circPLIN2 promotes clear cell renal cell carcinoma progression via the miR-199a-3p/ZEB1 pathway

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Article

Keywords: clear cell renal cell carcinoma (ccRCC), circular RNA (circRNA), circPLIN2, miR-199a-3p, ZEB1

Posted Date: July 5th, 2022

DOI: https://doi.org/10.21203/rs.3.rs-1761286/v1
Abstract

Recent evidence has indicated that circular RNAs (circRNAs), as a novel type of regulatory RNA, play important roles in the development and progression of various cancers. However, the potential regulatory roles and molecular mechanisms of circRNAs in clear cell renal cell carcinoma (ccRCC) remain largely unclear. Herein, we explored the expression profiles of circRNAs in 10 paired samples of RCC (including cancer tissues and surrounding tissues) from the Gene Expression Omnibus (GEO) datasets GSE124453 and GSE108735. We initially identified hsa_circ_0086457, designated circPLIN2, which was derived from exons 4 to 5 of the PLIN2 gene. circPLIN2 was significantly upregulated in ccRCC cells and tissues, and its overexpression was correlated with higher clinical stage and worse prognosis in ccRCC patients. We observed that circPLIN2 was preferentially located in the cytoplasm and had more stability than its linear counterpart PLIN2. Moreover, gain- and loss-of-function assays demonstrated that elevated circPLIN2 promoted ccRCC cell proliferation, migration and invasion in vitro and ccRCC tumor growth in vivo. Mechanistically, circPLIN2 competitively sponged miR-199a-3p to abolish the repressive effect of miR-199a-3p on ZEB1, which ultimately resulted in tumorigenesis and progression of ccRCC. Collectively, our results suggest that circPLIN2 may serve as a promising diagnostic and prognostic biomarker as well as a potential therapeutic target for ccRCC patients.

Introduction

Renal cell carcinoma (RCC) is one of the most common malignant tumors in humans, and its high morbidity and mortality, with 73,750 new cases and 14,830 deaths estimated for 2020 in the US, make it a growing global health problem [1]. Clear cell renal cell carcinoma (ccRCC) is the most common type of RCC, accounting for approximately 70–75% of RCC types [2]. Currently, the gold standard for the diagnosis and treatment of ccRCC patients is the early detection of microtumor lesions and radical surgical resection of localized ccRCC, which generally result in excellent long-term disease-free survival (DFS) [3, 4]. However, the prognosis for patients with advanced ccRCC is poor due to local tumor recurrence or distant metastasis even after radical nephrectomy [5]. Additionally, the majority of ccRCCs are resistant to both traditional chemotherapy and radiotherapy once they recur or metastasize, which leads to lower overall survival for advanced ccRCC patients [6, 7]. Therefore, it is urgent to elucidate the potential mechanisms in the pathogenesis of ccRCC and identify new effective therapeutic approaches for ccRCC.

Recently, circular RNAs (circRNAs) have been characterized by covalently closed loop structures, without a 5' cap and a 3' poly(A) tail, which are formed by back splicing events and have attracted the attention of many researchers [8–10]. circRNAs are widely involved in a variety of eukaryotes and have more stability and a stronger resistance to digestion by RNase R treatment than their linear counterpart mRNAs [8–10]. In addition, circRNAs have many important regulatory functions. For instance, circRNAs can function as competing endogenous RNAs (ceRNAs) to sponge miRNAs to regulate the expression of downstream genes [11–14] and can also interact with RNA-binding proteins to regulate protein functions [15–20]. In recent years, increasing evidence has shown that circRNAs can encode functional microproteins by the
cap-independent translation pathway [21–23] or m^6^A (N^6^-methyladenosine) modification [24–26].
circRNAs, as a novel type of regulatory RNA molecule, play important roles in the development and
progression of various cancers [27–31]. Meanwhile, the conserved, stable and spatiotemporal-specific
characteristics of circRNAs make them excellent biomarkers for tumor diagnosis and prognosis and
potential therapeutic targets for malignant tumors [32–34]. However, to date, the key regulatory roles and
underlying molecular mechanisms of circRNAs in the development and progression of ccRCC remain
largely unclear.

In this study, we investigated the expression profiles of circRNAs in 10 paired samples of RCC (including
cancer tissues and surrounding tissues) from the GEO datasets GSE124453 and GSE108735. We initially
identified hsa_circ_0086457, termed circPLIN2, derived from exons 4 to 5 of the PLIN2 gene. circPLIN2
was markedly upregulated in ccRCC cells and tissues, and its overexpression was correlated with higher
clinical stage and worse prognosis in ccRCC patients. circPLIN2 was preferentially located in the
cytoplasm and had more stability than its linear transcript PLIN2. Gain- and loss-of-function assays
indicated that elevated circPLIN2 promoted ccRCC cell proliferation, migration and invasion in vitro and
ccRCC tumor growth in vivo. Mechanistically, circPLIN2 competitively sponged miR-199a-3p to abolish
the repressive effect of miR-199a-3p on ZEB1, which ultimately resulted in tumorigenesis and progression
of ccRCC.

