNA as the internal standard and miRNA was analyzed with U6 as the internal standard. The reactions were prepared as follows: 7.5 µl SYBR Premix Ex Taq II, 0.25 µl ROX Reference Dye II, 0.125 µl forward primer, 0.125 µl reverse primer, 5 µl RNase-free water, and 2 µl cDNA. The thermocycling conditions were: one step at 95°C for 30 sec, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec, and a final step of 95°C for 15 sec, 60°C for 15 sec and 95°C for 15 sec. Primer sequences are listed in Table I; expression levels were quantified via the 2^ΔΔCq method (28).
Expression analysis of miR-548c-3p. Two methods were used to investigate the expression of miR-548c-3p among normal and tumor samples. The first was RT-qPCR, as detailed above. The second was in-silico analysis. The miRNA dataset of the esophageal carcinoma cohort from The cancer Genome Atlas (TCGA) project (29) was exploited. There were 13 normal samples and 184 tumor samples in this dataset. Normalized miRNA expressions of miR-548c-3p were compared between normal and tumor samples. Mann-Whitney U test was applied to test the significance.

Survival analysis. A Kaplan-Meier curve was used to examine the clinical relevance of miR-548c-3p levels in the patients' outcomes (30). Patients were separated into two groups according to the median expression of hsa-miR-548c-3p using TCGA clinical and expression dataset. Differences between groups were analyzed using log-rank test (31) and two-tailed P-values <0.05 were considered statistically significant.

Expression correlation of hsa-miR-548c-3p and its targeted genes. The miRNA and mRNA datasets of the esophageal carcinoma cohort from TCGA (29) were used for the correlation analysis. Common samples were isolated according to the sample barcodes. The Pearson correlation method was used to assess the expression association between hsa-miR-548c-3p and the targeted genes. Significance of association was determined by the R package cor.test (alternative='two.sided', method='pearson'). Then, P-values were corrected with Benjamini-Hochberg procedure for multiple testing.

Statistical analysis. All statistical analyses were generated using R (32). The Pearson correlation method was used to assess the expression association. Significances of associations were determined by the R package cor.test. Mann-Whitney U test was used for comparisons between two groups. Benjamini-Hochberg procedure was applied for multiple testing. Log-rank test was used for Kaplan-Meier survival curves. P<0.05 was considered to indicate a statistically significant difference.

Results

Identification of DE circRNAs in HCa. To identify DE circRNAs in HCa, circRNA sequencing (Seq) was performed using three matched normal and Hca tissue samples, and an average of 90 million reads was achieved for each sample. A total of 4,634 distinct circRNAs with at least two back-spliced reads across six samples using CIRI pipeline (13) were identified and the expressions of circRNAs were normalized and represented by reads per million mapped reads (RPM) values. Genetic distances across 6 samples were evaluated using PCA (Fig. 1A), and the normalized expression level (RPM) of circRNAs across the six samples is illustrated in Fig. 1B. Following statistical analysis, 71 and 102 circRNAs were determined to be significantly upregulated and downregulated, respectively
The DE circRNAs between tumor and adjacent normal samples were presented in a heatmap (Fig. 1C). To confirm the circRNA-Seq results, RT-qPCR was performed to assess the expression of 19 of the above DE circRNAs in both normal and tumor samples. The results confirmed that 12 of them were consistently upregulated or downregulated with the circRNA-Seq results (Fig. 2).

Next, the distribution of circRNAs in different DNA elements and chromosomes was examined. The bar diagram of Fig. 3A demonstrates the % of back-spliced junction reads on intron, intergenic, and exon areas. The majority of circRNAs belonged to exonic, followed by intronic and intergenic elements (Fig. 3B). These dysregulated circRNAs are widely distributed in all chromosomes, including sex chromosomes X (Fig. 3C).

Functional enrichment analysis of genes producing DE circRNAs. To reveal the dysregulated pathways underlying HCa, first KEGG pathway enrichment analyses were performed for genes that matched DE circRNAs. The results demonstrated that genes containing downregulated circRNAs were enriched in endocytosis, ubiquitin-mediated proteolysis, and Janus kinase (JAK)/signal transducer and activator of transcription (STAT) signaling pathways (Fig. 4A), whereas there were no KEGG pathways enriched with genes producing upregulated circRNAs.

