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ORIGINAL ARTICLE

circYap inhibits oral squamous cell carcinoma by arresting cell cycle

Xiao-Yun Zhang[a], Huifang Tang[b], Yanping Liu[c], Nan Du[b], Songbo Tian[b] and Yong-Qing Dou[a]

[a]College of Integrative Medicine, Hebei University of Chinese Medicine, Shijiazhuang, China; [b]Department of Oral Medicine, The Second Hospital of Hebei Medical University, Shijiazhuang, China; [c]Physical Examination Center, The Second Hospital of Hebei Medical University, Shijiazhuang, China;

ABSTRACT

Objective: Circular RNAs (circRNAs) involve in the development and progression of tumour. The mechanism of circRNAs in oral squamous cell carcinoma (OSCC) has remained unclear. This study aimed to investigate the role of circular Yes-associated protein (circYap) in OSCC.

Methods: Quantification reverse transcription-polymerase chain reaction (qRT-PCR) was applied to measure circYap expression in patients with OSCC tissues and cells. Flow cytometry was performed to evaluate cell cycle. circYap interaction with CDK4 was detected by RNA immunoprecipitation (RIP) and RNA pull-down. The interaction of Cyclin D1 and CDK4 was determined using co-immunoprecipitation (co-IP).

Results: We showed that circYap expression was downregulated in OSCC tissues. Using small interfering circular (Si-circYap) and overexpression plasmid, we found that circYap overexpression inhibited proliferation and arrested cell cycle in OSCC cells, while, circYap knockdown yielded the opposite result. Cyclin D1/CDK4 complexes and nuclear translocation is essential for cell cycle progression. We found that CDK4 interacted with circYap was increased when circYap overexpression, meanwhile, Cyclin D1/CDK4 complexes and of nuclear distribution were decreased.

Conclusions: Our findings suggest that circYap impedes progression of OSCC. Overexpression of circYap suppresses proliferation and cell cycle through binding to CDK4 to block formation and nuclear translocation of Cyclin D1/CDK4 complexes. Thus, circYap may serves as a valuable therapeutic target for OSCC.

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Introduction

Oral squamous cell carcinoma (OSCC) is one of the most common malignancies in the head and neck. There are more than 300,000 new cases of OSCC and more than 140,000 deaths every year. The incidence of OSCC is increasing year by year [1–3]. Although with the development of medical technology, mortality rates for OSCC have remained relatively unchanged. However, statistics show that the overall prognosis of OSCC is poor and the survival rate is about 55–65% [4]. The pathogenesis of OSCC is extremely complex, which may be the result of multiple factors such as dietary habits, smoking, local adverse stimulation, drinking, HPV virus infection and chewing betel nut [5,6]. Therefore, it is of great significance to further explore the molecular mechanism of the occurrence and development of OSCC and to find more accurate therapeutic targets and prognostic markers for improving the survival rate of OSCC patients.

Circular RNA (circRNA) is a special type of long non-coding RNA, which is a circular RNA formed by the reverse splicing of gene sequences from the head to the tail of precursor RNA molecules through a variety of reverse variable splicing methods [7]. With the further development of transcriptome sequencing and RNA-sequence technology, a large number of circRNAs have been screened and identified as having biological functions. Deregulated expression of some circRNAs affects tumour phenotypes and has important implications for tumour biology. Zhao et al [8] demonstrated that Hsa_circ_0001874 and Hsa_circ_0001971 in saliva can be used as potential biomarkers for the diagnosis of OSCC. Peng et al [9] confirmed that circ_0000140 plays a tumour-inhibiting role in OSCC through the Hippo signalling pathway via miR-31/LATS2 axis. The above studies suggested that differentially expressed circRNAs are involved in the occurrence and development of OSCC.

Hippo signalling pathway is highly conserved in the evolution of mammals. It is a signal network composed of several tumour suppressor genes and oncogenes, and plays a crucial role in regulating organ size, cell number and tissue homeostasis in many tissues and organs [10]. Yes-associated protein (Yap) is the most important member of Hippo signalling pathway, and its expression in various types of tumours and localization in the nucleus are significantly increased. Activated YAP can promote the proliferation of cancer cells, inhibit cell apoptosis and promote tumour metastasis [11]. circYap is a circRNA molecule derived from the parent gene of Yap. Studies have confirmed that circYap in breast cancer tissues can inhibit the initiation of Yap translation and significantly reduce the expression of Yap protein, thus inhibiting

CONTACT Yong-Qing Dou douyong139@sina.com College of Integrative Medicine, Hebei University of Chinese Medicine, Shijiazhuang, China

the proliferation of breast cancer cells [12]. Recent studies have shown that circYap promotes the binding of TMP4 and ACTG to inhibit microfilaments aggregation, fibrosis aggregation, and the fibrosis process of cardiomyocytes [13]. It can be seen that circYap plays different roles in different diseases. However, its role in OSCC has not been reported. In this study, we found that circYap was low expressed in OSCC tissues and cell lines. In addition, we examined the clinical relevance of circYap in OSCC, and characterized its potential function and mechanism of action.