Results

1. circPLIN2 is significantly upregulated in ccRCC cells and
tissues and participates in the progression of ccRCC

To explore the regulatory roles of circRNAs and their underlying molecular mechanisms in the
development and progression of human ccRCC, we first analyzed the expression profiles of circRNAs in
human ccRCC. We performed a joint analysis of the circRNA expression data for a total of 10 paired
samples of RCC (including cancer tissues and surrounding tissues) from the GEO datasets GSE124453
and GSE108735 (http://www.ncbi.nlm.nih.gov/geo) (Fig. 1A and Supplementary Table 1). A total of
12,299 circRNAs were identified (Fig. 1B and Supplementary Table 2). Among all circRNAs, 243 were
identified as differentially expressed circRNAs between RCC and normal samples, including 186
downregulated circRNAs and 57 upregulated circRNAs in RCC (Fig. 1C and Supplementary Table 2). It
was found that hsa_circ_0086457, designated circPLIN2, was significantly upregulated in RCC samples
(Fig. 1C). In situ hybridization staining was performed on a tissue microarray of human ccRCC, including
90 cases of tumor tissues and adjacent tissues, with probes specific for circPLIN2 to validate its
expression. Three representative cases of in situ hybridization staining for circPLIN2 expression in the
tissue microarray were shown (Fig. 1D). We found that circPLIN2 was significantly upregulated in ccRCC
tissues compared with surrounding normal tissues (Fig. 1E left), accounting for approximately 63%
(57/90) of 90 ccRCC specimens (Fig. 1E right). To further examine circPLIN2 overexpression in ccRCC, we
used a panel of four human ccRCC cell lines (786-O, ACHN, 769-P and OS-RC-2) and HK-2 cells (a
proximal tubule epithelial cell line) to test circPLIN2 expression by RT–qPCR. The results showed that circPLIN2 was observably overexpressed in ccRCC cells compared to HK-2 cells (Fig. 1F), which was consistent with the results of in situ hybridization staining assays (Fig. 1D-E).

Further analysis indicated that circPLIN2 levels were dramatically higher in ccRCC tissues at the advanced American Joint Committee on Cancer (AJCC) stages (AJCC 3–4 stages) than in ccRCC tissues at the AJCC early stages (AJCC 1–2 stages) (Fig. 1G). Additionally, we analyzed the correlation between circPLIN2 expression and clinicopathological characteristics in 90 ccRCC patients. The results showed that circPLIN2 expression was only significantly correlated with tumor differentiation, and the higher the expression level of circPLIN2 was, the worse the tumor differentiation and the higher the malignant grade of the tumor (Table 1). The survival curve analysis showed that ccRCC patients with high circPLIN2 expression had a markedly lower overall survival rate than ccRCC patients with low circPLIN2 expression (Fig. 1H). Moreover, the univariate Cox proportional hazard regression analysis showed that the differential expression of circPLIN2 was significantly correlated with overall survival in 78 ccRCC patients (P = 0.026) (Table 2), which was consistent with the results of the Kaplan–Meier analysis (Fig. 1H). However, the multivariate Cox proportional hazard regression analysis showed that the differential expression of circPLIN2 was not associated with overall survival in 78 ccRCC patients (P = 0.206) (Table 2), which may be explained by the fact that the number of patients involved in the study was small or there were some factors that interfered with the true results. The receiver operating characteristic curve (ROC) results indicated that the expression level of circPLIN2 showed excellent diagnostic performance for cancer and paracancer (Fig. 1I), AJCC 1–2 stages and 3–4 stages (Fig. 1J), and survival and death of ccRCC patients (Fig. 1K). Collectively, these results suggested that circPLIN2 was significantly upregulated in ccRCC cells and tissues and that its overexpression was correlated with higher clinical stage and worse prognosis in ccRCC patients.
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1. LN, Lymph node.

2. **, p < 0.01.
Table 2
Univariate and multivariate analyses of factors associated with overall survival in 78 ccRCC patients with significant high or low expression of circPLIN2.

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## Factors Overall survival

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<th>Multivariate analysis</th>
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<td>circPLIN2 expression</td>
<td>0.304, 0.106–0.869</td>
<td>0.480, 0.154–1.496</td>
</tr>
<tr>
<td></td>
<td>0.026*</td>
<td>0.206</td>
</tr>
<tr>
<td>High</td>
<td></td>
<td></td>
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<tr>
<td>Low</td>
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</table>

1. Statistical analysis, Cox proportional hazard regression model; 95% CI, 95% confidence interval; HR, Hazard ratio. LN, Lymph node.

2. *, p < 0.05; **, p < 0.01; ***, p < 0.001. which is considered as a significant difference.