Next, GO term enrichment analyses was performed for genes that produced aberrantly expressed circRNAs. Biological processes, such as the establishment of spindle orientation, response to fungicide, positive regulation of transcription, cell division were significantly enriched (Fig. 4B), whereas genes producing downregulated circRNAs were related to autophagy, mitochodrion organization actin cytoskeleton organization, membrane fission, and cell-cell adhesion pathways (Fig. 4C). These results suggested that multiple pathways may contribute to HCa pathogenesis and progression.

CircRNAs regulate the ErbB and Hippo pathways through a miRNA-CeRNA network. The role of circRNAs as a miRNA sponge is the main mechanism of circRNA function in tumor cells (11,33). Therefore, we further investigated the roles of circRNAs in HCa progression through establishing a ceRNA network. Firstly, the top 20 upregulated and top 20 downregulated circRNAs were isolated and were converted to circRNA ID using circBase database (34). Secondly, miRNAs targeting DE-circRNAs were isolated with the web server CircInteractome (23). Specifically, CircInteractome downloaded the mature sequences of all of the reported circRNAs from the UCSC browser, then to characterize miRNA-circRNA interactions, CircInteractome incorporated the ability to search using the TargetScan algorithm, which predicts miRNAs that target circRNA by surveying for 7-mer or 8-mer complementarity to the seed region as well as the 3'end of each miRNA (23). A total of 191 and 182 miRNAs were putatively identified as the targets of upregulated and downregulated circRNAs, respectively. Networks consisted of circRNAs and miRNAs were displayed using Cytoscape software (27). The results demonstrated extensive interactions...
| circRNA ID (CIRI)  | circRNA ID (circBase) | Adjusted P-value | FC       | Gene          |
|------------------|----------------------|------------------|----------|---------------|
| chr16:21973780-21987564 | hsa_circ_0005690 | 0.022002929 | 9.346453412 | UQCRC2       |
| chr2:24234324-242357524  | hsa_circ_0004924 | 0.042106003 | 4.23716918  | FARP2        |
| chr7:72873865-72884813   | hsa_circ_0004760 | 0.003579475 | 4.13619932  | BAZ1B        |
| chr5:133871547-133887899  | hsa_circ_0005608 | 0.036132329 | 3.80520016  | PHF15        |
| chr22:41979962-41980607  | hsa_circ_0005703 | 0.030406606 | 3.76275756  | PMM1         |
| chr3:48019354-48040369   | hsa_circ_0005255 | 0.039183919 | 3.736266721 | MAP4         |
| chr12:27521194-27523163  | hsa_circ_0009009 | 0.01165016  | 3.55331882  | ARNTL2       |
| chr9:117399269-117401006  | hsa_circ_0002318 | 0.041159198 | 3.381349856 | C9orf91      |
| chr1:165859440-165860559  | hsa_circ_0006758 | 0.041758958 | 3.36786062  | UCK2         |
| chr16:50321822-50322261  | hsa_circ_0000699 | 0.043912028 | 3.31487392  | ADCY7        |
| chr16:89484691-89497734  | hsa_circ_0000727 | 0.035411987 | 3.287797218 | ANKRd11      |
| chr9:89817580-98837381   | hsa_circ_0003214 | 0.018483558 | 3.272900498 | LAPTMB4      |
| chr19:48229068-48229481  | hsa_circ_0003146 | 0.006019062 | 3.268230068 | EH2D         |
| chr11:118003110-118045592 | hsa_circ_0002059 | 0.010572316 | 3.169435071 | MAN1A2       |
| chr14:92264128-922687655  | hsa_circ_0003296 | 0.040149042 | 3.130564666 | TC2N         |