Materials and methods

Clinical OSCC tissue specimens

Human OSCC tissues and para cancerous normal tissues from 43 patients with OSSC were collected from the Department of Stomatology, the Second Hospital of Hebei Medical University. All patients had not received any chemotherapy or radiation prior to surgery. Tissue specimens were rinsed with cold sterile saline and quickly placed into RNA preservation solution. Each patient gave informed consent. This study was approved by the Ethics Committee of the Second Hospital of Hebei Medical University.

Cell culture

Human normal oral epithelial keratinocytes cell line (HOK) and OSCC cell lines (SCC-4, SCC-9, Cal-27) used in this study were purchased from American Type Culture Collection (Manassas, VA, USA). The OSCC cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Gibco), and 100 U/ml penicillin and streptomycin (Invitrogen, Camarillo, CA, USA). Cells were maintained at 37 °C in an atmosphere filled with 5% CO_2.

Plasmid construction

The expression plasmid of circYap was created by the placement of human entire circYap sequence into pcDNA3.1 circRNA Mini Vector (Addgene). The partial fragment of GFP was amplified by PCR and cloned into pcDNA3.1 circRNA Mini Vector as a control plasmid.

Small interfering RNA (siRNA) transfection

The small interfering RNA (siRNA) targeting human circYap (si-circYap1 and si-circYap 2), 5′-CUGCUUCGGCAGGUCCU CUUU-3′ and 5′-GCUUCGGCAGGUCCUCUUCUU-3′, Scrambled siRNA (si-NC) 5′-UUCUCCGAACGUGUCACGUTT-3′served as a negative control. The siRNAs were transiently transfected into cells using Lipofectamine® RNAiMAX Transfection Reagent (Invitrogen) according to the manufacturer's protocol.

RNA isolation and quantitative reverse transcription-PCR (qRT-PCR)

Total RNAs from cell lysates were isolated using TRIzol reagent (Life Technologies). The nuclear and cytoplasmic fractions were extracted using Minute TM Cytoplasmic and Nuclear Extraction Kit (Invent Biotechnologies). To quantify the amount of circRNA, cDNAs were synthesized using the M-MLV First Strand Kit (Life Technologies), and quantitative PCRs were performed using SYBR Green qPCR SuperMix-UDG (Life Technologies). For quantification, all RNA expression was normalized to the amount of GAPDH using the $2^{-\Delta\Delta Ct}$ method. The primer sequence of circYap (Forward: 5′-GCAA GAACTGCTTCGGCAGGTCCT-3′, Reverse: 5′-GTTTATATAGTAAA TTTCTCCATC-3′)

Western blot and co-immunoprecipitation (co-IP) analysis

RIPA buffer was used to lyse cells (50 mM Tris–Cl, pH 7.5, 1% NP-40, 0.5% Na-deoxycholate, 150 mM NaCl supplemented with complete proteinase inhibitor, Roche Applied Sciences). Equal amounts of protein (30 ∼ 60 μg) were separated by 10% SDS-PAGE, and electrotransfered to a PVDF membrane. Membranes were blocked with 5% milk in TBS for 1 h at room temperature, and incubated with primary antibodies against GAPDH (Santa Cruz), CDK4 and Cyclin D1 (Abcam), Rb, p-Rb, AKT, p-AKT and Lamin A/C (Cell Signalling Technology), at 4 °C overnight, and then with the HRP-conjugated secondary antibody (Abcam) for 1 h. The blots were evaluated with GE ImageQuant™ LAS 4000 detection system. The protein bands of interest were quantified using Image Pro Plus 6.0 software, and the integrated signal densities were normalized to GAPDH (the loading control).

Lysate samples were precleared with Dynabeads Protein G (Life technology) to reduce non-specific binding. The supernatants were immuno-precipitated with indicated antibodies bound to Dynabeads Protein G at 4 °C, overnight. The beads were separated on a magnet, washed with the lysis buffer, and resuspended in sample buffer. Bound proteins were resolved by SDS-PAGE followed by Western blot analysis as described above.

Fluorescence in situ hybridization (FISH)

The cells were washed in PBS and fixed in 4% paraformaldehyde for 10 min and permeabilized overnight in 70% ethanol. Then the cells were rehydrated for 10 min in 50% formamide and 2 × SSC. The cells were incubated using specific probes of circYap. Hybridization was performed using fluorescence-labeled probes in hybridization buffer by incubation at 55 °C for 1 h. After stringent washing with SSC buffer, cell nuclei were counterstained with DAPI (Invitrogen). Images were acquired using a Confocal Laser Scanning Microscope Systems (Leica).

RNA immunoprecipitationn assay (RIP)

The cells were washed in ice-cold PBS, lysed in lysis buffer (20 mM Tris-HCl, pH 7.0, 150 mM NaCl, 0.5% NP-40, 5 mM

EDTA, with freshly added 1 mM DTT, 1 mM PMSF, and 2 U/μl RNase inhibitor), and then incubated with 5 μg the primary antibody at 4 °C for 2 h. 50 μl Dynabeads Protein G (Life technology) was added to each sample, and the mixtures were incubated at 4 °C for 4 h. The pellets were washed with PBS and resuspended in 1 ml TRizol Reagent (Invitrogen). The precipitated RNA in the aqueous solution was subject to quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis to demonstrate the presence of the binding products using the primer [14].