### 2. General characteristics of circPLIN2

circPLIN2 is a circular RNA molecule derived from exons 4 to 5 of the PLIN2 gene on human chromosome 9 (9p22.1) with a length of 369 nucleotides (Fig. 2A). The back-splice junction of circPLIN2 was amplified using divergent primers and confirmed by Sanger sequencing, and the result was consistent with the circBase database annotation (http://www.circbase.org) (Fig. 2A). Subsequently, PCR amplification assays and agarose gel electrophoresis assays using divergent and convergent primers further demonstrated that circPLIN2 and its linear isoform PLIN2 both truly existed in ccRCC cells (Fig. 2B). We next investigated the resistance of circPLIN2 to digestion by RNase R treatment, and the results indicated that circPLIN2 was more tolerant to RNase R digestion than the linear counterpart PLIN2 (Fig. 2C). In addition, actinomycin D, an inhibitor of transcription, was used to test the half-life of circPLIN2 in ccRCC cells, and the results showed that the content of circPLIN2 decreased slowly over time compared with the linear transcript PLIN2 in 786-O cells in the presence of 2 μg/mL actinomycin D, suggesting that circPLIN2 had more stability or a longer half-life than its linear counterpart PLIN2 (Fig. 2D). To explore the cellular localization of circPLIN2, we performed RT–qPCR analysis to determine the abundance of nuclear and cytoplasmic circPLIN2 in ccRCC cells. The results showed that circPLIN2 was preferentially located in the cytoplasm of ACHN (Fig. 2E) and OS-RC-2 (Fig. 2F) cells, which was consistent with the results of the fluorescence in situ hybridization (FISH) assays (Fig. 2G-H). Overall, circPLIN2, the back-spliced product of the parent gene PLIN2, was preferentially distributed in the cytoplasm of ccRCC cells and had a longer half-life and a stronger resistance to RNase R digestion than its linear counterpart PLIN2.

### 3. circPLIN2 promotes the proliferation, migration and invasion of ccRCC cells in vitro

To investigate whether changes in the expression of circPLIN2 affected the biological behaviors of ccRCC cells, two small interfering RNAs (circPLIN2-siRNA 1 and circPLIN2-siRNA 2) were designed and synthesized specifically targeting the back-splice junction of circPLIN2, and a circPLIN2 overexpression
vector was designed and constructed. The results of RT–qPCR assays showed that these two siRNAs could specifically knock down the expression level of circPLIN2 in ACHN and OS-RC-2 cells but had no effect on PLIN2 mRNA expression (Fig. 3A). Similarly, circPLIN2 was successfully overexpressed in ACHN and OS-RC-2 cells, while PLIN2 mRNA expression showed no obvious change (Fig. 3B). Then, we detected the effects of knockdown and overexpression of circPLIN2 on the proliferation of ccRCC cells. The results of the CCK-8 assays showed that knockdown of circPLIN2 significantly inhibited the proliferation of ACHN, OS-RC-2, 786-O and 769-P cells (Fig. 3C), while overexpression of circPLIN2 drastically promoted the proliferation of ACHN, OS-RC-2, 786-O and 769-P cells (Fig. 3D). Similar results were obtained in the colony formation assays. Knockdown of circPLIN2 markedly impaired the ability of ACHN and OS-RC-2 cells to form colonies (Fig. 3E), while overexpression of circPLIN2 notably enhanced the colony formation ability of ACHN and OS-RC-2 cells (Fig. 3F). Furthermore, wound-healing assays indicated that knockdown of circPLIN2 significantly suppressed the migration ability of ACHN (Fig. 3G) and OS-RC-2 (Fig. 3H) cells, while overexpression of circPLIN2 significantly promoted the migration of ACHN (Fig. 3I) and OS-RC-2 (Fig. 3J) cells. Additionally, Matrigel Transwell assays showed that knockdown of circPLIN2 obviously attenuated the invasion activities of ACHN and OS-RC-2 cells (Fig. 3K), and the opposite results were observed when circPLIN2 was overexpressed in ACHN and OS-RC-2 cells (Fig. 3L). Taken together, these results revealed that circPLIN2 significantly promoted the proliferation, migration and invasion of ccRCC cells in vitro.

4. circPLIN2 competitively sponges miR-199a-3p to abolish the repressive effect of miR-199a-3p on ZEB1