| chr2:21096882-211019335  | hsa_circ_0002617 | 0.020968937 | 3.121028088 | C2orf67      |
| chr15:48989365-494952495  | hsa_circ_0000660 | 0.032291249 | 3.092238962 | MCTP2        |
| chr19:48229068-48229481  | hsa_circ_0003146 | 0.006019062 | 3.268230068 | EH2D         |
| circRNA ID (CIRI) | circRNA ID (circBase) | Adjusted P-value | FC | Gene |
|------------------|----------------------|------------------|----|------|
| chr22:46125304-46136418 | hsa_circ_0001247 | 0.013526942 | 2.31185877 | ATXN10 |
| chr14:23419522-23421892 | hsa_circ_0005663 | 0.04538212 | 2.257072167 | HAUS4 |
| chr12:122773035-122801402 | #N/A | 0.015647694 | 2.25006903 | #N/A |
| chr3:3188323-3190820 | #N/A | 0.025850456 | 2.249708035 | #N/A |
| chr3:172363412-172365904 | hsa_circ_0007042 | 0.02689758 | 2.238856764 | NCEH1 |
| chr2:10799297-10808849 | hsa_circ_0008511 | 0.042758286 | 2.22912485 | NOL10 |
| chr10:70696697-70703013 | hsa_circ_0007097 | 0.040819924 | 2.219586601 | DDX50 |
| chrX:14886826-14877456 | hsa_circ_0006971 | 0.009211245 | 2.215797457 | FANcB |
| chr1:23356961-23385660 | hsa_circ_0007822 | 0.027240129 | 2.206406433 | KdM1A |
| chr20:13539654-13561628 | hsa_circ_0002001 | 0.017808498 | 2.188222168 | TASP1 |
| chr7:139741443-139757834 | hsa_circ_0004684 | 0.026813206 | 2.168759032 | PARP12 |
| chr7:122773035-122801402 | #N/A | 0.027697748 | 2.12527859 | #N/A |
| chr18:21644103-21663045 | hsa_circ_0047270 | 0.020649137 | 2.102327859 | #N/A |
| chr3:43341245-43345284 | hsa_circ_0004089 | 0.007985302 | 2.078191163 | PUM1 |
| chr2:10799297-10808849 | hsa_circ_0008511 | 0.042758286 | 2.221921485 | #N/A |
| chr10:70696697-70703013 | hsa_circ_0007097 | 0.040819924 | 2.219586601 | DDX50 |
| chrX:14886826-14877456 | hsa_circ_0006971 | 0.009211245 | 2.215797457 | FANcB |
| chr1:23356961-23385660 | hsa_circ_0007822 | 0.027240129 | 2.206406433 | KdM1A |
| chr20:13539654-13561628 | hsa_circ_0002001 | 0.017808498 | 2.188222168 | TASP1 |
| chr7:139741443-139757834 | hsa_circ_0004684 | 0.026813206 | 2.168759032 | PARP12 |
| chr7:122773035-122801402 | #N/A | 0.027697748 | 2.12527859 | #N/A |
| chr18:21644103-21663045 | hsa_circ_0047270 | 0.020649137 | 2.102327859 | #N/A |
| chr3:43341245-43345284 | hsa_circ_0004089 | 0.007985302 | 2.078191163 | PUM1 |
| chr2:10799297-10808849 | hsa_circ_0008511 | 0.042758286 | 2.221921485 | #N/A |
| chr10:70696697-70703013 | hsa_circ_0007097 | 0.040819924 | 2.219586601 | DDX50 |
| circRNA ID (CIRI) | circRNA ID (circBase) | Adjusted P-value | FC | Gene |
|-------------------|----------------------|-----------------|----|------|
| chr18:46858233-46906128 | hsa_circ_0002501 | 0.03573514 | 0.420126159 | DYM |
| chr1:246784730-246797889 | hsa_circ_0017311 | 0.028462249 | 0.41970008 | CNST |
| chr12:1399017-1481143 | hsa_circ_0024997 | 0.040174171 | 0.417295868 | ERC1 |
| chr12:27521194-27523163 | hsa_circ_0009009 | 0.024711114 | 0.414722924 | ARNTL2 |
| chr5:50055476-50059076 | hsa_circ_0006787 | 0.018488474 | 0.412395338 | PARP8 |
| chr3:17909612-17904147 | hsa_circ_0002219 | 0.029228228 | 0.403006229 | MFN1 |
| chr10:88203031-88206206 | #N/A | 0.022884862 | 0.399217754 | #N/A |
| chr17:26490568-26499644 | hsa_circ_0003638 | 0.013335258 | 0.397904838 | NLK |
| chr16:8952206-8953192 | hsa_circ_0000669 | 0.000715169 | 0.397910388 | CARHSP1 |