RNA pull-down assay

The cells were washed in ice-cold phosphate-buffered saline, lysed in 500 μl lysis buffer (20 mM Tris-HCl, pH 7.0, 150 mM NaCl, 0.5% NP-40, 5 mM EDTA, with freshly added 1 mM DTT, 1 mM PMSF, and 2 U/μl RNase inhibitor), and then incubated with 3 μg biotinylated DNA oligo probes against human circYap (5′-TCAGGAAGAGGACCTGCCGAAGCAGTTCTTGC) at 4 °C for 2 h. A total of 50 μL Dynabeads™ MyOne™ Streptavidin C1 magnetic beads (Invitrogen) were added to each binding reaction and further incubated at 4 °C for 2 h. The beads were washed with lysis buffer for three times. The bound proteins in the pull-down materials were analyzed by western blot [14].

Statistical analyses

All statistical analyses were performed with the SPSS 21.0 software (IBM Corporation, Armonk, NY, USA). The data are presented as means ± SD. Two groups were compared by Student's *T* tests. Differences among groups were analysed with one-way analysis of variance (ANOVA). For all statistical comparisons, $p<.05$ was considered significant.

Results

circYap was downregulated in OSCC tissues and cell lines

qRT-PCR analysis was used to detect the expression of circYap in OSCC tissues from 43 patients and cell lines. Result revealed that circYap was downregulated in OSCC tissues (Figure 1(A)). The low expression of circYap was closely correlated with tumour, nodes and metastases (TNM) stage and lymph node metastasis in OSSC (Table 1). In addition, Kaplan–Meier analysis indicated that low circYap expression in OSCC was associated with decreased overall survival (Figure 1(B)). Compared with that in HOK cells, circYap was also downregulated in SCC4, SCC9 and CAL-27 OSCC cell lines (Figure 1(C)). After RNase R treatment, YAP mRNA was significantly decreased, but circYap had no significant change (Figure 1(D)). FISH result showed that was mainly distributed in the cytoplasm of cells (Figure 1(E)). These imply circYap may be associated with OSCC progression.

circYap inhibits OSCC cells proliferation

To investigate the role of circYap in OSCC cells, circYap overexpression plasmid was constructed. Human circYap cDNA was synthesized and cloned into pcDNA3.1 circRNA Mini