Increasing evidence has shown that circRNAs can function as sponges for miRNAs to regulate the expression of genes by the competing endogenous RNA (ceRNA) mechanism [11-14]. Given that circPLIN2 was preferentially distributed in the cytoplasm (Fig. 2E-H), we investigated whether circPLIN2 might also function by a ceRNA mechanism. We first made predictions through the circBank (http://www.circbank.cn/index.html) database and selected 10 miRNAs that might be sponged by circPLIN2 for further validation (Fig. 4A). The dual-luciferase reporter assays showed that miR-199a-3p had a particularly significant inhibitory effect on the luciferase activity of circPLIN2, suggesting that circPLIN2 might sponge miR-199a-3p (Fig. 4B). To further verify that circPLIN2 sponged miR-199a-3p, we constructed a circPLIN2 dual-luciferase reporter with the mutated miR-199a-3p binding site (Supplementary Fig. 1A). The results of dual-luciferase reporter assays showed that the wild-type (WT) circPLIN2 luciferase activity was significantly inhibited by miR-199a-3p, while the mutated (MUT) circPLIN2 luciferase activity was not affected (Fig. 4C). In addition, the results of RNA immunoprecipitation assays showed that circPLIN2 was drastically enriched on AGO2 protein compared with the control IgG, and the enrichment of circPLIN2 on AGO2 protein was further increased when miR-199a-3p was added (Fig. 4D). These data revealed that circPLIN2 sponged miR-199a-3p (Fig. 4A-D).
(https://mrmicrot.imsi.athenarc.gr/), miRmap (https://mirmap.ezlab.org/), and PITA (https://genie.weizmann.ac.il/pubs/mir07/index.html) databases. We found a total of 88 target genes that coappeared in these five databases (Fig. 4E). We further performed enrichment analysis of molecular function (GO_MF enrichment) on these 88 target genes of miR-199a-3p via the DAVID tool (https://david.ncifcrf.gov/) and found that the P value of the “transcription corepressor activity” term was the most significant (P = 0.000351) (Fig. 4F). There were seven target genes of miR-199a-3p appearing in the “transcription corepressor activity” term, including AEBP2, CITED2, MEIS2, RUNX1, ZEB1, ZHX1 and ZHX2. RT–qPCR showed that knockdown of circPLIN2 drastically suppressed the expression of ZEB1 but had no effect on the expression levels of AEBP2, CITED2, MEIS2, RUNX1, ZHX1 and ZHX2 (Fig. 4G). Similarly, overexpression of circPLIN2 significantly increased the expression of ZEB1, while the expression levels of AEBP2, CITED2, MEIS2, RUNX1, ZHX1 and ZHX2 showed no obvious changes (Fig. 4H). The results of western blot assays also indicated that circPLIN2 could regulate the expression of the target gene ZEB1 of miR-199a-3p (Fig. 4I-J and Supplementary Fig. 2-5). Moreover, wild-type (WT) and mutated (MUT) ZEB1 dual-luciferase reporters targeting the miR-199a-3p binding site were constructed to detect the binding of ZEB1 and miR-199a-3p (Supplementary Fig. 1B). The results of the dual-luciferase reporter assays showed that the addition of miR-199a-3p significantly inhibited the wild-type ZEB1 luciferase activity, while the mutated ZEB1 luciferase activity was not affected, suggesting that ZEB1 sponged miR-199a-3p (Fig. 4K).

Next, we considered whether there was a ceRNA mechanism among circPLIN2, miR-199a-3p and ZEB1. The RT–qPCR results showed that miR-199a-3p significantly reduced the expression level of ZEB1, while overexpression of circPLIN2 abolished the repressive effect of miR-199a-3p on ZEB1 expression (Fig. 4L-M). Additionally, the results of the dual-luciferase reporter assays indicated that overexpression of circPLIN2 significantly increased wild-type ZEB1 luciferase activity, while knockdown of circPLIN2 markedly decreased wild-type ZEB1 luciferase activity (Fig. 4N). Moreover, the mutated ZEB1 luciferase activity was not affected by circPLIN2 overexpression or knockdown (Fig. 4N). These results revealed that there was an endogenous RNA competition relationship between circPLIN2 and ZEB1 for miR-199a-3p. Collectively, these results suggested that circPLIN2 competitively sponged miR-199a-3p to abolish the repressive effect of miR-199a-3p on ZEB1.

5. circPLIN2 exerts its carcinogenic effects on ccRCC cells via the miR-199a-3p/ZEB1 axis in vitro

Next, we investigated whether the circPLIN2/miR-199a-3p/ZEB1 molecular signaling pathway participated in the development and progression of ccRCC. The results of CCK-8 cell viability assays showed that knockdown of circPLIN2 significantly repressed the proliferation of ACHN and OS-RC-2 cells, and the proliferation of ACHN and OS-RC-2 cells was further inhibited when miR-199a-3p was added (Fig. 5A-B). Overexpression of ZEB1 rescued the inhibition of circPLIN2 knockdown and addition of miR-199a-3p on the proliferation of ccRCC cells (Fig. 5A-B). Similar results were obtained in the colony formation assays. Overexpression of ZEB1 drastically rescued the long-term suppression of circPLIN2 knockdown
and addition of miR-199a-3p on the proliferation of ccRCC cells (Fig. 5C-D). Furthermore, the wound-healing assays indicated that knockdown of circPLIN2 markedly reduced the wound-healing speeds of ACHN and OS-RC-2 cells, and the wound-healing speeds of ACHN and OS-RC-2 cells were slower when miR-199a-3p was added, while overexpression of ZEB1 significantly rescued the inhibition of circPLIN2 knockdown and the addition of miR-199a-3p on the migration of ccRCC cells (Fig. 5E-F). Moreover, the results of Matrigel Transwell assays showed that overexpression of ZEB1 drastically rescued the repression of circPLIN2 knockdown and the addition of miR-199a-3p on the invasion of ccRCC cells in vitro (Fig. 5G-H). Overall, our data suggested that the circPLIN2/miR-199a-3p/ZEB1 molecular signaling pathway was involved in the proliferation, migration and invasion of ccRCC cells.