| chr2:145946065-185964557 | hsa_circ_0005633 | 0.009131314 | 0.397798992 | #N/A |
| chr11:8569570-85695016 | hsa_circ_0006629 | 0.01991069 | 0.396956177 | PIcALM |
| chr7:91980263-91991587 | hsa_circ_0006787 | 0.018488474 | 0.396956177 | PIcALM |
| chr14:35519989-35522657 | hsa_circ_0006424 | 0.04322634 | 0.387507944 | FAM177A1 |
| chr12:42768664-42792796 | hsa_circ_0003961 | 0.014465456 | 0.386034041 | PHLH1N |
| chr1:246021797-246093239 | hsa_circ_0017289 | 0.011181456 | 0.3845259 | SMYD3 |
| chr10:27431315-27434519 | hsa_circ_0005633 | 0.009131314 | 0.384171219 | YMEIL1 |
| chr11:129299319-129299615 | hsa_circ_0000462 | 0.006941025 | 0.38095649 | SLC15A4 |
| chr4:103644027-103647840 | hsa_circ_0006007 | 0.02570568 | 0.37992038 | MAN1C2 |
| chr6:55966269-56006781 | hsa_circ_0006629 | 0.00220417 | 0.379828753 | MAN1C2 |
| chr8:17123415-17126465 | hsa_circ_0008592 | 0.040012263 | 0.37509744 | FAM177A1 |
| chr10:99915849-99923154 | hsa_circ_0004419 | 0.032103617 | 0.372519687 | #N/A |
| chr16:3900297-3901010 | hsa_circ_0006629 | 0.00220417 | 0.379828753 | MAN1C2 |
| chr10:99915849-99923154 | hsa_circ_0004419 | 0.032103617 | 0.372519687 | #N/A |
| chr11:1307231-1317024 | hsa_circ_0003310 | 0.0259611 | 0.359726565 | MANBA |
| chr6:55966269-56006781 | hsa_circ_0006629 | 0.00220417 | 0.379828753 | MAN1C2 |
| chr8:18619432-18624147 | hsa_circ_0006733 | 0.037595523 | 0.35338689 | #N/A |
| chr14:52977957-53011089 | hsa_circ_0003193 | 0.01966747 | 0.351937505 | TXNDC6 |
| chr21:37710767-37717005 | hsa_circ_0001189 | 0.010082196 | 0.351304877 | MRC3 |
| chr1:94685813-94697199 | hsa_circ_0003310 | 0.0259611 | 0.35119971 | #N/A |
| chr3:47136562-47180680 | hsa_circ_00065159 | 0.020521933 | 0.350064342 | SETD2 |
| chr8:11726317-11728999 | hsa_circ_0005524 | 0.04966353 | 0.34303411 | DROSHA |
| chr11:1307231-1317024 | hsa_circ_0008301 | 0.018942131 | 0.342187543 | TOLLIP |
| chr6:108242132-108243113 | hsa_circ_0003310 | 0.012934139 | 0.34093459 | #N/A |
| chr19:48229068-48229481 | hsa_circ_0003146 | 0.020521933 | 0.340720207 | EHD2 |
| chr3:37107053-37190529 | hsa_circ_0003264 | 0.013159432 | 0.336803278 | LRRFIP2 |
| chr7:65705311-65751696 | hsa_circ_0006041 | 0.039274732 | 0.313502465 | SETD2 |
| chr13:96409897-96416207 | hsa_circ_0005524 | 0.04966353 | 0.34303411 | DROSHA |
| chr8:68200189-68214701 | hsa_circ_0006773 | 0.003935998 | 0.328463848 | HIBADH |
| chr7:27668989-27689252 | hsa_circ_0006408 | 0.043640249 | 0.325878207 | KIF5B |
| chr7:7310965-73101425 | hsa_circ_0005588 | 0.042073587 | 0.318524548 | WBSCR22 |
| chr7:72873865-72884813 | hsa_circ_0004670 | 0.043342921 | 0.316315928 | BAZ1B |
| chr2:422284206-242283312 | hsa_circ_0005906 | 0.007810999 | 0.315723287 | SEPT2 |
| chr3:47139444-47144913 | hsa_circ_0001289 | 0.039274732 | 0.313502465 | SETD2 |
between miRNAs and upregulated (Fig. 5A), and downregulated circRNAs (Fig. 5B). Then, KEGG pathway enrichment analysis was performed for the miRNAs targeted by the top 40 DE circRNAs, in order to explore the altered biological processes using mirPath 3.0 (26). Genes targeted by miRNAs were significantly enriched in multiple signaling pathways, including the ErbB, the Hippo, the Ras, the transforming growth factor (TGF)-β, the phosphoinositide 3-kinase/AKT serine/threonine kinase and the Wnt signaling pathways (Fig. 5C).