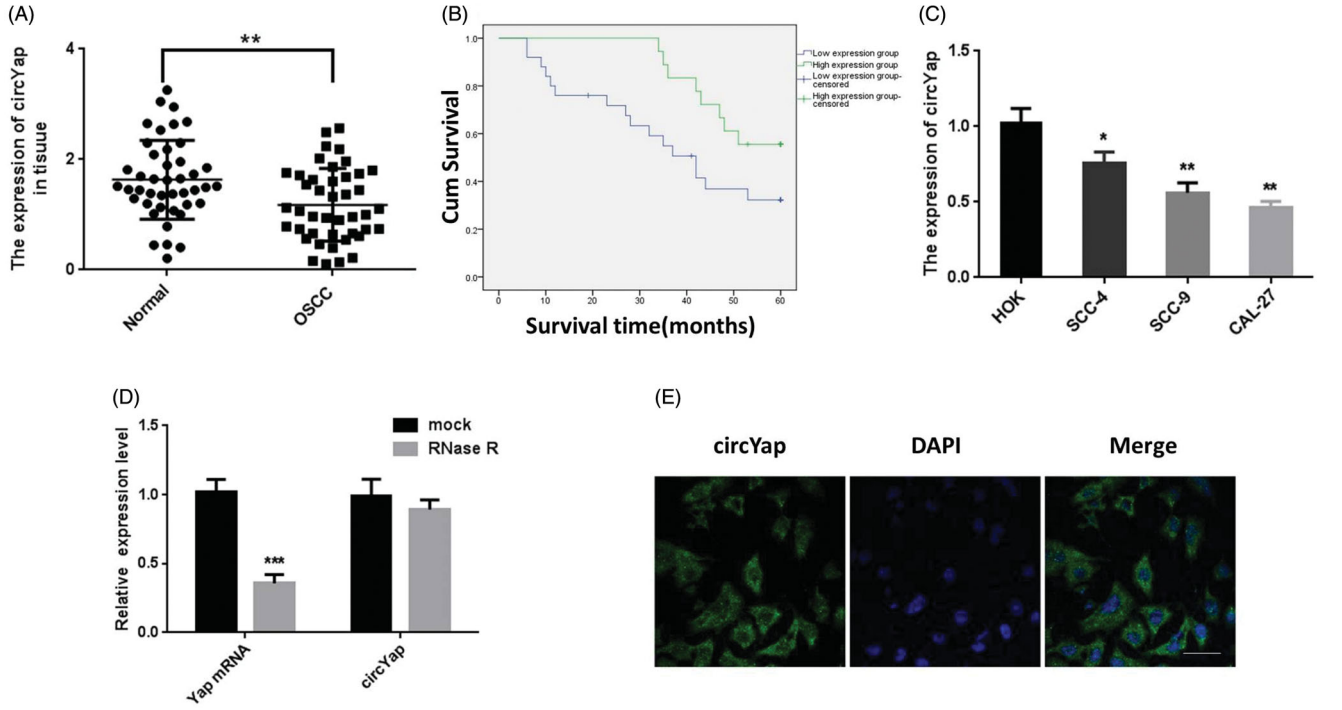

Figure 1. circYap was downregulated in OSCC tissues and cell lines. (A) Levels of circYap were measured by qRT-PCR in cancer tissues and paracancerous normal tissues from 43 patients with OSSC. (B) Kaplan-Meier curves for OSCC patients comparing circYap expression and overall survival ($p < .05$). (C) qRT-PCRs for circYap expression in difference types of cells. (D) Total RNAs were digested with RNase R followed by qRT-PCRs detection of circYap and Yap mRNA expression in HOK cells. (E) FISH for subcellular localization of circYap in the HOK cells. Scale bars = 100 μm. Data represent mean ± SD. Student's *t*-test, one-way ANOVA: $^{*}p < .05$, $^{**}p < .01$, $^{***}p < .001$ versus the corresponding control.

Vector. qRT-PCR analysis showed that circYap expression was significantly up-regulated by 4-fold (Figure 2(A)). Using BrdU incorporation, we showed that overexpression of circYap significantly reduced the proliferation activity of CAL-27 cells (Figure 2(B)). Moreover, overexpression of circYap suppressed the level of PCNA and C-MYC (Figure 2(C)). To further confirm the inhibitory effect of circYap on proliferation of OSCC cell lines, two siRNAs against circYap (si-circYap −1 and si-circYap −2) were transfected with SCC4 cells, we found that two siRNAs both significantly decreased circYap (Figure 2(D)), and si-circYap-1 had more effect in two siRNAs, thus it was selected for subsequently experiments. Knockdown of circYap significantly increased the proliferative capacities of SCC4 cells (Figure 2(E)), and Western blot analysis found that protein levels of PCNA and C-MYC were increased (Figure 2(F)). These findings indicate that circYap inhibits proliferation activity of OSCC cells.

Table 1. The relationship between the expression of circYap and OSCC patients clinical pathological features.

			circYap Expression(*n*)		
Pathological features	Group	Number	Low(25)	High(18)	*p*
Sex	Male	29	19	10	.16
	Female	14	6	8	
Age	<60	23	14	9	.70
	≥60	20	11	9	
Smoking	No	16	9	7	.85
	Yes	27	16	11	
Drinking	No	19	10	10	.31
	Yes	24	15	8	
Tumour size	<4 cm	15	8	7	.64
	≥4 cm	28	17	11	
Lymphatic metastasis	No	21	8	12	.02*
	Yes	22	17	6	
TNM stage	I–II	16	6	10	.03*
	III–IV	27	19	8	

The differences were compared using the chi-square test (*$p < .05$).

circYap suppresses cell cycle progression of OSCC cells

To further study the biological role of circYap in OSCC cells, we treated CAL-27 and SCC-4 cells with either circYap overexpression plasmid or si-circYap, and analysed the effects on cell cycle progression. circYap overexpression plasmid induced a strong and reproducible block of the G1/S transition in CAL-27 cells (Figure 3(A)). While, circYap knockdown promoted the G1/S transition in SCC-4 cells (Figure 3(B)). We also investigated some well-known protein factors regulating G1-S transition and cell proliferation, Retinoblastoma (Rb) and Akt proteins. Hypo-phosphorylated Rb can block to activate S-phase genes and so causing G1-S arrest [15]. In CAL-27 cells of circYap overexpression, a reduction of the total amount of Rb protein was observed (Figure 3(C)), moreover, protein decrease was accompanied by a strong decrease of its phosphorylated form, leading to a ratio between p-Rb and Rb down to approximately 40% compared to control conditions, meanwhile, the phosphorylated Akt (p-Akt), which has a crucial role in sustaining cell proliferation [16], was reduced, p-Akt/total Akt ratio was also decreased