6. circPLIN2 promotes ccRCC tumor growth in vivo

To examine the effect of circPLIN2 on the growth of ccRCC cells in vivo, we constructed subcutaneous xenograft tumors of ACHN with stable low or high expression of circPLIN2 in BALB/c nude mice. Photographs of the tumors at necropsy showed that stable knockdown of circPLIN2 significantly inhibited the growth of ACHN cells in vivo (Fig. 6A), while stable overexpression of circPLIN2 drastically promoted the growth of ACHN cells in vivo (Fig. 6B). In addition, the volumes of subcutaneous xenograft tumors indicated that stable knockdown of circPLIN2 markedly decreased the volumes of tumors in nude mice compared with the control group (Fig. 6C), whereas stable overexpression of circPLIN2 suggested the opposite results (Fig. 6D), which was consistent with the results of weight measurement of subcutaneous xenograft tumors (Fig. 6E-F). Collectively, these results revealed that circPLIN2 promoted the growth of ccRCC cells in vivo.

Discussion

In this study, we proved the oncogenic roles of circPLIN2 and determined its underlying mechanism in the development and progression of ccRCC. We first explored the expression profiles of circRNAs in 10 paired samples of RCC from GSE124453 and GSE108735 in the GEO database. We initially identified hsa_circ_0086457, designated circPLIN2, which was derived from exons 4 to 5 of the PLIN2 gene. circPLIN2 was significantly upregulated in ccRCC cells and tissues, and its overexpression was correlated with higher clinical stage and worse prognosis in ccRCC patients. We identified the characteristics of circPLIN2 in ccRCC cells and found that circPLIN2 was preferentially distributed in the cytoplasm of ccRCC cells and had a longer half-life and a stronger resistance to digestion by RNase R treatment than its linear counterpart PLIN2. Intriguingly, depletion of circPLIN2 significantly attenuated the proliferation, migration and invasion of ccRCC cells in vitro and the tumor growth of ccRCC in vivo, whereas overexpression of circPLIN2 resulted in the opposite effects, suggesting that elevated circPLIN2 may be a cancer-promoting event in ccRCC. Mechanistically, circPLIN2 competitively sponged miR-199a-3p to abolish the repressive effect of miR-199a-3p on ZEB1, which ultimately resulted in tumorigenesis and progression of ccRCC. Together, these findings indicated the oncogenic function of circPLIN2 and its
potential molecular mechanism in which elevated circPLIN2 participated in the development and progression of ccRCC by binding miR-199a-3p to regulate ZEB1 expression (Fig. 6G).

Recent evidence has shown that circRNAs play vital roles in the development and progression of ccRCC [35–38]. For example, circZNF609, which is highly expressed in various ccRCC cell lines, acts as a sponge for miR-138-5p to upregulate the expression of FOXP4 and promote the growth and invasion of ccRCC [35]. Intriguingly, it has been shown that circZNF609 in myoblasts can be translated into a functional small protein [39], which is regulated by its own m6A modification [40]. Hence, we considered whether circZNF609 has a translational protein and regulatory role in ccRCC, which requires further exploration in the future. As another example showed, circTLK1 was not merely drastically upregulated in ccRCC cells and tissues but was related to the distant metastasis of tumors and the prognosis of ccRCC patients [36]. Moreover, circTLK1 upregulated the expression of CBX4 by competitively sponging miR-136-5p to exert its oncogenic activity [36]. Although these circRNAs have been shown to be involved in the development and progression of ccRCC, their key regulatory roles and molecular mechanisms have not been fully clarified. In addition, novel circRNAs need to be further identified in ccRCC.

In this study, to more accurately detect the expression profiles of circRNAs in RCC, we selected a total of 10 paired RCC samples of circRNA expression data from GSE124453 and GSE108735 in the GEO database for joint analysis, which can reduce the bias of RCC sample selection in two different studies and expand the RCC sample size to make circRNA expression data more reliable. We found that circPLIN2, as an oncogene, was significantly highly expressed in ccRCC cells and tissues, and its overexpression was correlated with higher clinical stage and worse prognosis in ccRCC patients. Furthermore, elevated circPLIN2 promoted ccRCC cell proliferation, migration and invasion in vitro and ccRCC tumor growth in vivo. This is similar to the performance and function of circTLK1, circSDHC and circPRRC2A in ccRCC [36, 41, 42]. However, unlike circPLIN2, circRAPGEF5 and circAKT3 were significantly expressed at low levels in ccRCC and inhibited the malignant progression of ccRCC [43, 44]. Overall, these conflicting results surrounding circRNA performance in ccRCC can be partly explained by the fact that circRNAs participate in different molecular signaling pathways.