Table II. Continued.

| circRNA ID (CIRI) | circRNA ID (circBase) | Adjusted P-value | FC    | Gene            |
|------------------|-----------------------|------------------|-------|-----------------|
| chr2:43655238-43657441 | hsa_circ_0054309     | 0.038540875      | 0.309703944 | THADA          |
| chr21:46275124-46281186 | hsa_circ_00010200    | 0.0474882        | 0.302025085 | PTG11P         |
| chr5:179976930-179980471 | hsa_circ_00088383    | 0.028790905      | 0.292442462 | CNOT6          |
| chr1:87185189-87190088 | hsa_circ_00130843    | 0.01567065       | 0.280991627 | SH3GL1B        |
| chr19:53577392-53578436 | hsa_circ_00074802    | 0.031784533      | 0.275005495 | ZNF160         |
| chr16:53289511-53297009 | #/N/A                | 0.003705027      | 0.260024152 | #/N/A          |
| chr22:29090000-29091861 | hsa_circ_00049811    | 0.008438851      | 0.252361922 | CHEK2          |
| chrX:11771869-117724265 | hsa_circ_00913824    | 0.032765014      | 0.247214419 | DOCK11         |
| chr11:12899340-128997200 | hsa_circ_00050273    | 0.041341275      | 0.246885645 | ARHGAP32       |
| chr15:34342498-34343258 | hsa_circ_00343467    | 0.039707643      | 0.246690583 | SLC12A6        |
| chr10:70152894-70154208 | hsa_circ_00002393    | 0.008512372      | 0.236281903 | RUFI2          |
| chr1:236966727-236979843 | #/N/A                | 0.036146024      | 0.222019071 | #/N/A          |
| chr19:33604672-33605325 | hsa_circ_00082875    | 0.042162087      | 0.207707068 | GPC3          |
| chr2:16892000-168931741 | hsa_circ_00032793    | 0.001767352      | 0.196582978 | STK39          |
| chr1:17908721-179091002 | #/N/A                | 0.019369988      | 0.169662407 | #/N/A          |
| chr18:9524591-9525849 | hsa_circ_00051587    | 0.043927311      | 0.166193525 | RALBP1         |
| chr22:36737414-36745300 | hsa_circ_00044700    | 0.042494836      | 0.147382721 | MYH9           |

The criteria for the differential expression were: Adjusted P<0.05 and FC>2 or FC<0.5. The top 20 upregulated and downregulated genes are presented in bold. circRNA, circular RNA; FC, fold change.