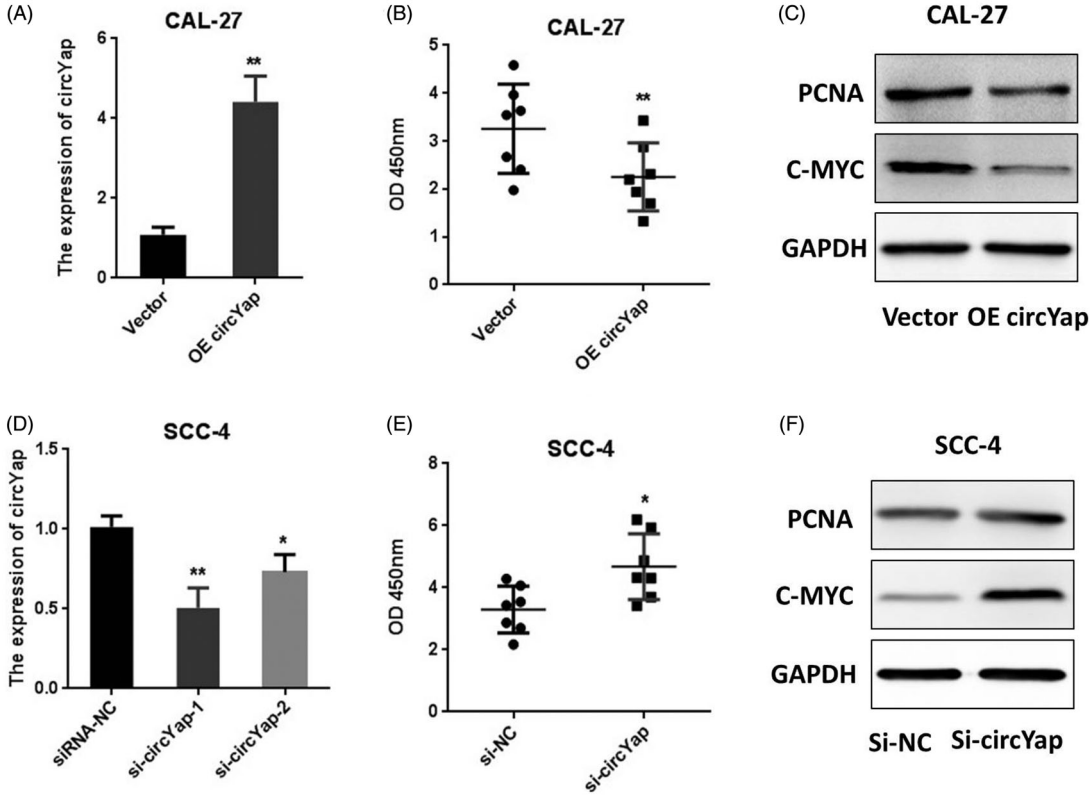

Figure 2. circYap inhibits OSCC cells proliferation. (A) Expression of circYap after the cells transiently transfected with circYap overexpression plasmid or vector in CAL-27 cells. (B) The relative activity of proliferation by BrdU incorporation in CAL-27 cells of circYap overexpression. (C) Western blot analysis of PCNA and C-MYC. (D) circYap expression in SCC-4 cells transfected with siRNA NC or two sicircYap. (E) The relative activity of proliferation by BrdU incorporation in SCC-4 cells of circYap knockdown. (F) Western blot analysis of PCNA and C-MYC. All the experiments were performed with three independent repeats. Data represent mean ± SD. Student's *t*-test: *$p < .05$, **$p < .01$ versus the corresponding control.