In addition, we found an underlying ceRNA mechanism in which circPLIN2 competitively sponged miR-199a-3p to abolish the repressive effect of miR-199a-3p on ZEB1. Subsequent rescue assays further showed that the circPLIN2/miR-199a-3p/ZEB1 molecular signaling pathway participated in the development and progression of ccRCC. Intriguingly, ZEB1, as a transcriptional repressor, inhibits the transcription of E-cadherin by recruiting BRG1 and promotes epithelial-mesenchymal transition (EMT) and tumor progression [45]. Hence, we speculate that EMT may be involved in the circPLIN2-regulated development and progression of ccRCC, which needs to be further evaluated. Moreover, ZEB1 suppresses the expression of stemness-inhibiting miR-200 and miR-203 and promotes tumor proliferation and progression [46]. Therefore, we speculate that miR-200 and miR-203 may also participate in circPLIN2-mediated ccRCC progression, which also needs to be further confirmed.
In conclusion, our study suggested that circPLIN2 functioned as an oncogene and participated in the development and progression of ccRCC. Additionally, our results revealed that circPLIN2 sponged miR-199a-3p to abolish the repressive effect of miR-199a-3p on ZEB1, which ultimately resulted in tumorigenesis and progression of ccRCC. These data revealed that circPLIN2 may serve as a promising diagnostic and prognostic biomarker as well as a potential therapeutic target for ccRCC patients.

**Materials And Methods**

**Bioinformatics analysis of the expression profile of circRNAs in RCC**

We first retrieved circRNA expression data in RCC from the GEO database (http://www.ncbi.nlm.nih.gov/geo) and obtained GSE124453 and GSE108735 data. Then, we downloaded the raw data of GSE124453 and GSE108735 from the SRA database (https://www.ncbi.nlm.nih.gov/sra) and converted them into FASTQ format using Sratoolkit software (version 2.9.2) (https://hpc.nih.gov/apps/sratoolkit.html). The FASTQ files were aligned onto the human hg38 reference using STAR software (version 2.7.1a) (https://github.com/alexdobin/STAR) [47]. circRNAs were subsequently calculated and identified using DCC software (https://github.com/dieterich-lab/DCC) with default parameters [48]. Next, the circRNAs identified were filtered by read count more than 5 and expressed samples over 30%. The function and identities of circRNAs were then annotated by the circBase database (http://www.circbase.org) [49]. DESeq2 was used to read the raw count matrix after filtration, and normalization was performed by using the variance stabilizing transformation algorithm [50]. Significantly differentially expressed circRNAs between RCC and normal samples were screened with the criteria of adjusted p value less than 0.05 and absolute value of log$_2$(fold change) more than 2. The results of the bioinformatics analysis were eventually visualized as a heatmap and a volcano plot.

**Plasmid construction and cell transfection assay**

Referring to the method for constructing the circTP63 overexpression vector described in a previous study [51], we successfully constructed the circPLIN2 overexpression vector by homologous recombination using the pLCDH-ciR plasmid. For cell transfection assays, briefly, cells were first seeded on 6-well plates to a confluence of approximately 50%. Next, cells were transfected with circPLIN2 or vector using Lipofectamine 2000 reagent according to the manufacturer’s protocol and then cultured at 37 °C with 5% CO₂ for 48~72 h. Finally, the expression level of circPLIN2 was assessed by RT–qPCR. Additionally, the overexpression vector of ZEB1 was designed and constructed by GENE (Shanghai, China) using the GV658 plasmid. The primers used for plasmid construction are listed in Supplementary Table 3.

**Statistical analysis**
The IBM SPSS package (version 23.0) and GraphPad Prism software (version 6.0) were used for statistical analysis. All data in this study are shown as the means ± S.D. of the values from triplicate assays. Two-tailed Student’s t test was used to compare two independent groups. Spearman’s test was performed to analyze the correlations for categorical variables. The Kaplan–Meier test was performed for the univariate analysis of overall survival, and the Cox proportional hazards regression model was used for the multivariate analysis of overall survival. *p < 0.05, **p < 0.01 and ***p < 0.001 were considered statistically significant.

Declarations

Acknowledgements

We would like to thank the Biomedical Research Institute of Shenzhen Peking University - The Hong Kong University of Science and Technology Medical Center for platform support and technical help. This work was supported by grants from the National Natural Science Foundation of China (81874249), Guangdong Basic and Applied Basic Research Foundation (2020A1515011125, 2021A1515011558), and Shenzhen Basic Research Grants (JCYJ20180223181224405, JCYJ20180507182657867, JCYJ20210324110008023) and Scientific Research Foundation of Peking University Shenzhen Hospital (KYQD2021038).

Author contributions

Bin Z performed most of experiments and data analysis and wrote the manuscript. Cong H performed animal experiments and participated in data analysis and discussion for results. Jie P and Xiaofan C revised manuscript. Hao H, Xiaojuan L, Kaoyuan Z, Fenli Z, Xin S, Jun W and Bo Y participated in data analysis and discussion for results. Bin Z, Xiaofan C and Wei Z designed the overall study, supervised the experiments and discussed the results.

Conflict of interest

The authors declare that they have no competing interests.