Figure 2. Reverse transcription-quantitative polymerase chain reaction analysis. Twelve of 19 circRNAs were demonstrated to be consistently regulated with the circRNA-sequencing results. circRNAs, circular RNAs.
To get further insight into the function of circRNAs in the ErbB and Hippo signaling pathways, miRNA-ceRNA networks were constructed corresponding to the two pathways using Cytoscape. For the miRNA-ceRNA network regulating the ErbB pathway, there were 33 circRNAs, 43 miRNAs and 74 ErbB pathway genes (Fig. 6A). In the ErbB miRNA-ceRNA network, the highest number of circRNAs and miRNAs was found in the ErbB pathway genes, indicating a strong interaction between these two pathways. The miRNA-ceRNA network revealed a complex interplay between circRNAs and miRNAs, suggesting that circRNAs may act as sponges to sequester miRNAs, thereby regulating the expression of target genes. This finding highlights the importance of circRNAs in post-transcriptional gene regulation and the potential role of circRNAs in the regulation of ErbB and Hippo signaling pathways.
network, we isolated a subnetwork consisting of circRNAs (hsa_circ_0008287 and hsa_circ_0005027), miRNAs (hsa-miR-548c-3p) and 38 ErbB pathway genes which had the most interaction between miRNAs and targeted genes (Fig. 6B). Hsa_circ_0008287 and hsa_circ_0005027 were significantly downregulated in tumor samples compared with normal (Figs. 2 and 6C). In a similar manner, the miRNA-ceRNA network regulating the Hippo pathway was constructed, consisting of 33 circRNAs, 43 miRNAs and 110 Hippo pathway genes (Fig. 7A). In the Hippo miRNA-ceRNA network, we also isolated a subnetwork consisting of circRNAs (hsa_circ_0008287 and hsa_circ_0005027), miRNAs (hsa-miR-548c-3p) and 61 Hippo pathway genes, which had the most interaction between miRNAs and targeted genes (Fig. 7B).

To further investigate the important role of this subnetwork in tumor progression, the miRNA and mRNA datasets of the esophageal carcinoma cohort from TCGA (29) were exploited. The esophageal carcinoma cohort contains 13 normal samples and 184 tumor samples. In this cohort, the miRNA hsa-miR-548c-3p expression between normal and tumor samples was detected, and its clinical relevance to patient survival was analyzed. The results suggested that hsa-miR-548c-3p was highly expressed in tumor samples compared with normal samples (Fig. 8A and B), and its high expression was significantly associated with lower survival in patients with esophageal carcinoma (Fig. 8C). These findings suggested that hsa-miR-548c-3p is an oncogenic miRNA, which is consistent with the hypothesis that in tumor samples circRNAs were downregulated resulting in more oncogenic hsa-miR-548c-3p being released, and highly expressed hsa-miR-548c-3p may promote HCa progression through downstream target genes. To confirm the negative regulation of hsa-miR-548c-3p on the ErbB and Hippo pathway genes, the expression correlation of hsa-miR-548c-3p and its targeted genes were also analyzed. Many of the targeted genes were negatively correlated with hsa-miR-548c-3p levels, which supported a negative regulatory role of hsa-miR-548c-3p on the ErbB and Hippo pathways (Table III). The present results demonstrated that circRNAs regulate HCa progression through multiple pathways and identifying a miRNA-ceRNA network that regulated the ErbB and Hippo signaling pathways.
Discussion

HCA is clinically difficult to diagnose and has a poor prognosis, therefore, identifying early stage molecular biomarkers has become urgent. CircRNAs, which are stable and easier to extract and detect, are considered ideal candidates for early-stage biomarkers. This is the first report on the expression profile of circRNAs in HCA. In the present study, a number of aberrantly expressed circRNAs in HCA samples were identified. Pathway enrichment results revealed that circRNAs may regulate HCA progression through multiple signaling pathways, especially the ErbB and Hippo signaling pathways. These results provided several potential biomarkers and therapeutic targets for HCA.