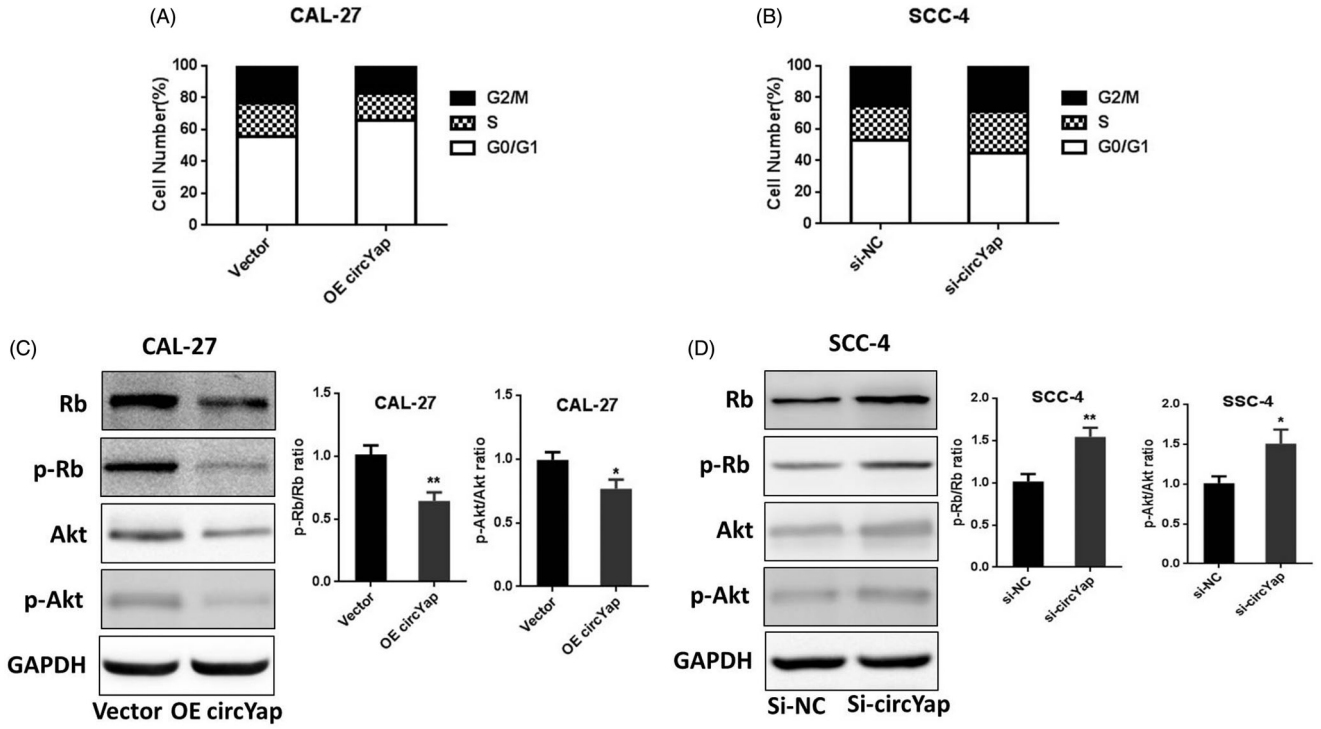

Figure 3. circYap suppresses cell cycle progression of OSCC cells. (A) Cell cycle analysis by flow cytometry in CAL-27 cells of circYap overexpression. $N = 3$. (B) Cell cycle analysis by flow cytometry of SCC-4 cells upon circYap knockdown. $N = 3$. (C) Western blot in CAL-27 cells of circYap overexpression (GAPDH was used as loading control). Corresponding ratio of pRb/Rb and p-Akt/Akt protein levels (relative to GAPDH) are shown. (D) Western blot in SCC-4 cells upon circYap knockdown (GAPDH was used as loading control). Corresponding ratio of pRb/Rb and p-Akt/Akt protein levels (relative to GAPDH) are shown. All the experiments were performed with three independent repeats. Data represent mean ± SD. Student's *t*-test: $^{*}p < .05$, $^{**}p < .01$ versus the corresponding control.

(Figure 3(C)). Conversely, the increase in Rb, p-Rb and p-Akt protein levels were observed, accompanied by increase of pRb/Rb and pAkt/Akt ratio upon circYap knockdown in SCC-4 cells (Figure 3(D)). In conclusion, these experiments indicate that circYap arrests cell cycle progression in OSCC cells.

circYap suppresses cyclin D1/CDK4 complexes formation by interacting with CDK4

Cyclin D1/CDK4 complexes is essential for G1-S transition. RPIseq predicted that circYap could bind to Cyclin D1 and CDK4 respectively. To confirm that circYap influences formation of Cyclin D1/CDK4 complexes, we further examined the potential interactions of circYap with Cyclin D1 and CDK4 using RIP and RNA pull-down assays, respectively. We demonstrated that circYap was retrieved by CDK4 antibody (Figure 4(A)), and CDK4 pulled down by the circYap probe was enhanced in CAL-27 cells overexpressing circYap (Figure 4(B)). Unfortunately, the interaction between circYap and Cyclin D1 was not observed. To investigate whether circYap influences formation of Cyclin D1/CDK4 complexes, we performed co-IP using Cyclin D1 antibody to detect the binding between of Cyclin D1 and CDK4. We showed that overexpression of circYap significantly decreased the binding of Cyclin D1 to CDK4 (Figure 4(C)). Using Western blot after separation of cytoplasm and nucleus, we showed that nuclear translocation of Cyclin D1 and CDK4 was significantly inhibited in CAL-27 cells with circYap overexpression (Figure 4(D)). To further prove that circYap inhibits proliferation of OSCC cells by CDK4, we overexpressed circYap and CDK4 in CAL-27 cells (Figure 4(E)). BrdU incorporation assay confirmed that CDK4 overexpression partially reverse the inhibitory effects of circYap on proliferation activity of CAL-27 cells (Figure 4(F)). Taken together, our data demonstrate that circYap, as a competitive inhibitor, inhibits formation of Cyclin D1/CDK4 complexes, resulting in repressing complexes to translocate to the nucleus (Figure 4(G)), and inhibits proliferation of OSCC cells.

Discussion

A large number of studies have been devoted to exploring the role of circRNAs in OSSC. It has been found that abnormal expression circRNAs are closely related to the OSCC clinical characteristics, suggesting that circRNAs can be used as biomarkers for OSSC [17]. YAP protein, formed by transcription and translation of YAP gene, plays an important role in promoting proliferation of hippo signaling. CircYap is derived from parent gene YAP gen. Studies have reported that the expression of circYap is significantly reduced in colorectal cancer tissues, which is closely associated with poor prognosis of patients [18]. To further explore the role of circYap in OSSC, we detect the expression of circYap in cancer tissues and para cancerous normal tissues from patients with OSSC. qRT-PCR results showed that the expression of circYap in cancer tissues was significantly lower than adjacent normal tissues. The low expression of circYap was closely correlated with TNM stage and lymph node metastasis in OSSC, but there were no correlated with age, gender, smoking and other factors. These results suggest that the differential