References


Figures
Figure 1

circPLIN2 is upregulated in ccRCC cells and tissues and participates in the progression of ccRCC. A The flowchart delineates the steps for exploring circRNA expression profiling in 10 paired samples of RCC by meta-analysis of the GSE124453 and GSE108735 datasets from GEO. B Heatmap of circRNA expression in 10 paired samples of RCC. C Volcano plot for differentially expressed circRNAs in 10 paired samples of RCC. D Representative three cases of in situ hybridization (ISH) for circPLIN2 expression in the ccRCC.
tissue microarray. Scale bar, 20 μm. E circPLIN2 ISH staining scores in 90 pairs of cancerous and paracancerous tissues are shown on the left, and the expression profiles of circPLIN2 in 90 patients with ccRCC are shown on the right. F RT–qPCR analysis of the relative expression levels of circPLIN2 in a panel of four human ccRCC cell lines (786-O, ACHN, 769-P, OS-RC-2) and an immortalized proximal tubule epithelial cell line (HK-2). The relative circPLIN2 expression level was normalized to GAPDH. G circPLIN2 ISH staining scores in ccRCC tissues (n = 90) in different AJCC stages. H Overall survival curve of ccRCC patients with high circPLIN2 expression (n=57) or low circPLIN2 expression (n=21). Statistical significance was determined by the Kaplan–Meier test. I-K The receiver operating characteristic curve (ROC) analysis for cancer and paracancer (I), AJCC stage 1-2 and 3-4 (J), survival and death (K) in 90 ccRCC patients based on the circPLIN2 ISH staining scores. Two-tailed Student’s t test (E-G). The error bars represent S.D. *p < 0.05; **p < 0.01; ***p < 0.001.
Figure 2

General characteristics of circPLIN2. A Genomic localization of circPLIN2. circPLIN2 is derived from exons 4 to 5 of the parental PLIN2 gene and has a length of 369 nucleotides. The back-splice junction of circPLIN2 was identified by Sanger sequencing. Divergent primer for circPLIN2 and convergent primer for PLIN2. B PCR and agarose gel electrophoresis analysis of circPLIN2 and its linear isoform PLIN2 in the cDNA of OS-RC-2 cells. circPLIN2, 128 bp; PLIN2, 90 bp; GAPDH, 197 bp. bp, base pair. GAPDH served as a positive control. C RT–qPCR analysis of the abundance of circPLIN2 and PLIN2 in 786-O cells treated
with RNase R. D RT–qPCR analysis of the levels of circPLIN2 and PLIN2 in 786-O cells treated with actinomycin D (2 μg/mL) at the indicated time points. E-F RT–qPCR analysis of the abundance of circPLIN2 in the nuclear and cytoplasmic fractions of ACHN (E) and OS-RC-2 (F) cells. GAPDH served as a cytoplasmic positive control, and U6 served as a nuclear positive control. G-H Fluorescence in situ hybridization (FISH) analysis of circPLIN2 levels in the nuclear and cytoplasmic fractions of ACHN (G) and OS-RC-2 (H) cells. All probes are labeled with Cy3. 18S was used as a cytoplasmic positive control, and U6 was used as a nuclear positive control. Two-tailed Student’s t test (C). The error bars represent S.D. (n=3). ns, no significance; ***p < 0.001.
**Figure 3**

circPLIN2 promotes the proliferation, migration and invasion of ccRCC cells in vitro. 

A RT–qPCR analysis of the relative expression levels of circPLIN2 and PLIN2 in ACHN and OS-RC-2 cells transfected with circPLIN2-siRNA 1/2 or circPLIN2-NC. 

B RT–qPCR analysis of the relative expression levels of circPLIN2 and PLIN2 in ACHN and OS-RC-2 cells transfected with circPLIN2 or vector. 

C CCK-8 cell viability assays for ACHN, OS-RC-2, 786-O and 769-P cells transfected with circPLIN2-siRNA 1/2 or circPLIN2-NC. 

D CCK-8
cell viability assays for ACHN, OS-RC-2, 786-O and 769-P cells transfected with circPLIN2 or vector. 

E Colony formation assays for ACHN and OS-RC-2 cells transfected with circPLIN2-siRNA 1/2 or circPLIN2-NC. The number of colonies was determined (right). 

F Colony formation assays for ACHN and OS-RC-2 cells transfected with circPLIN2 or vector. The number of colonies was determined (right). 

G-H Wound-healing assays for ACHN (G) and OS-RC-2 (H) cells transfected with circPLIN2-siRNA 1/2 or circPLIN2-NC. The wound closure rate was calculated (right). Amplification, 40x. 

I-J Wound-healing assays for ACHN (I) and OS-RC-2 (J) cells transfected with circPLIN2 or vector. The wound closure rate was calculated (right). Amplification, 40x. 

K Matrigel Transwell assays for ACHN and OS-RC-2 cells transfected with circPLIN2-siRNA 1/2 or circPLIN2-NC. The cell number per field are quantified (right). Scale bar, 100 μm. 