The ceRNA hypothesis was described as a way that RNAs communicate with each other, via competing for...
Figure 7. Involvement of circRNAs in the Hippo signaling pathway. (A) miRNA-ceRNA network of Hippo signaling pathway. (B) Subnetwork consisting of circRNAs (hsa_circ_0008287 and hsa_circ_0005027)/miRNAs (hsa-miR-548c-3p) and Hippo pathway genes. circRNAs, circular RNAs; miRNA, microRNA; ceRNA, competing endogenous RNA.
binding to miRNAs and regulating the expression of each other to construct a complex post-transcriptional regulatory network (35,36). mRNAs and long non-coding (Inc) RNAs may all serve as ceRNAs (37). It has been demonstrated that circRNAs can also function as miRNA sponges (6,11). The present study demonstrated that aberrantly expressed circRNAs have extensive interactions with miRNAs, and those miRNAs exerted their effect on multiple cancer-related pathways. These data indicated that the circRNA-associated ceRNA network may have crucial roles in Hca progression.

The activation of ErbB oncogenes has been described in various types of human tumors, including hypopharynx carcinomas, and it has been correlated with a poor prognosis. For example, one study describing the molecular alterations in hypopharynx carcinomas demonstrated that ErbB1 was amplified in 29% of patients with hypopharyngeal squamous cell carcinomas (38). In addition, ErbB1 amplification is correlated with a hypopharyngeal primary site (39). Another study reported that v-erbB stained positively in 62.5% of hypopharyngeal squamous cell carcinomas samples but negatively in normal mucosa (40). The present ceRNA network analysis demonstrated that a circRNA (hsa_circ_0008287 and hsa_circ_0005027)/miRNA (hsa-miR-548c-3p) axis may have important roles in ErbB-mediated tumor progression (Fig. 6).

Another pathway that is likely to be associated with hypopharynx carcinomas is the Hippo signaling pathway. The Hippo pathway has generated considerable interest in recent years because of its involvement in several key hallmarks of cancer progression and metastasis (41). Regulation of Hippo signaling can be an attractive alternative strategy for cancer treatment (42-44). Previously, ACTL6A and p63 were demonstrated to cooperatively promote head and neck squamous cell carcinoma, through activation of the Hippo/Yes-associated protein 1 (YAP) pathway and YAP activation can predict poor patient survival (45). The present ceRNA network analysis demonstrated that a circRNA (hsa_circ_0008287 and hsa_circ_0005027)/miRNA (hsa-miR-548c-3p) axis may have important roles in Hippo-mediated tumor progression (Fig. 7).

Extensive evidence has suggested that miRNAs have important roles in breast cancer. The miR-548 family has been demonstrated to be involved in the pathogenesis of several cancers. For example, miR-548-3p was significantly downregulated in breast cancer and overexpression of miR-548-3p inhibited the proliferation and promoted the apoptosis of breast cancer cells (46). Overexpression of miR-548c-3p was also confirmed in prostate epithelial stem cells and in castration-resistant prostate cancer cells (45). Overexpression of miR-548c-3p in differentiated cells induced stem-like properties and radio-resistance (45). Re-analyses of published studies further revealed that miR-548c-3p is significantly overexpressed.
in castration-resistant prostate cancer cells and is associated with poor recurrence-free survival, suggesting that miR-548c-3p is a functional biomarker for prostate cancer aggressiveness (47). The present results demonstrated that miR-548c-3p may have important roles in Hca progression through modulating the ErbB and Hippo pathways. Due to the crucial roles of miR-548c-3p in multiple types of cancer, development of novel gene therapies based on miR-548c-3p might be encouraged.

Taken together, the present study indicated that hsa_circ_0008287 and hsa_circ_0005027 were downregulated in Hca and competitively bound miR-548c-3p with ErbB and Hippo signaling pathway genes. Further studies are warranted on the roles of hsa_circ_0008287, hsa_circ_0005027, and miR-548c-3p as potential diagnostic biomarkers and therapeutic targets for Hca.

Acknowledgements

Not applicable.

Funding

This work was funded by Yunnan Applied Basic Research Projects (grant no. 2016FB038).

Availability of data and materials

The sequencing data have been deposited in the Gene Expression Omnibus (GEO) database under the accession number GSE111423.
The authors declare that they have no competing interests.

Written informed consent was obtained from all the participants in the study.

The study was approved by the Ethics Committee of the First Affiliated Hospital of Kunming Medical University (Kunming, China). Written informed consent was obtained from all the participants in the study.

The authors declare that they have no competing interests.

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