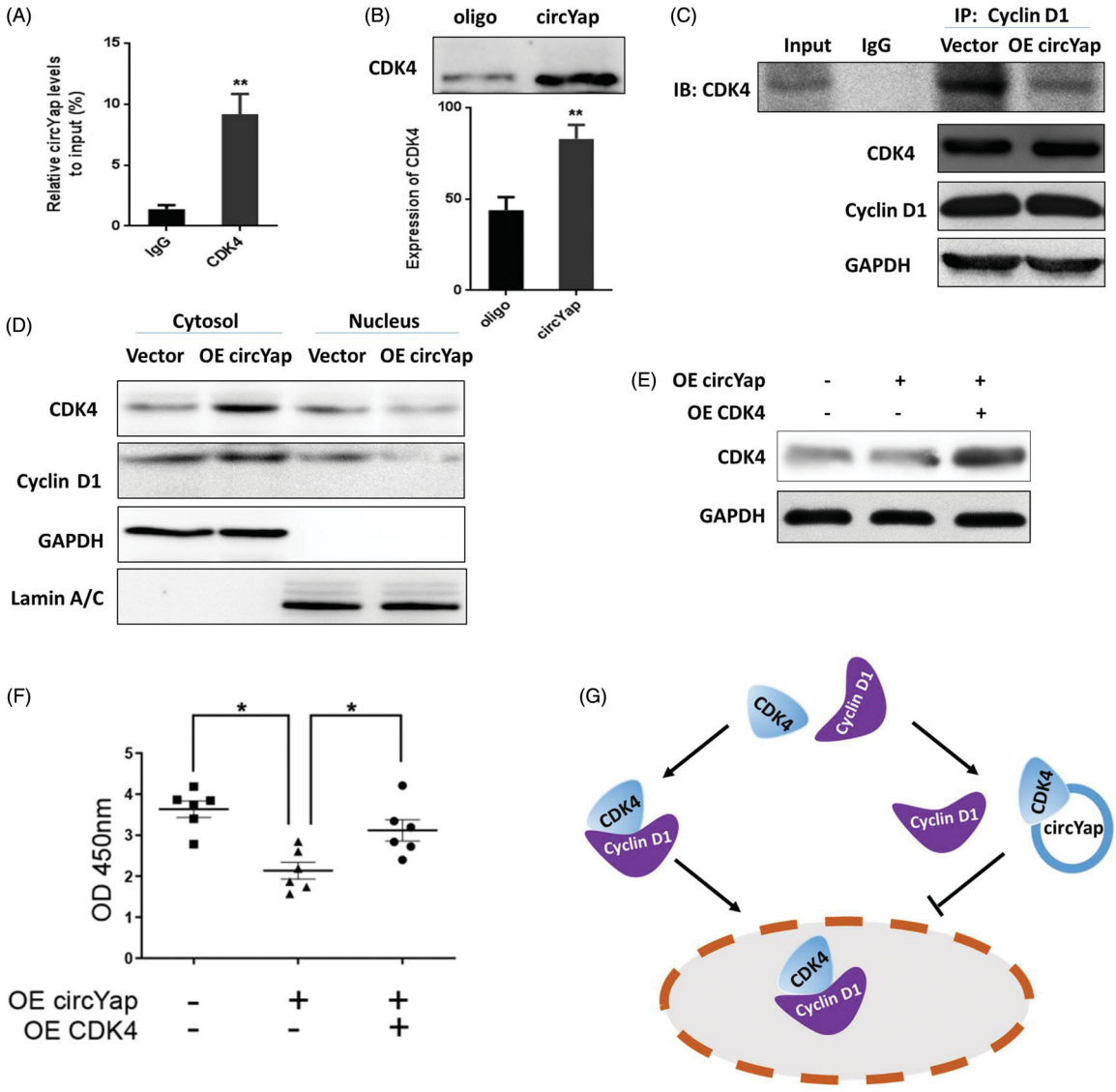

Figure 4. circYap suppresses Cyclin D1/CDK4 complexes formation by interacting with CDK4. CAL-27 cells were transfected with vector or circYap for 24 h. (A) RIP assay was performed using CDK4 antibodies. (B) RNA pull-down assay was performed using the probe. Western blot was used to validate the interactions between circYap and CDK4. (C) Co-IP assay for the interactions of Cyclin D1 with CDK4. (D) The cytoplasmic and nuclear expression of Cyclin D1 and CDK4. (E-F) CAL-27 cells were transfected with vector or CDK4 for 24 h. (E) Western blot in CAL-27 cells (GAPDH was used as loading control). (F) The relative activity of proliferation by BrdU incorporation in CAL-27 cells. All the experiments were performed with three independent repeats. Data represent mean ± SD. Student's *t*-test, one-way ANOVA: $^{*}p < .05$, $^{**}p < .01$ versus the corresponding control. (G) Schematic representation of a working model by which circYap inhibits the Cyclin D1/CDK4 complex formation through interacting with CDK4, which exerts an anti-proliferation effect.

expression of circYap may be involved in the occurrence and development of OSSC.