L Matrigel Transwell assays for ACHN and OS-RC-2 cells transfected with circPLIN2 or vector. The cell number per field was quantified (right). Scale bar, 100 μm. Two-tailed Student’s t test (A-L). The error bars represent S.D. (n=3). *p < 0.05; **p < 0.01; ***p < 0.001.
**Figure 4**

circPLIN2 competitively sponges miR-199a-3p to abolish the repressive effect of miR-199a-3p on ZEB1. A sketch map was drawn to show circPLIN2 sponging 10 miRNAs predicted in the circBank database. B Dual-luciferase reporter assays for the luciferase activity of circPLIN2 in 293T cells treated with different miRNAs. Luciferase activity was normalized to firefly luciferase activity. C Dual-luciferase reporter assays for the luciferase activity of circPLIN2 in 293T cells treated with pmiR-circPLIN2-WT or pmiR-circPLIN2-
MUT and mimics NC or miR-199a-3p mimics. Luciferase activity was normalized to firefly luciferase activity. D RNA immunoprecipitation analysis for the fold enrichment of circPLIN2 with anti-AGO2 antibody or anti-IgG antibody in 293T cells treated with mimics NC or miR-199a-3p mimics. The IgG group served as the control. AGO2, Argonaute 2. E Venn diagram showing the downstream target genes of miR-199a-3p predicted by the TargetScan, PicTar, microT, miRmap and PITA databases. F GO_MF enrichment analysis for 88 downstream target genes of miR-199a-3p commonly predicted in the TargetScan, PicTar, microT, miRmap and PITA databases. GO, Gene Ontology. MF, Molecular function. G RT–qPCR analysis of the relative expression levels of AEBP2, CITED2, MEIS2, RUNX1, ZEB1, ZHX1 and ZHX2 in ACHN cells treated with circPLIN2-siRNA 1/2 or circPLIN2-NC. H RT–qPCR analysis of the relative expression levels of AEBP2, CITED2, MEIS2, RUNX1, ZEB1, ZHX1 and ZHX2 in ACHN cells treated with circPLIN2 or vector. I Western blot analysis of the levels of ZEB1 in ACHN (left) and OS-RC-2 (right) cells treated with circPLIN2-siRNA 1/2 or circPLIN2-NC. GAPDH served as the loading control. J Western blot analysis of the levels of ZEB1 in ACHN (left) and OS-RC-2 (right) cells treated with circPLIN2 or vector. GAPDH was used as the loading control. K Dual-luciferase reporter assays for the luciferase activity of ZEB1 in 293T cells treated with pmiR-ZEB1-3'UTR-WT or pmiR-ZEB1-3'UTR-MUT and mimics NC or miR-199a-3p mimics. Luciferase activity was normalized to firefly luciferase activity. L-M RT–qPCR analysis of the relative expression levels of ZEB1 in ACHN (L) and OS-RC-2 (M) cells treated with mimics NC or miR-199a-3p mimics and circPLIN2 or vector. N Dual-luciferase reporter assays for the luciferase activity of ZEB1 in 293T cells treated with pmiR-ZEB1-3'UTR-WT or pmiR-ZEB1-3'UTR-MUT and circPLIN2 or vector as well as circPLIN2-siRNA 1 or circPLIN2-NC. Luciferase activity was normalized to firefly luciferase activity. Two-tailed Student’s t test. The error bars represent S.D. (n=3). ns, no significance; *p < 0.05; **p < 0.01; ***p < 0.001.
Figure 5

circPLIN2 exerts its carcinogenic effects on ccRCC cells via the miR-199a-3p/ZEB1 axis in vitro. A-B CCK-8 cell viability assays for ACHN (A) and OS-RC-2 (B) cells transfected with circPLIN2-siRNA 1 or circPLIN2-NC and ZEB1 or vector and miR-199a-3p mimics or mimics NC. C-D Colony formation assays for ACHN (C) and OS-RC-2 (D) cells transfected with circPLIN2-siRNA 1 or circPLIN2-NC and ZEB1 or vector and miR-199a-3p mimics or mimics NC. The number of colonies was determined. E-F Wound-healing assays for ACHN (E) and OS-RC-2 (F) cells transfected with circPLIN2-siRNA 1 or circPLIN2-NC and ZEB1 or vector and miR-199a-3p mimics or mimics NC. The wound closure rate was calculated. Amplification, 40x. G-H Matrigel Transwell assays for ACHN (G) and OS-RC-2 (H) cells transfected with circPLIN2-siRNA 1 or circPLIN2-NC and ZEB1 or vector and miR-199a-3p mimics or mimics NC. The cell
number per field was quantified. Scale bar, 100 μm. Two-tailed Student’s t test (A-H). The error bars represent S.D. (n=3). *p < 0.05; **p < 0.01; ***p < 0.001.

Figure 6

circPLIN2 promotes ccRCC tumor growth in vivo. A Following subcutaneous injections of ACHN cells treated with lentivirus-circPLIN2-shRNA 1/2 or lentivirus-circPLIN2-NC in athymic nude mice and tumor
growth for 35 days, photographs of the tumors were obtained at necropsy. **B** Following subcutaneous
injections of ACHN cells treated with lentivirus-circPLIN2 or lentivirus-vector in athymic nude mice and
tumor growth for 35 days, photographs of the tumors were obtained at necropsy. **C-D** The volumes of
subcutaneous xenograft tumors of ACHN cells were measured every 5 days. **E-F** Boxplot analysis of the
weights of xenograft tumors of ACHN cells isolated from nude mice 35 days after subcutaneous
injection. **G** Hypothesis diagram illustrating the function and mechanism of circPLIN2 in ccRCC
progression. Two-tailed Student's t test (**C-F**). The error bars represent S.D. (n=6). ***p < 0.001.

**Supplementary Files**

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