In order to further explore the possible molecular mechanism of circYap in OSSC, We detected the expression of circYap in OSSC. The results showed that the expression of circYap in four oral squamous cell cells was significantly lower than normal oral squamous epithelial cells, and the decrease was most significant in CAL-27 cells. Afterwards, HOK cells were treated with RNase R, YAP mRNA was significantly decreased, while circYap expression was not significantly changed, which proved the stability of circYap. The subcellular localization of circRNA is closely related to its function. CircRNAs can be mainly located in the cytoplasm, with a higher enrichment degree than linear, or can be mainly located in the nucleus to play a cis regulatory role, and the differences in their subcellular localization may be related to the molecular types [19,20]. Therefore, RNA in cytoplasm and nucleus of cells were extracted and analyzed respectively, and FISH assay to confirm that circYap was mainly located in cytoplasm, suggesting that circYap may play a regulatory role in cytoplasm.

Then circYap were overexpressed in CAL-27 cells and knocked down in SSC-4 cells. BrdU assay confirmed that overexpression of circYap could significantly inhibit the proliferation of CAL-27 cells, while knockdown of circYap could significantly promote the proliferation of SSC-4. The results proved that circYap inhibited the proliferation of OSSC cells. Proliferating Cell Nuclear Antigen (PCNA) can regulate cell proliferation and is a prognostic marker for malignant tumors [21]. C-MYC protein, the translation product of oncogene c-MYC gene, promotes cell division and regulates cell proliferation [22]. We detected the expression of two proteins. The

results showed that overexpression of circYap significantly inhibited the expression of PCNA and C-MYC proteins, while knockdown of circYap significantly promoted the expression of PCNA and c-MYC proteins. The results further confirmed the inhibitory effect of circYap on the proliferation of OSSC cells.

The active proliferative ability of tumor cells is closely related to the mitosis process. We then examined the effect of circYap on the cell cycle of OSSC cells. The results showed that overexpression of circYap inhibited G1/S transformation in CAL-27 cells, while knockdown of circYap promoted G1/S transformation in SCC-4 cells. Rb protein regulates the G1/S cell cycle by regulating the transcription factor E2F1, which induces the expression of S phase genes. In particular, low-phosphorylated Rb can bind to E2F1, thus blocking its ability to activate S phase genes, leading to G1-S stagnation [15]. Akt and phosphorylated Akt (p-Akt) are also known to be important regulators of the cell cycle. We detected the effect of circYap on the changes of Rb and Akt proteins and their phosphorylation levels. The results showed that overexpression of circYap inhibited the two proteins and their phosphorylation levels, reducing the phosphorylation ratio, respectively. Knocking down circYap yielded the opposite result. The above experimental results confirmed that circYap inhibited the G1/S phase transformation of OSSC cells and then inhibited cell proliferation.

Cyclin D1 promotes progression through the G1 phase of the cell cycle. Over expression of cyclin D1 has been reported in various tumours like oesophageal carcinoma, hepatocellular carcinoma, lung carcinoma, and head and neck carcinoma. It is reported that cyclin D1 protein expression is significantly altered from epithelial dysplasia to oral squamous cell carcinomas [23]. CDK4 is one of the main players in cell cycle. Progression from G1 phase to S phase of the mammalian cell cycle is controlled by Cyclin D1 in conjunction with their catalytic partners CDK4 [24]. Cyclin D1/CDK4 complexes form in cytoplasm and are then translocated to the nucleus to promote G1/S transition and are responsible for inactivation of Rb proteins [25]. We predicted whether circYap could bind to Cyclin D1 or CDK4 by RPIseq. The scores indicate circYap may interact with Cyclin D1 and CDK4, respectively. We hypothesized that circYap may bind CDK4 and Cyclin D1 to influence the cell cycle. Next, we used RIP and RNA pull-down assays to confirm the above conjecture. We found that circYap could only bind to CDK4, but could not bind to Cyclin D1. Further experiments showed that overexpression of circYap significantly reduced the interaction between CDK4 and Cyclin D1. Therefore, we demonstrated that circYap may bind CDK4, thereby inhibiting the formation of Cyclin D1 and CDK4 complex, resulting in the retention of Cyclin D1 in the cytoplasm, and leading to cell cycle arrest. Thus, we clarify the mechanism that Cyclin D1 involves in OSCC from another aspect.

However, due to the short observation period, whether it can be used as an independent predictor of poor prognosis in OSSC patients remains to be further confirmed. And, whether circYap could suppresses OSCC *in vivo* by transfected to animal to need further research.

Conclusions

The results of the study reveal that the significantly low expression of circYap in OSSC cancer tissues. circYap overexpression inhibits proliferation of OSSC cells. It is closely related to TNM staging, lymph node metastasis and other clinicopathological factors. *In vitro* studies have confirmed that circYap can suppress the formation of Cyclin D1 and CDK4 complex to inhibit cell proliferation by binding CDK4. Our findings provide not only novel insight into the molecular mechanism, but also new therapeutic target of OSCC.

Disclosure statement

No potential conflict of interest was reported by the author(s).

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